ORIGINAL ARTICLES

Effect of diet on the fatty acid composition of the major phospholipids of infant cerebral cortex

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Abstract

The fatty acid compositions of the major cerebral cortex phospholipids, phosphatidylcholine, phosphatidylethanolamine, and phosphatidylserine were measured in 16 term and one preterm 'cot death' infants fed exclusively either breast milk or one of two formulas. Docosahexaenoic acid (DHA; C22:6n-3) content in cerebral cortex phosphatidylethanolamine and phosphatidylserine of breast fed infants was greater than in both formula groups with significances varying between $p < 0.01$ and $p < 0.001$. Compensation for this deficiency in DHA in the formula fed infants was largely achieved by increased incorporation of docosapentaenoic acid (C22:5n-6) in the cerebral cortex of term infants and Mead (C20:3n-9) and dihomio Mead acids (C22:3n-9) in the preterm infant.

As the phospholipids most affected are known to perform an important role in membrane function and are possibly integral to neurotransmission it is recommended that breast milk substitute infant formulas should contain n-3 and n-6 series polyunsaturated fatty acids in proportions similar to those of human milk.

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At term the newborn human infant brain weighs about 350 g, which is approximately 10% of the total body weight. During the first year brain weight increases by about 750 g so that the brain remains approximately 10% of the body weight. This threefold increase of weight from birth is achieved largely by nerve cell growth with concurrent dendritic arborisation, glial cell (astrocyte and oligodendrocyte) proliferation, and axon myelination. Maximal glial cell proliferation in term infants takes place in the first six postnatal months.¹ The cerebral cortex (grey matter) is composed largely of neurones and astrocytes and makes up about 45% of the total brain weight. Much of the increase in the grey matter weight is due to the development of the complex arborisations and synaptosome formation which subserve neuronal function

and the learning processes. Myelination also proceeds rapidly after birth and in this process neuroglial cells envelop the axons of cortical neurones with sheaths of myelin which speed the rate of transmission of electrical messages between neurones, other central nervous system cells, and end organs such as muscle and skin.

Approximately 60% of the total energy intake of the infant during the first year is utilised by the brain and much of this energy is used to synthesise neuronal membrane and deposit myelin. Fatty acids from human milk or infant formulas provide not only a source of hydrocarbon for energy production but help synthesise the complex hydrocarbon structures necessary for the creation of neurotransmitter membranes.

Breast fed infants have significantly greater concentrations of the long chain polyunsaturated fatty acid (PUFA), docosahexaenoic acid (DHA; C22:6n-3) in their cerebral cortex phospholipids than infants fed current infant formulas.^{2,3} Cerebral cortex neuronal membrane phospholipids are composed of phosphatidylcholine, phosphatidylethanolamine, phosphatidylserine, and phosphatidylinositol. While the membrane receptor and secondary messenger characteristics of phosphatidylinositol and its metabolites have been extensively investigated^{4–6} the functions of other phospholipids are less certain. Phosphatidylcholine is known to confer a stabilising influence within the neuronal membrane.⁷ The carboxyl groups of phosphatidylserine function as ion exchange sites⁸ while both phosphatidylserine and phosphatidylethanolamine have an important influence on the distribution of protein molecules in the membrane.^{7,9} Incorporation of proteins into the phosphatidylserine and phosphatidylethanolamine rich areas of membrane is critically dependent on the chain length, degree of unsaturation, and hence configuration of the two fatty acids attached to each phosphatidylethanolamine moiety.¹⁰ The major DHA containing phospholipids of synaptosomes are phosphatidylserine and phosphatidylethanolamine and the polyunsaturated DHA of these moieties will preferentially cross link to proteins. The degree of unsaturation present in neuronal phospholipid fatty acids can mediate the activities of

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Table 1 Infant details and milk essential fatty acid compositions in relation to diet

