Age- and Dietary-Related Distributions of Hepatic Arachidonic and Docosahexaenoic Acid in Early Infancy

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ABSTRACT

A dietary-related deficiency of docosahexaenoic acid [C22:6(n-3)] in infant cerebral cortex has been identified. Absence or very low rates of hepatic synthesis from the essential fatty acid precursor, α-linolenic acid [C18:3(n-3)], in early life may have been a contributory factor. We have analyzed liver total lipid fatty acid compositions in 27 term (37–42 wk gestation) and 4 preterm (30–33 wk gestation) infants who died within the first 6 mo of life from sudden infant death syndrome. The infants were fed exclusively either human or formula milks. Formula-fed infants were subdivided into two groups, one receiving SMA milk with an α-linolenic acid content at 1.5% of total fatty acids and the other a composite group fed milks with low α-linolenic acid concentrations (<0.1% to 0.4%). The hepatic content of arachidonic acid [C20:4(n-6)] and docosahexaenoic acid was generally lower in both formula-fed groups than in the human milk-fed group. The age-related distributions of docosahexaenoic acid showed that coincident minimum levels were present in both formula groups in the third month of life. This may indicate that the hepatic enzymes involved in the final stage (Δ4-desaturation) conversion of α-linolenic acid to docosahexaenoic acid could be inactive in the first months of life. Emphasis must be on provision of preformed dietary docosahexaenoic acid and possibly arachidonic acid as well as their essential fatty acid precursors, to both term and preterm infants for at least the first 16 wk of life. (*Pediatr Res* **38: 361–365, 1995**)

Abbreviations

DHA, docosahexaenoic acid
AA, arachidonic acid
LCPUFA, long-chain polyunsaturated fatty acid

It has now been established that the LCPUFA DHA [C22:6(n-3)] and AA [C20:4(n-6)] should be supplied in formula milks fed to both term and preterm infants (1–5). What is uncertain, however, is the optimum duration for such supplementation. To estimate this would probably require us to define the age at which DHA and AA are synthesized in the infant liver by enzymatic conversion of their essential fatty acid precursors α-linolenic [C18:3(n-3)] and linoleic acid [C18:2(n-6)], respectively. Desaturation of these essential fatty acids is performed in a series of reactions located primarily in the hepatic microsomes of the endoplasmic reticulum. Conversion of α-linolenic acid to DHA and linoleic acid to AA proceeds via successive desaturation and elongation reactions involving Δ6- and Δ5-desaturase enzymes and Δ4-desaturation, with *in vitro* studies initially implicating Δ6-desaturase as the rate-limiting step (6). Measurement of the Δ6- and Δ5-desaturases has been made in human tissue (7, 8), although most research

in this area has inevitably been confined to animal models (9–12). We considered that as the complex multistage enzyme systems involved are unstable and rapidly inactivated in biopsy material (7), analysis of the hepatic LCPUFA products of these reactions might be more informative as to their overall *in vivo* activities (13, 14). In the rat administered radiolabeled linoleic and α-linolenic acids have been subsequently identified in liver triglyceride and phospholipid fractions as both the parent and LCPUFA derivative (15). Any investigation of liver fatty acids would therefore need to take into account this widespread distribution of LCPUFA. However, no *in vitro* enzymatic assays will ever adequately represent the complexity of lipid interactions associated with the intact liver. The most recently published results for human neonatal hepatic microsomal desaturases (7) appear to indicate enzyme activities of an order that may be sufficient to provide a limited supply of both DHA and AA to neuronal tissue to enable repair and renewal but inadequate for the large amounts of these LCPUFA required by the rapidly growing brain during this early period of development (2, 16, 17). In addition, as the hepatic desaturase activities are known to be dependent on initial substrate concentrations, we recognized that examination of tissue from infants having

received a diet low in α-linolenic acid may be particularly informative.

