Effect of linoleic acid-rich infant formula feeding on brain synaptosomal lipid accretion and enzyme thermotropic behavior in the piglet

Nina Hrboticky,* Murray J. MacKinnon,† Martin L. Puterman,† and Sheila M. Innis[1,*]

Department of Pediatrics,* Faculty of Medicine, University of British Columbia, Vancouver, and Statistical Consulting Service,† The Children's Hospital, Vancouver, British Columbia, Canada

Abstract The effects of a vegetable oil-based infant formula, virtually devoid of n-6 and n-3 long chain polyenoid fatty acids (LCP) and high in 18:2(n-6) and 18:2(n-6)/18:3(n-3) ratio, on brain synaptosome lipid composition and enzyme thermotropic behavior were studied in neonatal piglets. Term gestation piglets were fed either sow milk (SMF) or formula (FF) from birth for 5, 10, 15, or 25 days. Synaptosomal cholesterol, total lipid phosphorus, and phospholipid class composition did not differ between SMF and FF piglets. Synaptosomal fatty acid composition, however, was influenced by diet. The proportion of n-3 LCP, especially 22:6(n-3), was decreased, while the n-6 LCP, especially 22:4(n-6) and 22:5(n-6), were increased in FF compared to SMF piglets. These diet-related changes were most pronounced in the ethanolamine glycerophospholipid fraction and increased with the duration of feeding. FF thus reversed an apparent developmental increase in the synaptosomal n-3/n-6 LCP ratio. The monoene content, especially 18:1, was also reduced in the synaptosomes of FF compared to SMF pigs. FF had no effect on the activity of synaptosomal acetylcholinesterase. However, higher transition temperatures for this enzyme, indicating decreased membrane fluidity, were found in the FF compared to SMF piglets. The data suggest that exclusive feeding of proprietary formulae, devoid of LCP and high in 18:2(n-6) and/or the 18:2(n-6)/18:3(n-3) ratio, may compromise normal fatty acid accretion and physical properties of brain synaptosomal membranes.—**Hrboticky, N., M. J. MacKinnon, M. L. Puterman, and S. M. Innis.** Effect of linoleic acid-rich infant formula feeding on brain synaptosomal lipid accretion and enzyme thermotropic behavior in the piglet. *J. Lipid Res.* 1989. **30:** 1173-1184.

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Clinical evidence of differences in erythrocyte phospholipid fatty acid composition between formula and breast-fed term (1) and pre-term (2) infants has raised concern over the extent to which the fatty acid composition of infant formula may perturb normal fatty acid accretion in structural lipids of biomembranes. Currently available infant formulae vary widely in their fatty acid composition. Unlike human milk, all are almost completely devoid of long chain (20 and 22 carbon) n-3 and n-6 polyunsaturated fatty acids (LCP), the in vivo biosynthesis of which may be limiting in early neonatal life (3). Further, formulae based on corn or safflower oil blends may contain 18:2 (n-6)/18:3(n-3) ratios similar to diets that produce depletion of n-3 LCP in the brain and its subcellular structures of rodents (4-13). A correspondence in the response of the erythrocyte and brain pospholipid fatty acid composition tion to diet fat has been demonstrated (5). Thus, formula feeding may alter normal fatty acid accretion in cerebral membranes, which in the human occurs predominantly during late fetal and early neonatal life (3,14).

Brain synaptosomal membranes are especially enriched in LCP. Numerous studies have shown that membrane lipid constituents are important in the development and regulation of synaptic functions. These include the activity and thermodynamic properties of membrane-associated enzymes (15-19), the regulation of neurotransmitter and drug receptor binding (20), and the initiation, consolidation, and assembly of synaptic contacts during synaptogenesis (21). In turn, development and learning have been related to changes in synaptic density and morphology (22). Furthermore, learning behavior is altered in rodents fed diets that deplete brain n-3 LCP levels (7,13).

The perinatal brain growth velocity (23) and fatty acid accretion (24) of the pig and human appear similar. Results presented in this report also demonstrate that sow milk resembles human milk in its LCP, 18:2(n-6), and 18:3(n-3) content. The piglet thus appears suitable for study of effects of early lipid nutrition on developmental

Abbreviations: EPG, ethanolamine glycerophospholipid; SPH, sphingomyelin; PC, phosphatidylcholine; PI, phosphatidylinositol; PS, phosphatidylserine; TLC, thin-layer chromatography; LCP, long chain polyenoic fatty acids; TT, transition temperature; SMF, sow milk-fed; FF, formula-fed; PigIG, pig immunoglobulin; UI, unsaturation index.