	Diet		
	Breast milk	SMA	CGOST
Infant data			
Mean (SD) birth weight (g)	3235 (574)	3011 (593)	3044 (475)
Mean (SD) gestational age (weeks)	39.8 (1.5)	39.2 (1.3)	39.5 (1.7)
Mean (range) age (weeks)	15.8 (6–38)	20.5 (6–43)	21.8 (9–40)
Range Apgar score			
1 min	7–9	8–9	8–9
5 min	9–10	9	9–10
Male/female	2/3	6/0	3/2
Milk essential fatty acids (wt%)*			
Linoleic (C18:2n-6)	11.9 (6.3–15.7)	16.0	14.5
α -Linolenic (C18:3n-3)	0.8 (0.5–1.2)	1.5	0.4
Arachidonic (C20:4n-6)	0.5 (0.3–0.8)	ND	ND
Docosahexaenoic (C22:6n-3)	0.4 (0.1–0.5)	ND	ND

*Results given as median (range) for 13 mature human breast milks; results for SMA and CGOST are mean values provided by milk manufacturers. ND=not detected or <0.05% of total fatty acids.

membrane bound enzymes.^{11 12} We present an analysis of cerebral cortex phosphatidylcholine, phosphatidylethanolamine, and phosphatidylserine of infants fed human or formula milks in the first months of life and consider how the biochemical findings might relate to neuronal structure and function.

Methods

SUBJECTS, TISSUE SAMPLES, AND DIETS

The subjects investigated were infants who had died of 'cot death' in the Greater Glasgow Health Board area within the first year of life. No neuropiloidosis,^{13–15} mitochondrial,^{16 17} or peroxisomal¹⁸ fatty acid β oxidation defects were evident at necropsy. Retrospective examination of gestational ages, birth weights, and Apgar scores ensured that no infants were included in whom adverse prenatal or perinatal events such as intrauterine growth retardation were evident. Detailed dietary histories were also obtained and cerebral cortex tissues, taken from the parietal lobe, allocated to the group appropriate to the type of milk feed received exclusively by the infant. This resulted in five infants who had been breast fed, six fed SMA (Wyeth), and five fed either Cow and Gate Premium (Cow and Gate) or Farley's Osterfeed (Crookes Healthcare) formulas. The Cow and Gate and Osterfeed groups were combined as a designated CGOST group because of the similarity in fatty acid composition of both milks at the time of the study. The very nature of the population involved precluded specific age matching of individuals in each feeding group. Previous workers have not reported age related variations in cerebral cortex phospholipid fatty acids of term infants, initially breast fed (two months) before receiving non-specified formula milks.¹⁹ However, we sought to ensure mean age comparability between groups, together with near equivalence in age ranges. Cortical tissue from a 10 week old, 30 week gestation infant fed SMA was also examined. To ensure that the breast fed infants were not receiving milks with a fatty acid composition unique to the catchment area of the study we subsequently analysed 13 random mature human milk samples and compared the results with published data.^{20 21}

LIPID EXTRACTION AND FATTY ACID ANALYSES
Phospholipids were isolated from the parietal cortex as described.² These were separated by two dimensional silica gel thin layer chromatography (Silica Gel G, Merck) initially in chloroform:methanol:water (65:25:4 v/v/v) followed by development in n-butanol:acetic acid:water (60:20:20 v/v/v). On a second plate the phospholipid standards (10 mg/ml in chloroform:methanol, 95:5 v/v), L- α -phosphatidylcholine, dipalmitoyl; L- α -phosphatidylethanolamine, dipalmitoyl; L- α -phosphatidyl-L-serine (bovine brain); L- α -phosphatidylinositol; sphingomyelin (bovine brain); tripalmitin; cholesteryl stearate (all purchased from Sigma Chemical Co) were separated by the same procedure. After drying, the plates, sprayed with 2',7'-dichlorofluorescein (1 mg/ml methanol), were viewed under ultraviolet light and the lipids in the specimen extracts identified by comparison with the standards. The areas of silica corresponding to the phospholipids phosphatidylcholine, phosphatidylethanolamine, and phosphatidylserine were scraped off the plate and added directly to the derivatisation reagent (2 ml, boron trifluoride:methanol, 14% w/v). The fatty acid methyl esters (FAME) formed were separated by gas chromatography.² Identification of the fatty acids extracted from the human milk samples was achieved using a method previously employed in analysing subcutaneous fat triglycerides.²²

DATA AND STATISTICAL ANALYSIS

The composition of each cerebral cortex phospholipid FAME was expressed as a weight percentage of the total fatty acids identified. For each phospholipid only the eight most abundant fatty acids were considered (the results being rounded to 100%), leading to the exclusion of fatty acids contributing <0.6% to the total. The percentage content of essential fatty acids and their metabolites, arachidonic acid and DHA in breast milk were given without exclusion of fatty acids contributing <0.6% to the total.