METHODS

The population investigated were infants diagnosed as sudden infant death syndrome by experienced pediatric pathologists. They were aged less than 6 mo and had lived in the Greater Glasgow Health Board area during the period of 6 y from May 1987 to July 1993. Liver specimens were taken at necropsy from the right lobe and stored at −60°C before analysis. Pathologic examination excluded any specimens in which significant fatty infiltration or deposition had been evident. Liver samples from 27 term infants were subsequently assigned to one of three feeding groups, the prerequisite of which being that the infant had received exclusively a milk diet of fatty acid composition defined by that group. The age-equivalent dietary group compositions and details are provided in Table 1. Group 1 infants were breast fed, receiving both LCPUFA and their essential fatty acid precursors. Group 2 were fed exclusively SMA formula devoid of LCPUFA (C20 and C22) but with an α-linolenic acid content of 1.5% of total fatty acids, a composition similar to that found in most U.S. and European formulations. Group 3 infants had been fed formulas both lacking in these LCPUFA but also with low concentrations of α-linolenic acid varying between <0.1% and 0.4% of total fatty acids, which were unique to the milks outlined in Table 1. In spite of the extended collection period, few significant changes occurred in the lipid contents of the formula milks in respect of the nonessential saturated and mono-unsaturated fatty acids. During this time formula linoleic acid contents remained relatively constant at between 12 to 18% of total fatty acids. Liver tissue from two preterm infants (30 and 32 wk of gestation) aged 10 and 17 wk, respectively, fed diet 2 and a further two 13-wk-old preterm infants (31 and 33 wk of gestation) fed diet 3 were examined.

Tissue analysis. On thawing, core samples (250–500 mg) were taken from the liver, and extraneous blood was removed by washing with distilled water. On drying, the tissue was weighed, lipid extraction was performed as previously described (1), and the weight of lipid isolated was noted to calculate the percentage of recovery. Fatty acids were derivatized in 2-mL boron trifluoride:methanol (14% wt/vol) to which 200 μL of chloroform had been added, enabling the phospholipids, triglycerides, and cholesterol esters to be transesterified in a one-step procedure to give fatty acid extracts of total hepatic lipid. The derivatized fatty acid methyl esters were separated and identified by gas chromatography (1).

To correct for the non-fatty acid component of these lipids toward recoveries, extracts were assayed for total cholesterol (cholesterol 352-20, Sigma Chemical Co., UK), triglyceride glycerol (triglyceride UV, 334-A, Sigma Chemical Co., UK) and phospholipid choline phosphate (18). To verify homogeneous distribution of liver fatty acids tissue samples were assayed from two different parts of each specimen. The assessment of stability of hepatic fatty acids with storage of tissue at −60°C was evaluated by reassaying specimens after intervals of between 6 mo and 1 y.

STATISTICAL ANALYSIS

The 10 most abundant fatty acids accounted for >98% of total lipid fatty acids (Table 2). Results were expressed, with reference to lipid recovery, as g/kg wet tissue. In all groups

Table 1. *Term infant details, dietary groups, and milk fatty acid compositions*

		Group		
Diet		1 HM*	2 SMA†	3 MIX‡
Infant data§				
Birthweight (g)		3380 (492)	3133 (365)	3011 (384)
Liver weight (g)		201 (37)	205 (41)	191 (71)
Gestational age (wk)		40.6 (1.2)	39.8 (1.1)	39.4 (1.5)
Age (wk)		11.3	11.6	12.9
Age range (wk)		2–21	2–22	4–21
Male:female ratio		2:5	7:4	7:2
Milk fatty acid ranges¶				
Myristic	C14:0	3.8–11.5	6.0–11.4	5.2–12.5
Palmitic	C16:0	20.3–29.0	13.0–22.8	12.4–22.8
Palmitoleic	C16:1 (n-7)	1.5–3.9	1.1–1.6	0.5–0.8
Stearic	C18:0	4.6–9.5	3.9–8.2	3.6–6.5
Oleic	C18:1 (n-9)	31.3–43.4	34.0–36.6	29.5–46.7
Linoleic	C18:2 (n-6)	6.3–15.7	16.0–17.8	12.1–15.5
α-linolenic	C18:3 (n-3)	0.5–1.2	1.4–1.6	<0.1–0.4
Dihomo-γ-linolenic	C20:3 (n-6)	0.2–0.8	ND	ND
Arachidonic	C20:4 (n-6)	0.3–0.8	ND	ND
Docosahexaenoic	C22:6 (n-3)	0.1–0.5	ND	ND