[1]Address reprint requests to: S. M. Innis, Ph.D., Faculty of Medicine, Department of Paediatrics, The Research Centre, 950 W. 28th Avenue, Rm. 179, Vancouver, B. C., Canada, V5Z 4H4.

brain fatty acid accretion as it pertains to the human. This study compared developmental changes in brain synaptosomal membrane lipid composition of neonatal piglets fed a high linoleic acid, corn-coconut oil infant formula to piglets raised on sow milk. Associated diet effects on membrane fluidity and function were investigated through the assay of activity and transition temperature (TT) of synaptosomal acetylcholinesterase (EC3.1.1.7).

MATERIALS AND METHODS

Animals

Male Yorkshire piglets of normal term gestation (116-118 days) were obtained from the University of British Columbia, Animal Science and from Pitt Ineffable Growers Ltd., Pitt Meadows, B.C. Sow milk-fed (SMF) piglets were kept on the farm in unculled litters and were suckled by their natural mothers. The sows were fed a typical 16% protein lactation feed (Ritchie-Smith Inc., Abbotsford, B.C.). Piglets were taken at random from different litters at 5, 10, 15, or 25 days post partum, or at birth prior to suckling. All animals were killed between 0900 and 1000 h, and within 1.5 to 2 h of removal from the sow.

All animals designated for formula feeding (FF) were taken from the sow immediately after birth, prior to receiving colostrum. Three to four animals from the same litter were transported in sterilized cages and reared in a germ-free animal containment unit. For the first 48 h, the piglets were housed in a single cage, and hand-fed every 3 h. Subsequently, they were housed individually and fed every 3 h from 0700 to 2400 h from feeders attached to each cage. Heating was provided with spot heat lamps secured above each cage. Animals were killed on days 5, 10, 15, or 25 post partum, at 0900 to 1000 h and within 1.5 to 2 h of the last feed. Only one piglet per litter was studied at each age.

Diets

SMF piglets were raised exclusively on sow milk throughout the suckling period, with the addition of 5 to 10 g of solid prestarter feed (22% dried milk protein, Ritchie-Smith Inc.) from days 21 to 25 post partum. This practice, common to pig rearing, facilitates the transition to adult pig feed. FF piglets were raised exclusively on a corn-coconut oil infant formula, modified to resemble sow milk in its macronutrient density (**Table 1**), and to meet NRC nutrient requirements for the growing piglet (27). The nutrient compositions of the formula and sow milk differed essentially in their lipid components (Table 1). The amount of formula fed was adjusted daily to achieve an average growth of 200 to 250 g/day, equivalent to that of sow milk-fed piglets. Passive immunity was provided to the colostrum-deprived FF piglets by supplementation with pig serum-derived immunoglobulin (pigIG) during the first 5 days of life (28). On day 1, pigIG was fed at 37 mg/ml formula, and provided an additional 0.065 mg total fatty acids, containing 0.013% and 0.003% n-6 and n-3 LCPs, respectively. From day 2 to 5, pigIG supplementation was reduced to 7.4 mg/ml formula per day, ie., one-fifth of the first day dose. Both FF and SMF animals received 100 mg of an iron-dextran complex (Pigtran 200, Tuco Products Co., Orangeville, Ont.) intramuscularly on day 3 post partum. No health problems were experienced by any of the piglets.

TABLE 1. Macronutrient and fatty acid composition of sow milk and formula

Composition	Sow Milk[a]	Formula[b]
Protein (g/l)	55	54.5
Fat (g/l)	50-60	60.1
Lactose (g/l)	58	72
Energy (kcal/l)	1000	1050
Fatty acids (% total wt)		
8:0	n.d.	3.7
10:0	0.1 ± 0.4	2.9
12:0	0.4 ± 0.1	22.9
14:0	3.1 ± 0.4	9.4
16:0	27.6 ± 3.0	10.4
16:1	9.2 ± 1.0	n.d.
18:0	5.5 ± 0.2	2.8
18:1	32.0 ± 3.2	17.3
18:2(n-6)	13.0 ± 1.1	29.4
18:3(n-3)	0.6 ± 0.1	0.8
20:0	0.2 ± 0.1	0.4
n-6 LCP	2.3 ± 0.1	n.d.
n-3 LCP	0.7 ± 0.0	n.d.
18:2(n-6)/18:3(n-3)	24.5 ± 4.5	36.8
P/S ratio	0.2 ± 0.0	0.4