All group results are presented for each phospholipid as means and standard deviations with statistical significance calculated by the two tailed, Student's *t* test with eight or nine degrees of freedom. It was apparent that the distribution of the CGOST docosapentaenoic acid (DPA; C22:5n-6) results in phosphatidylserine was skewed and these data

Table 2 Fatty acid profile in cerebral cortex phosphatidylcholine related to infant diet

Fatty acid*	Diet		
	Breast milk	SMA	CGOST
C14:0	1.9 (0.4)	2.0 (0.5)	1.8 (0.7)
C16:0	53.8 (1.8)	54.4 (1.8)	53.3 (2.8)
C16:1n-7	4.3 (1.3)	4.4 (0.4)	4.4 (1.3)
C18:0	9.4 (1.0)	9.7 (1.2)	9.9 (1.6)
C18:1n-9	25.8 (0.7)	24.5 (1.3)	24.4 (1.8)
C18:2n-6	0.7 (0.2)	0.9 (0.1)	1.0 (0.2)
C20:3n-6	0.9 (0.2)	1.0 (0.2)	1.1 (0.1)
C20:4n-6	3.2 (0.7)	3.3 (1.4)	3.9 (0.6)
Total (n-6)	4.7 (0.8)	5.1 (1.7)	5.9 (0.8)

*Mean (SD) weight percentage of total fatty acids present.

Table 3 Fatty acid profile in cerebral cortex phosphatidylethanolamine related to infant diet

Fatty acid*	Diet		
	Breast milk	SMA	CGOST
C16:0	8.4 (2.1)	6.4 (0.9)	7.6 (1.0)
C18:0	30.8 (2.6)	31.4 (1.6)	28.8 (1.7)
C18:1n-9	8.8 (1.5)	9.2 (0.8)	9.2 (1.0)
C20:3n-6	1.5 (0.1)	2.0 (0.4)¶	1.9 (0.4)†
C20:4n-6	17.6 (0.7)	20.1 (0.8)‡	19.6 (1.3)¶
C22:4n-6	12.0 (0.8)	12.6 (1.5)	14.3 (1.0)§
C22:5n-6	3.2 (0.6)	4.8 (0.7)§	7.0 (1.9)§
C22:6n-3	17.7 (1.3)	13.4 (1.2)‡	11.6 (1.6)‡
Total (n-6)	34.3 (1.7)	39.6 (2.3)§	42.9 (2.6)‡
Total (n-6+n-3)	52.0 (2.1)	53.0 (2.1)	54.5 (1.9)

*Mean (SD) weight percentage of total fatty acids present.
†p<0.05; ‡p<0.001; §p<0.01; ¶p<0.02 all calculated by Student's *t* test.

were therefore also analysed by the non-parametric Mann-Whitney U test. A similar tendency was noted in the breast milk results which are given as median and ranges.

Results

The infant characteristics and milk essential fatty acid compositions are illustrated in table 1. The essential fatty acid content of human milk from our local population of mothers was very similar to those reported by other workers.^{20,21} No significant differences were found between feeding groups in the major cerebral cortical phosphatidylcholine fatty acids (table 2). Analysis of the phosphatidylethanolamine and phosphatidylserine fractions also revealed no significant differences between groups in the content of saturated and monounsaturated fatty acids (tables 3 and 4). DHA was greater in the phosphatidylethanolamine of the breast fed group than both formula fed groups ($p<0.001$) and the distribution of results can be seen in fig 1. Formula fed infants 'compensated' by increased incorporation of all the major n-6 series PUFAs (table 3), such that in all feeding groups PUFAs accounted for over 50% of total phosphatidylethanolamine fatty acids. In phosphatidylethanolamine the only significant difference between the formula fed infants was a greater DPA in the CGOST group ($p<0.05$) as shown in fig 2. The phosphatidylserine fraction represents about 15% of total cerebral cortex phospholipids in the first year of life and the breast fed group has a greater DHA con-