* HM = human milk.

† SMA (Wyeth, UK) = Gold or White Cap formulas.

‡ MIX = Cow&Gate Premium (Cow&Gate, UK—pre-1991 formula) or Farley's (Heinz, UK) Ostermilk (pre-1991), Ostermilk 1 or Ostermilk 2 (both post 1991).

§ Infant details are given as means with standard deviations in parentheses.

¶ Milk fatty acid contents of the formulas (as weight percent of total fatty acids) represent manufacturers' published data confirmed by the authors and those for human milk are the authors' findings from a local population of mothers. ND = not detected or <0.05% of total fatty acids.

Table 2. *Profile of fatty acids in the term infant hepatic total lipid fraction in relation to diet*

Diet	Group		
	1	2	3
Lipid*			
Recovery	50.3 (40.6–108.1)	45.5 (28.8–124.3)	47.9 (35.5–58.8)
Cholesterol	2.5 (2.0–3.9)	2.1 (1.4–4.3)	2.2 (1.6–2.7)
Triglyceride	12.5 (5.4–34.1)	6.1 (1.9–47.1)	11.4 (3.0–15.0)
Fatty acid†			
C14:0	0.66 (0.22–1.44)	0.42 (0.13–1.11)	0.55 (0.23–1.05)
C16:0	11.36 (8.39–25.94)	9.37 (6.06–25.94)	11.67 (8.71–13.11)
C16:1 (n-7)	0.91 (0.36–2.58)	0.47 (0.29–2.24)	0.83 (0.34–0.98)
C18:0	4.40 (3.68–7.13)	4.36 (2.92–7.32)	3.73 (2.60–4.44)
C18:1 (n-9)	12.43 (7.62–31.68)	9.94 (4.26–38.47)	11.46 (8.18–17.23)
C18:2 (n-6)	6.03 (3.77–11.63)	6.89 (4.49–19.48)	5.37 (4.32–7.86)
C18:3 (n-3)	0.16 (0.03–0.38)‡	0.17 (0.04–1.07)§	0.02 (0.01–0.15)
LCPUFA¶			
C20:3 (n-6)	0.48 (0.33–0.52)	0.47 (0.30–0.71)	0.50 (0.33–0.65)
C20:4 (n-6)	3.05 (2.83–4.09)	2.58 (1.90–3.43)	2.36 (1.75–2.65)
C22:6 (n-3)	1.99 (1.39–2.80)	1.01 (0.60–1.23)	0.49 (0.24–1.69)

All results are expressed as g per kg of tissue (wet weight) and given as medians and ranges.

* No between-group statistical analysis performed.

† Between group significances calculated by the two-tailed Mann-Whitney U test.

‡ $p < 0.01$, group 1 vs 3.

§ $p < 0.002$, group 2 vs 3.

both AA and DHA exhibited age-dependent results' distributions, which were subjected to regression analysis and optimal curve fit (dependent on identifiable maxima and minima), computed by the Cricket Graph program (Computer Associates, San Diego, CA). The derived second (group 1) and third (groups 2 and 3) degree polynomials and calculated coefficients of determination (r^2) are shown (Figs. 1 and 2).