Sow milk lipid was extracted with chloroform-methanol. Total lipid fatty acids were transmethylated with methanolic HCl (5:1, v/v, 100°C, 60 min) and analyzed by GLC. Means ± SEM of four milk samples, collected between 8 and 28 days of lactation, are given; n.d., not detectable.

[a]Macronutrient values for sow milk are average values (25, 26).

[b]Macronutrient and fatty acid values for formula were obtained from Ross Laboratories (Columbus, Ohio).

Experimental procedures

Tissue isolation and acetylcholinesterase assay. Animals were killed by cardiac injection of 10 ml KCl (20 mEq/10 ml) and decapitated. The heads were immediately put on ice and transferred to a cold room (4°C) for further processing. Whole brains were removed within 15 min of death, weighed, and minced. The minced tissue was homogenized in 5 vol/wt tissue of 0.32 M sucrose-Tris-HCl buffer (15 mM Tris-CHl, 1 mM EDTA, 1 mM MgCl, 1.5 mM glutathione, pH 7.4), in a glass Dounce hand-homogenizer, with five strokes of the loose and eight strokes of the tight pestle. A vol equivalent to 30 g initial brain tissue was used for isolation of synaptosomal plasma membrane, following the method of Gurd et al. (29), where membrane purity has been characterized. Harvested synaptosomal

membrane was resuspended in 5 ml of the above Tris-HCl buffer without sucrose and acetylcholinesterase was assayed in a 1-ml aliquot after overnight storage at 4°C. The remaining synaptosomal membrane was immediately frozen and stored at −80°C for subsequent lipid analysis.

Synaptosomal acetylcholinesterase activity was measured using the colorimetric method of Ellman et al. (30), with the modifications of Whittaker (31), and using ethopropazine (10^{-4} M, Sigma Chemical Co., St. Louis, MO) as a cholinesterase inhibitor. Activity was determined over a temperature range of 8° to 37°C, in a SP8-400 Pye Unicam spectrophotometer equipped with a circulating water-bath cuvette chamber. Reaction rates at each temperature were measured for 5 min, in duplicate, and the actual temperature (± 0.2°C) was determined with a digital thermoprobe. Synaptosomal protein content was assayed using the method of Lowry et al. (32) with bovine serum albumin as standard.

Lipid and fatty acid composition analysis. Synaptosomal membrane preparations were thawed on ice, and total lipids were extracted (33). The crude lipid was redissolved in chloroform–methanol 2:1 (v/v), and aliquots were taken for determination of whole membrane cholesterol, lipid phosphorus, and total lipid fatty acid composition. Cholesterol was measured enzymatically (kit #225-26, Diagnostic Chemicals Ltd., Charlottetown, P.E.I.) following reconstitution in isopropyl alcohol. Total lipid inorganic phosphorus was assayed after digestion with 72% perchloric acid (34). Synaptosomal total fatty acids were transmethylated in 1 ml methanolic–HCl (5:1, v/v, 100°C, 90 min) and the methyl esters were partitioned twice with 3 ml 0.9% saline and 4 ml pentane. The pooled pentane layers were dried under N_2 and stored at −80°C.

Phospholipids were resolved into individual classes by chromatography on silica gel 60 HPTLC plates (2 mm, 10 × 20 cm, Merck, Darmstadt, West Germany), using a one-dimensional solvent system (35). The bands were visualized with 2', 7'-dichlorofluorescein (Supelco Inc., Bellefonte, PA), scraped, and eluted from the silica with chloroform–methanol 2:1 (v/v). Fatty acid methyl esters were prepared with 1 ml 14% boron trifluoride–methanol (BDH, Canada). Phosphatidylcholine (PC), phosphatidylinositol (PI), and phosphatidylserine (PS) were refluxed at 100°C, 10 min. Ethanolamine glycerophospholipid (EPG), containing both phospholipid and plasmalogen, was refluxed at 100°C, 30 min (36). Sphingomyelin (SPH) was transmethylated in 1 ml methanolic–HCl (5:1, v/v) at 100°C, overnight. Methyl esters were recovered by pentane and saline partition, as above.