Table 4 Fatty acid profile in cerebral cortex phosphatidylserine related to infant diet

Fatty acid*	Diet		
	Breast milk	SMA	CGOST
C16:0	2.7 (1.1)	2.2 (0.9)	2.8 (1.3)
C18:0	44.6 (1.7)	44.6 (1.5)	43.7 (0.9)
C18:1n-9	6.3 (2.6)	6.8 (0.8)	8.9 (2.0)
C20:3n-6	1.7 (0.3)	2.0 (0.4)	2.3 (0.5)†
C20:4n-6	7.9 (1.6)	9.0 (1.4)	9.0 (1.1)
C22:4n-6	7.9 (1.1)	8.5 (0.9)	9.3 (1.2)
C22:5n-6	5.3 (1.5)	7.7 (0.9)¶	10.4 (3.1)¶
C22:6n-3	23.5 (1.3)	19.3 (1.8)§	14.4 (1.5)‡
Total (n-6)	22.8 (2.9)	27.1 (1.9)¶	31.0 (3.5)§
Total (n-6+n-3)	46.4 (2.8)	46.5 (0.9)	45.4 (3.1)

*Mean (SD) weight percentage of total fatty acids present.
†p<0.05; ‡p<0.001; §p<0.01; ¶p<0.02 all calculated by Student's *t* test.

Figure 1 Distribution of cerebral cortex phosphatidylethanolamine DHA (C22:6n-3) in relation to infants' diet and age.

tent than both the SMA ($p<0.01$) and CGOST ($p<0.001$) groups (table 4). The individual, age related DHA concentrations are shown in fig 3. A 'compensatory' effect was again evident with increased insertion of DPA into the phosphatidylserine fraction of both formula fed groups (fig 4), such that total PUFA content was similar in each group (table 4). The one significant difference between the formula fed infants was a greater DHA in the SMA group than in the CGOST group ($p<0.001$). The lowest DHA content encountered in the phosphatidylethanolamine fraction (7.6%) was from the 10 week old preterm, SMA fed infant (fig 1). In the phosphatidylserine the DHA at 12.9% (fig 3) was as for the phosphatidylethanolamine about 6% lower than that found in the term SMA fed individuals. In this preterm infant overall total PUFA concentrations were, however, maintained at about 50% in each phospholipid by the incorporation of n-9 series Mead acid (C20:3) and dihomio Mead acid (C22:3) in the phosphatidylserine (1.1% and 1.6%) and phosphatidylethanolamine fractions (2.5% and 1.7%) respectively. Application of non-parametric, two tailed Mann-Whitney U test statistics to the phosphatidylserine DPA concentrations confirmed that both the formula fed groups, SMA ($p<0.05$) and CGOST ($p<0.02$), contained higher percentage contents than the breast fed group.

Figure 2 Distribution of cerebral cortex phosphatidylethanolamine DPA (C22:5n-6) in relation to infants' diet and age.

Figure 3 Distribution of cerebral cortex phosphatidylserine DHA (C22:6n-3) in relation to infants' diet and age.

Discussion

All membranes whether they are cell surface or form part of an intracellular organelle such as mitochondrion or peroxisome are composed of phospholipid bilayers. Phospholipids are amphiphilic (like both water and lipid) because they have chemical groupings at one end which are hydrophilic (like water) and at the other end hydrophobic (dislike water).¹ This property allows these molecules to permit interaction between a wide range of water soluble and fat soluble substances while limiting movement of water and other substances between the outside and inside of the membrane. Membrane lipid provides a flexible and adaptable structure into which are inserted proteins and glycoproteins such as enzymes, transmembrane transporter proteins or receptors. The main membrane phospholipids contain two fatty acids and a substituted (amino) alcohol attached to a glycerol phosphate backbone which is hydrophilic. The nature of the alcohol head group and the attached fatty acids have major effects on the biophysical function of that membrane.²³ Saturated fatty acids and those containing *trans* double bonds tend to adopt a straighter and more rigid configuration. Ethylenic double bonds in monounsaturated and polyunsaturated fatty acids provide a site of chemical reactivity. Thus membranes with phospholipids containing higher concentrations of *cis* double