The essential and nonessential fatty acid contents did not display a similar covariation with age (Minitab 10, Minitab Inc., PA) and were not normally distributed. Their group differences were therefore analyzed by the nonparametric, two-tailed Mann-Whitney U test with significance levels set at

$p < 0.01$ inasmuch as multigroup comparison was undertaken. The small number of observations ($n = 4$) on preterm infants and their absence from the breast-fed group precluded between group statistical analysis; however, visual interpretation of their LCPUFA results with respect to the corresponding term individuals was made.

RESULTS

The results of the hepatic total lipid fatty acid analyses are best subdivided into three categories, namely the nonessential (saturated and mono-unsaturated), the essential (linoleic and α-linolenic), and their LCPUFA derivatives. There were no significant differences between feeding groups in the nonessential fatty acids (Table 2). The hepatic essential fatty acid, α-linolenic, content was significantly lower in group 3 than in both groups 1 ($p < 0.01$) and 2 ($p < 0.002$). In general, human milk-fed infants had greater concentrations of the LCPUFA AA and DHA than those fed either of the formula diets, with results' ranges (Table 2) and distributions illustrated (Figs. 1 and 2). Hepatic AA content in group 1 nevertheless fell gradually over the 5-mo period from 4 to 3 g/kg tissue (Fig. 1). Polynomial equations demonstrated that both formula groups had minimal liver AA content (2 g/kg tissue) in the third month of life (8–10 wk) with a subsequent rapid increase. The curves for groups 1 and 2 intersected at 16 wk and those of groups 1 and 3 became tangential at approximately 18 wk before again diverging. In comparison with breast-fed infant liver the reduction in absolute amounts of AA in the liver of formula-fed infants throughout the first months of life was on average approximately 1 g/kg tissue or approximately 0.15–0.20 g AA per liver. No obvious differences were evident in AA concentrations between term and preterm infants of similar chronologic age.

Although the pattern of results for hepatic DHA (Fig. 2) was similar to that found for AA, with the minima for the formula
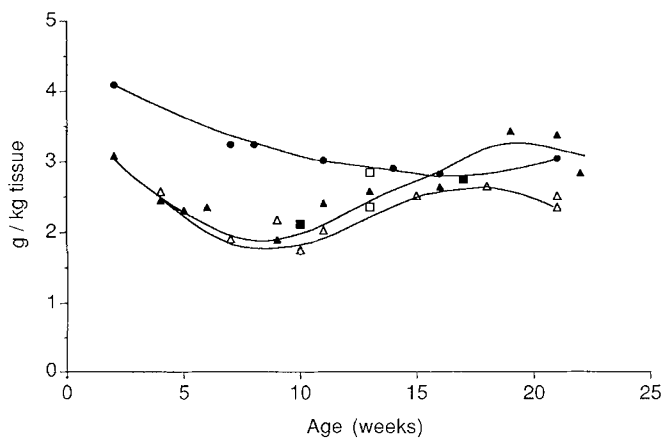

Figure 1. Hepatic total lipid AA [C20:4(n-6)] in relation to infants' diet and age. AA contents for a given age (x) are defined by the following polynomial equations.

Group 1 = $4.448 - 0.206x + 0.0067x^2$; $r^2 = 0.99$
Group 2 = $4.123 - 0.604x + 0.0542x^2 - 0.00131x^3$; $r^2 = 0.84$
Group 3 = $4.740 - 0.784x + 0.0675x^2 - 0.00168x^3$; $r^2 = 0.84$