Fatty acid methyl esters were separated and quantified by gas–liquid chromatography (GLC), using a dual column Varian 6000 GLC equipped with flame ionization detection and a Varian Vista 402 data system (Varian Canada Ltd., Georgetown, Ontario, Canada). Separation was achieved on 30 m × 0.25 mm I.D. nonbonded, fused silica capillary SP 2330 columns (Supelco Inc.). Helium was the carrier gas at a column flow of 1 ml/min and inlet pressure of 15 psi. The inlet splitter was set at 30 to 1. Samples were injected at 80°C and the oven temperature was programmed to remain at 80°C for 2 min, then increase to 180°C (20°C/min), stabilize for 19.5 min, rise to 200°C (20°C/min), and stabilize for 20 min. The column was then heated to 240°C for 2–5 min prior to subsequent analyses. The injectors and detectors were set at 240° and 260°C, respectively. Fatty acid methyl esters were identified by comparison of retention times with those of authentic standards (Supelco Inc.; Nu-Chek-Prep Inc., Elysian, MN; Laridan Fine Chemicals, Malmo, Sweden) and quantified on the basis of heptadecanoic acid (Sigma Chemical Co.) as internal standard. Total amounts of each phospholipid class were computed as the sum of fatty acids detected in the fraction by GLC and appropriate correction factors.

Statistical analysis

Arrhenius plots of the logarithm of acetylcholinesterase reaction rate versus the reciprocal of the incubation temperature (°K) were constructed. Estimates of TT and the energy of activation (E_a) above and below TT were obtained by application of nonlinear regression to a bent-hyperbolic transition model (37). This procedure, which provides formal tests of the adequacy of a biphasic, rather than a single straight line or curvilinear fit, confirmed the biphasic temperature dependence of acetylcholinesterase (37).

Diet group differences and age effects on synaptosomal membrane lipid composition and enzyme parameters were investigated using two-way analysis of variance (ANOVA), with diet (d) and age (a) as the two factors. A significant interaction between diet and age (d × a) indicated that the effects of diet and age were interdependent. Formal tests of differences between diets at each age level and age levels within each diet were based on least squares means, and standard errors were calculated from ANOVA. Bonferroni corrections within a fatty acid were used to determine which of these differences were significant. All hypothesis tests for differences between means were two-sided. Relationships between selected membrane lipid components and acetylcholinesterase TT were determined by univariate regression. All calculations were performed using SAS Statistical Software PROC GLM (38). Tables 2–7 report means and standard errors computed in the usual manner for the piglets after 5 or 25 days feeding (data for piglets after 10 or 15 feeding not shown). The significance tests reported therein were based on ANOVA as described above. Data on synaptosomal composition and enzyme parameters of one newborn piglet were collected for reference and are also provided, but not used in any of the above statistical analyses.

RESULTS

Synaptosomal membrane cholesterol and phospholipid content

Total cholesterol and lipid phosphorus, cholesterol to PL molar ratio, and PL class distribution were similar in SMF and FF piglets at all ages studied, and did not change with increasing age (**Table 2**). EPG constituted the largest PL class (ca. 42% of PL recovered). PC contributed ca. 30% to the total, PS ca. 14%, while SPH and PI were minor PL components, at 10% and 7%, respectively.

Synaptosomal membrane total fatty acids

The synaptosomal membrane fatty acid compositions of SMF and FF piglets at 5 and 25 days of age are given in **Table 3**. *P* values of the overall effect of diet, age, and d × a interactions obtained from the ANOVA of all piglets, as well as significant mean comparisons of SMF and FF piglets at each age, are provided. Developmental, diet-independent changes, represented by a significant *p* value for the effect of age without a d × a interaction, were small and included a decrease in 14:0 and an increase in 18:0. In contrast, the type of feeding had a substantial effect on the membrane fatty acid composition. Diet-related, age-independent changes (represented by a significant *P* value for the effect of diet without a d × a interaction), were a decrease in 16:1, 18:1 and 22:5(n-3), and an increase in 14:0, 18:2(n-6), 20:4(n-6), 22:4(n-6), and mean chain length (MChL) in FF compared to SMF piglets. In addition, significant d × a interactions obtained for 20:3(n-6) and 22:5(n-6) indicated an age-dependent increase, and for 22:6(n-3) a decrease, in FF compared to SMF piglets.