Figure 4 Distribution of cerebral cortex phosphatidylserine DPA (C22:5n-6) in relation to infants' diet and age.

bonds are more flexible and more permeable. It is the presence of high concentrations of DHA on phosphatidylethanolamine and phosphatidylserine, particularly at the inner aspect of the phospholipid bilayer that allows rapid and repeated complex biochemical activities to take place at the neuronal synaptosomes.^{10 23 24} DHA has a highly specific distribution and is the predominant membrane fatty acid of synaptosomes, retinal photoreceptors, mitochondria, and spermatozoa. Membrane thickness, elasticity, porosity, and ability to support or transmit other molecules also depends on the organic bases such as choline, ethanolamine, serine, and inositol which are attached to the phosphoglycerides.²³ The control mechanism that determines the siting and type of phospholipid in the lipid bilayers of membranes is unknown. Whereas protein synthesis will cease if essential amino acid supplies are deficient, incorporation of fatty acids into membrane phospholipids will proceed and it appears from the data presented that the next nearest available fatty acid is substituted thus altering the properties of that membrane. Stability of mammalian membranes is crucially dependent upon the presence of long chain PUFAs.²⁵ In severe fatty acid deficiency states PUFAs of the n-3 and n-6 series are replaced by PUFAs of the n-9 family.²⁶ The membrane becomes metabolically unstable and more permeable to water. Where there is a lesser deficiency of n-3 series, then n-6 fatty acids alone may substitute for them.

The reductions in the DHA content of phosphatidylserine and phosphatidylethanolamine between the breast and artificially fed infants are of a magnitude which, *in vitro*, would be sufficient to alter membrane function and could therefore critically affect the resultant responses to electrical and chemical stimulation and alter membrane and neurotransmitter function. It can be seen from figs 2 and 4 that there is a preferential replacement of DHA in the phospholipid membrane by DPA. This selective substitution, although reported in animal studies,²⁷⁻²⁹ has not hitherto been identified in man. It appears that it is only from about the fourth month of life (figs 2 and 4) that DHA is specifically replaced by DPA and this is particularly prominent in the CGOST group. Synthesis of both DHA and DPA from the parent essential fatty acids, α -linolenic and linoleic respectively, are probably ultimately dependent on a peroxisomal β -oxidation reaction (' $\Delta 4$ -desaturase'),³⁰ which may not function in early infancy. This may be the major reason why DHA is initially replaced by less unsaturated n-6 series PUFAs (tables 3 and 4).

It appears that the DHA content of phosphatidylserine in cortical neuronal membranes is 'safeguarded' and preferentially incorporated when compared with the degree of substitution of DHA with DPA seen in phosphatidylethanolamine. The subcutaneous fat reserves of the parent essential fatty acid α -linolenic acid are negligible at birth and those reserves of DHA which are present are rapidly exhausted.²² From the data presented

in tables 3 and 4 it can be seen that the DHA concentrations in the phosphatidylserine of the SMA group are nearer to those of the breast fed infants, although still significantly lower (table 4). This increased DHA must have been synthesised from dietary α -linolenic acid and preferentially distributed to phosphatidylserine as no significant differences in DHA were found between the formula fed groups in the phosphatidylethanolamine fraction. From fig 3 it can be seen that there is one breast fed infant with the highest DHA content (25.8%). This infant had a very high concentration of α -linolenic acid (0.9%) in subcutaneous triglyceride.²² It appears that optimal synthesis of DHA does not occur until an α -linolenic substrate concentration in excess of 1% of total fatty acids is present in the diet, although this value was found in only 30% of breast milks analysed. With human milk, however, DHA is provided directly from the milk feed so that the need for synthesis of DHA from α -linolenic acid is not so critical in the breast fed infant. Preformed long chain PUFAs are incorporated into the developing rat brain with a greater than tenfold efficiency when compared with those synthesised from the parent essential fatty acids.³¹ The brain preferentially uses the preformed long chain fatty acids and not those synthesised from the parent essential fatty acids. It is important to note that there are major differences in the DHA content of phosphatidylethanolamine and phosphatidylserine between breast and artificially fed infants even at 40 weeks after birth in term infants.