with C20:4(n-6) contents in g/kg wet tissue and x in weeks. ●, Group 1; ▲, group 2; △, group 3; ■, preterm—group 2; □, preterm—group 3.
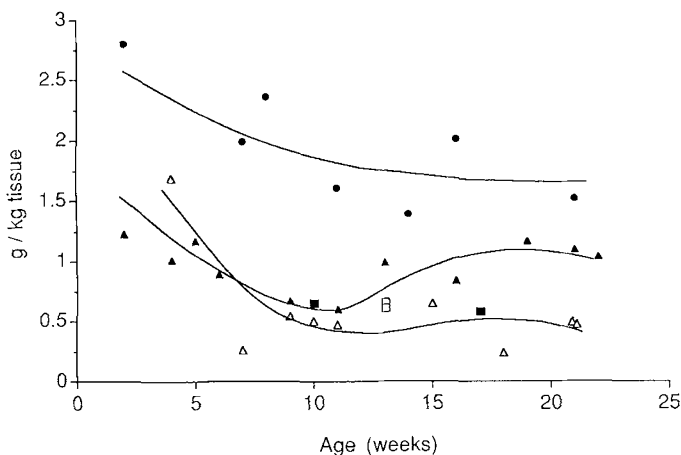
Figure 2. Hepatic total lipid DHA [C22:6(n-3)] in relation to infants' diet and age. DHA contents for a given age (x) are defined by the following polynomial equations.

Group 1 = 3.105 − 0.168x + 0.0046x^2; r^2 = 0.73
Group 2 = 1.688 − 0.221x + 0.0163x^2 − 0.00034x^3; r^2 = 0.63
Group 3 = 3.872 − 0.791x + 0.0573x^2 − 0.00131x^3; r^2 = 0.74

with C22:6(n-3) contents in g/kg wet tissue and x in weeks. Symbols as in Figure 1.

groups 2 and 3 again coincident during the third month, the major differences were that the human milk fed compositions were more randomly distributed, and in group 3 no increase in liver DHA concentrations was detected at the end of the third month such that the reduction with respect to the group 1 infants at 21 wk was still of the order of 1 g/kg tissue (Fig. 2). In group 2 an increase in DHA levels in the older infants (>12 wk) was apparent (Fig. 2) but they did not impinge on the group 1 curve at any stage. The hepatic DHA compositions of the preterm infants were not substantially different from their term counterparts, although there was no evidence of an increase in DHA in the 17-wk-old infant of 32 wk of gestation. No significant differences were found between the results of the duplicate tissue analyses nor in those reassayed after long-term (up to 12 mo) storage.

DISCUSSION

At first sight it may seem impossible to unravel and evaluate the many individual contributions toward hepatic fatty acid concentrations in early life. To reduce the complications in such a process we sought to ensure that our experimental procedures would estimate total fatty acids derived from the three major lipid components, the phospholipids, triglycerides, and cholesterol esters. Hepatic metabolism of these fatty acids involves several cell organelles, initially mitochondrial synthesis from malonyl and acetyl CoA or breakdown by β-oxidation predominantly confined to the nonessential saturated and mono-unsaturated fatty acids and specific for those up to a 16-carbon chain length (palmitic acid).

From Tables 1 and 2, it can be seen that there is a degree of correlation between the levels of hepatic and dietary nonessential fatty acids. Similarly it was probably to be expected that the essential fatty acids, linoleic and α-linolenic, would reflect dietary intake (Table 2); however, although they themselves

contribute little to neuronal structures (19, 20), they are of critical importance as substrates in the hepatic synthesis of the LCPUFA AA and DHA, respectively. Formerly thought to be restricted to the microsomes, new evidence suggests that synthesis of DHA also has a peroxisomal component (21). Some evaluation of the individual contributions toward the AA and DHA age-related distributions may be possible. The universal reduction in these LCPUFA in the first weeks of life (Figs. 1 and 2) possibly represents the cumulative effects of both dilution through tissue growth and maturation and some degree of mobilization from the adipose and endogenous hepatic stores. Easier to delineate could be that of dietary input, which during our study was confined to those infants receiving human milk and may be reflected in the differences between the corresponding group curves (Figs. 1 and 2).