EPG fatty acid composition

EPG constituted the most highly unsaturated PL class, with a mean unsaturation index (UI) of 280 (**Table 4**). Diet-independent, age-specific changes were an increase in 16:0 and 16:1 and a decrease in the UI. As in the whole membrane lipid, an age-independent reduction in 18:1 and 22:5(n-3) occurred in FF compared to SMF pigs. Significant d × a interactions, obtained for 22:4(n-6), 22:5(n-6), and 22:6(n-3), again indicated that the elevation of n-6 LCP and the reduction in 22:6(n-3) in FF versus SMF piglets were age-dependent. Thus, the magnitude of the compositional differences between the two diet groups increased from day 5 to 25 post partum.

PC fatty acid composition

Synaptosomal PC was the least unsaturated PL class, with a mean UI of 75 (**Table 5**). An age-specific, diet-independent decrease in 16:0 and increase in 18:0 occurred in this phospholipid. A diet-specific increase was found in 14:0, 18:2(n-6), 22:5(n-6), 18:3(n-3), and 22:5(n-3) in FF versus SMF piglets. On the other hand, a decrease in 16:0, 16:1, and 18:1 occurred in the PC of FF compared to SMF animals.

PS fatty acid composition

The synaptosomal PS fraction had a mean UI of 180 and contained levels of n-3 LCP comparable to EPG (**Table 6**). The level of 18:1 and 20:3(n-6) increased with age independently of the diet fed. A diet-associated decrease in 16:0 and 18:1, along with an increase in 22:5 (n-6) and the MChL length, occurred in FF versus SMF piglets. PS 20:4 (n-6) also appeared to be elevated in FF compared to SMF piglets. The value for this fatty acid, however, may contain variable amounts of 22:0, which

TABLE 2. Synaptosomal membrane cholesterol and phospholipid content

Content	Newborn (n = 1)	5 Days SMF (n = 4)	5 Days FF (n = 4)	25 Days SMF (n = 5)	25 Days FF (n = 4)	*P* Values Diet	*P* Values Age	*P* Values d × a
Cholesterol (μmol/mg protein)	0.98	1.14 (0.05)	1.04 (0.04)	1.00 (0.06)	1.02 (0.06)	0.22	0.24	0.59
Phospholipid (μmol/mg protein)	1.51	1.62 (0.05)	1.54 (0.04)	1.62 (0.08)[b]	1.62 (0.08)	0.22	0.44	0.79
EPG (μmol/mg protein)[a]	0.38	0.41 (0.02)	0.46 (0.02)	0.45 (0.03)	0.43 (0.03)	0.67	0.13	0.41
PC (μmol/mg protein)	0.34	0.31 (0.01)[c]	0.30 (0.01)	0.29 (0.01)	0.30 (0.01)	0.37	0.83	0.22
PS (μmol/mg protein)	0.15	0.13 (0.01)[c]	0.15 (0.01)	0.15 (0.01)[c]	0.14 (0.01)	0.29	0.92	0.47
SPH (μmol/mg protein)	0.084	0.07 (0.00)[c]	0.10 (0.01)	0.11 (0.01)	0.101 (0.00)	0.39	0.35	0.18
PI (μmol/mg protein)	0.090	0.065 (0.006)[c]	0.068 (0.003)	0.063 (0.005)[c]	0.074 (0.003)	0.90	0.64	0.31
Ratio phospholipid/cholesterol	0.65	0.70 (0.03)[c]	0.67 (0.01)	0.64 (0.03)[b]	0.63 (0.01)	0.70	0.16	0.82

Synaptosomal total lipid was extracted with chloroform-methanol. Cholesterol was measured with a commercial enzymatic kit. Total lipid phosphorus was determined following perchloric acid digestion. Phospholipids were separated by TLC, and quantified on the basis of total fatty acid methyl ester by GLC. Data are means (± SEM). Piglets were fed sow milk (SMF) and formula (FF) from birth for 5, 10, or 15 (data not shown) or 25 days. *P* values for the effect of diet, age and diet × age (d × a) interaction, derived from ANOVA, are provided.