The finding of significant quantities of Mead acid and dihomio Mead acid in the phosphatidylserine and phosphatidylethanolamine fractions of the infant born preterm is very worrying. These fatty acids are very unstable and are most unlikely to allow the membranes to function normally.

There is clustering of phosphatidylserine, phosphatidylethanolamine, and phosphatidylcholine head groups into membrane areas known as domains. The phosphatidylserine and phosphatidylethanolamine domains are critically important for neurotransmitter function. It has long been recognised that there are structural similarities between phosphoserine, which is the hydrophilic moiety of phosphatidylserine, and the major central nervous system activatory neurotransmitter glutamate (or aspartate) and the polar phosphoethanolamine of phosphatidylethanolamine, which resembles the inhibitory amino acid neurotransmitter gamma amino-butyric acid (GABA). The phospholipids phosphatidylethanolamine and phosphatidylserine are known to exchange polar head groups directly in vivo and may also be interconverted in a simple irreversible decarboxylation reaction similar to that which neutralises the effect of glutamate by its conversion to GABA.³² Although we have no direct evidence that phosphatidylserine functions in tandem with glutamate it is known that L-AP4 (L-2-amino-4-phosphonobutanoic acid), a phosphorus containing molecule structurally similar to phosphoserine is a

proved L-glutamate agonist at presynaptic terminals and retinal bipolar receptors.³³⁻³⁵ It may be that phosphatidylserine and phosphatidylethanolamine have a neurotransmitter function that could be independent or work in parallel with the glutamate-GABA system.

We have, therefore, demonstrated major differences in the fatty acid composition of phosphatidylethanolamine and phosphatidylserine in the brains of artificially fed infants compared with those who have been breast fed. What if anything is the clinical consequence of these findings? Lucas and his colleagues demonstrated that preterm infants fed human milk have a higher developmental status at 18 months and higher intelligence quotient in later childhood than those fed infant formulas.³⁶⁻³⁷ Preterm infants fed standard formulas have significantly different electroretinographic patterns indicating a delayed rod photoreceptor maturation compared with those fed human milk or given supplementary DHA. Visual cortical functions as measured by pattern reversal, visually evoked potential, and forced choice preferential looking visual acuity response are also better in breast fed or fish oil supplemented preterm infants.³⁸ In a study of experimental infant formulas providing 0.2% DHA, Carlson *et al* have shown that DHA supplemented preterm infants have greater red blood cell phosphatidylethanolamine DHA content and better visual acuity than standard formula fed preterm infants.³⁹ DHA supplemented infants performed significantly better than controls on the Bayley mental scales. It has also been shown that term infants' visual responses at five months after birth correlate with erythrocyte DHA concentrations⁴⁰ and that human milk fed infants have better stereoacuity and better matching ability at 3 years of age than do formula fed children.⁴¹ The British Paediatric Association Standing Committee on Nutrition has recently reviewed the differences in cognitive function between breast and bottle fed infants.⁴² In spite of the difficulties of interpretation of many confounding variables in the published evidence their conclusion was that there is a factor in breast milk that if fed for four or more months is likely to be responsible for the greater cognitive function in breast fed infants. They further suggest that DHA might be that factor. The evidence we present suggests possible mechanisms for altered neurotransmission in DHA deficient brains of formula fed infants. Infants have no reserve pool of DHA and there is limited ability to synthesise DHA from the essential fatty acid precursor α -linolenic acid, therefore DHA should be considered an essential fatty acid for at least the first four months of life in term infants and possibly longer in preterm infants. There must now be little doubt that there is a 'short term' effect of DHA deficiency on efficiency of synaptic transmission during a critical period of brain development and learning. There is so much 'redundancy' and 'reserve' in the efficiency of central nervous function that the biochemical differences demonstrated in the brains of these infants appears to convey only a

'marginal' handicap on artificially fed infants. However, these short term effects and indeed the long term effects on neuronal integrity that might predispose to adult neurodegenerative disease⁴³⁻⁴⁶ warrant urgent attention.

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