The contributions to liver AA and DHA contents provided by enzymatic desaturation and elongation of the essential fatty acids would in normal circumstances be the most difficult to differentiate. However, even with what appears a wholly inadequate provision of α-linolenic acid precursor in the third diet, it was observed that both formula group curves for DHA were virtually superimposable during the first 3 mo of life (Fig. 2). This may indicate an absence or reduced supply of synthetic DHA to the infants in these formula groups. Subsequently there appears to be evidence of a relative increase in the hepatic DHA of group 2 compared with group 3 (Fig. 2). It may be that only after approximately 12 wk is optimal hepatic synthesis of DHA evinced and that irrespective of dietary provision of α-linolenic acid, Δ4-desaturation is poorly or nonfunctioning in early life. This appears to corroborate recent observations on cord plasma fatty acids which seem to demonstrate that Δ4-desaturation and not Δ6 or Δ5 may be rate-limiting in the formation of hepatic DHA (22). If as indicated Δ4-desaturation is achieved through microsomal chain elongation of docosapentaenoic acid [C22:5(n-3)] followed by Δ6-desaturation then the final peroxisomal β-oxidation may be the true rate-limiting step to DHA production in the infant liver (21). This has profound consequences for the supply of essential fatty acids and their LCPUFA derivatives to infants in the immediate postnatal period when brain growth and development are most vulnerable to reduced provision of these fatty acids. As we have now established that subcutaneous tissue reserves are insufficient (2) and hepatic Δ4-desaturation probably at best suboptimal, we again emphasize the need of dietary DHA for all formula-fed infants. It would appear that from the fourth month onward some conversion of α-linolenic acid to DHA occurs when the substrate is present in sufficient concentration such that synthetic DHA may represent approximately 0.6 g/kg tissue in the liver at 5 mo (Fig. 2). Within the human milk-fed group DHA results, that of the 16-wk-old infant at first appeared to be an outlier until it was recognized that uniquely this infant exhibited a subcutaneous tissue α-linolenic acid concentration similar to that of an SMA-fed infant (2). This hepatic DHA composition (Fig. 2) may correspond to the summation of both a dietary and synthetic component.

From Figure 1 the age-related distributions of AA can be seen. However, the minimum levels, which as with DHA occur simultaneously at 8–10 wk in both formula-fed groups, repre-

sent amounts which at approximately 2 g/kg tissue are four times greater than that for DHA. It seems that in term infants no prolonged reduction in hepatic AA is present perhaps as no peroxisomal β-oxidation stage is required in its formation (only $\Delta6$- and $\Delta5$-desaturation), and indeed formula-fed infants have the capacity to incorporate increased amounts of AA (and other n-6 series LCPUFA) into neural membranes in apparent response to reduced DHA supply (1). This may be explained further by the contrast with the DHA distribution curves, which for the formula-fed groups at no point intersect that of the human milk-fed group, whereas those for AA are at least equivalent by 16 wk and that of group 2 for a period ultimately in excess of group 1 concentrations (Fig. 1).

The AA results for the preterm infants all lie close to their corresponding term group curves (Fig. 1), indicating that their $\Delta6$- and $\Delta5$-desaturase enzymes may also be relatively active in the first weeks of life. The preterm infant appears to possess enzyme activities that are appropriate to chronologic age but liver growth and development related to gestational age, therefore although their LCPUFA production per kg of tissue is similar to that of term infants of the same age, the total hepatic content of LCPUFA in preterm infants of gestational ages from 30 to 33 wk are on average 20–30% lower. Nonetheless, as it is present in breast milk, it would seem prudent to provide dietary AA to all formula-fed infants in the first 4 mo of life.

In summary, although use of necropsy material necessarily precludes investigation of the dynamics of the processes involved in hepatic fatty acid metabolism, nevertheless we believe that we have clearly demonstrated that reliance on hepatic peroxisomal (or microsomal) synthesis of DHA in the first months of life has been misplaced. With formula milks we would advise that they should contain the range of LCPUFA found in human milk if neonatal neurologic and cardiovascular structural and possibly functional integrity is not to be compromised.

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