[a] Sum of phosphatidylethanolamine and ethanolamine plasmalogen.

[b] n = 4.

[c] n = 3.

TABLE 3. Synaptosomal total lipid fatty acid composition

Fatty Acid	Newborn (n = 1)	5 Days SMF (n = 4)	5 Days FF (n = 4)	25 Days SMF (n = 4)	25 Days FF (n = 4)	*P* Values Diet	*P* Values Age	*P* Values d × a
				% of total fatty acids				
Saturated								
14:0	0.7	0.5 (0.07)	0.8 (0.15)	0.4 (0.02)	0.5 (0.04)	0.01*	0.03*	0.52
16:0	24.5	23.1 (1.34)	22.0 (0.67)	23.3 (0.67)	22.4 (0.69)	0.09	0.96	0.99
18:0	24.1	24.3 (0.49)	24.1 (0.15)	25.7 (0.19)	25.4 (0.18)	0.93	0.001*	0.45
Monoene								
16:1[a]	2.2	1.7 (0.11)	1.6 (0.08)	1.5 (0.06)	1.3 (0.05)	0.01*	0.46	0.43
18:1[a]	14.0	14.1 (0.31)	12.9 (0.22)**	14.5 (0.19)	13.6 (0.09)**	0.0001*	0.13	0.29
n-6 Series								
18:2	0.3	0.9 (0.11)	0.9 (0.07)	0.7 (0.03)	1.1 (0.13)**	0.0003*	0.70	0.08
20:3	0.2	0.5 (0.02)	0.5 (0.03)	0.5 (0.03)	0.64 (0.05)**	0.0001*	0.01*	0.02*
20:4	9.9	9.5 (0.67)	10.0 (0.46)	8.9 (0.45)	9.8 (0.09)	0.02*	0.81	0.88
22:4	4.8	5.7 (0.17)	5.9 (0.12)	5.2 (0.22)	6.2 (0.08)**	0.0001*	0.26	0.15
22:5	3.7	3.2 (0.17)	3.7 (0.35)	2.6 (0.14)	5.3 (0.25)**	0.0001*	0.03*	0.0002*
n-3 Series								
18:3	0.4	0.2 (0.11)	0.2 (0.09)	0.1 (0.05)	0.2 (0.12)	0.08	0.77	0.58
20:5	0.2	0.1 (0.02)	0.3 (0.07)	0.0 (0.03)	0.1 (0.07)	0.07	0.19	0.34
22:5	0.4	0.5 (0.05)	0.4 (0.11)	0.6 (0.10)	0.3 (0.06)	0.001*	0.90	0.59
22:6	11.5	12.8 (0.22)	12.4 (0.23)	12.8 (0.14)	9.6 (0.39)	0.0001*	0.001*	0.001*
UI[b]	171	179 (4)	184 (2)	172 (3)	174 (2)	0.10	0.08	0.86
MChL[c]	18.6	18.7 (0.06)	18.7 (0.06)	18.6 (0.04)	18.7 (0.04)	0.04*	0.57	0.81

Piglets were fed sow milk (SMF) or formula (FF) from birth for 5, 10, or 15 (data not shown) or 25 days. Synaptosomal membrane total lipid was extracted with chloroform-methanol. Fatty acids were transmethylated and quantified by GLC. Mean values (± SEM) and *P* values for the effect of diet, age, and diet × age interaction (d × a) are provided: *$P < 0.05$; **FF and SMF values within age group are different by LSM comparison with Bonferroni correction.

[a]Sum of isomers.

[b]UI, Unsaturation index.

[c]MChL, mean chain length.

TABLE 4. Synaptosomal ethanolamine glycerophospholipid fatty acid composition

Fatty Acid	Newborn (n = 1)	5 Days SMF (n = 4)	5 Days FF (n = 4)	25 Days SMF (n = 4)	25 Days FF (n = 4)	*P* Values Diet	*P* Values Age	*P* Values d × a
				% of total fatty acids				
Saturated								
16:0	7.4	7.0 (0.61)	7.2 (0.49)	7.6 (0.17)	7.5 (0.16)	0.86	0.01*	0.52
18:0	23.1	23.7 (0.66)	25.0 (0.59)	24.0 (0.33)	24.0 (0.23)	0.85	0.82	0.31
Monoene								
16:1[a]	0.8	0.8 (0.05)	0.7 (0.04)	0.8 (0.05)	0.7 (0.04)	0.32	0.01*	0.18
18:1[a]	10.6	9.6 (0.75)	8.4 (0.40)	8.6 (0.23)	8.3 (0.22)	0.02*	0.72	0.77
n-6 Series								
18:2	0.4	0.8 (0.24)	0.7 (0.04)	0.8 (0.15)	0.8 (0.04)	0.23	0.63	0.26
20:3	0.3	0.5 (0.03)	0.4 (0.03)	0.5 (0.04)	0.6 (0.01)	0.17*	0.30	0.40
20:4	16.4	16.5 (0.34)	16.5 (0.32)	16.4 (0.43)	17.1 (0.31)	0.38	0.20	0.76
22:4	9.2	10.5 (0.21)	10.7 (0.39)	9.6 (0.15)	11.7 (0.12)**	0.0005*	0.44	0.04*
22:5	4.8	4.2 (0.21)	4.8 (0.41)	3.5 (0.13)	7.8 (0.35)**	0.0001*	0.004*	0.0001
n-3 Series								
18:3	0.3	0.1 (0.03)	0.2 (0.03)	0.2 (0.04)	0.3 (0.04)	0.68	0.37	0.43
20:5	0.2	0.2 (0.04)	0.3 (0.04)	0.2 (0.02)	0.3 (0.02)	0.08	0.08	0.54
22:5	0.3	0.9 (0.17)	0.4 (0.02)	1.1 (0.18)	0.4 (0.03)**	0.0001*	0.72	0.41
22:6	18.7	19.7 (0.32)	20.2 (0.52)	21.7 (1.24)	15.0 (0.98)**	0.0001*	0.06	0.003*
UI[b]	265	275 (2)	277 (3)	280 (4)	270 (3)	0.67	0.03	0.51
MChL[c]	19.4	19.6 (0.07)	19.6 (0.05)	19.6 (0.03)	19.6 (0.06)	0.41	0.20	0.90

Mean values (± SEM) and *P* values for the effect of diet, age, and diet × age (d × a) interaction are provided: *$P < 0.05$; **FF and SMF values within age group are different by LSM comparison with Bonferroni correction.

[a]Sum of isomers.

[b]UI, unsaturation index.

[c]MChL, mean chain length.

TABLE 5. Synaptosomal phosphatidylcholine fatty acid composition

Fatty Acid	Newborn (n = 1)	5 Days SMF (n = 3)	5 Days FF (n = 4)	25 Days SMF (n = 4)	25 Days FF (n = 4)	*P* Values Diet	*P* Values Age	*P* Values d × a
				% of total fatty acids				
Saturated								
14:0	1.6	1.4 (0.05)	1.5 (0.40)	0.8 (0.16)	3.0 (1.03)	0.01*	0.67	0.15
16:0	45.9	49.2 (0.27)	45.2 (0.52)	43.8 (1.28)	43.7 (0.13)	0.005*	0.03*	0.26
18:0	18.8	12.0 (0.27)	13.2 (0.49)	14.4 (0.67)	14.2 (0.81)	0.11	0.05*	0.53
Monoene								
16:1[a]	3.1	3.8 (0.19)	3.2 (0.04)	2.7 (0.05)	2.8 (0.22)	0.05*	0.84	0.17
18:1[a]	19.9	22.2 (0.13)	22.8 (0.39)	24.4 (0.35)	22.1 (0.51)	0.04*	0.29	0.06
n-6 Series								
18:2	0.8	1.4 (0.14)	1.8 (0.04)**	1.3 (0.04)	1.9 (0.10)**	0.0001	0.20	0.61
20:3	0.1	0.3 (0.06)	0.3 (0.01)	0.4 (0.01)	0.3 (0.04)	0.52	0.28	0.76
20:4	3.8	3.8 (0.37)	4.1 (0.04)	3.9 (0.06)	3.9 (0.08)	0.67	0.99	0.81
22:4	0.4	0.8 (0.16)	0.8 (0.13)	1.3 (0.19)	0.9 (0.08)	0.67	0.06	0.05*
22:5	0.3	0.3 (0.06)	0.6 (0.06)	0.5 (0.06)	0.8 (0.05)**	0.0001*	0.13	0.88
n-3 Series								
18:3	0.5	0.2 (0.04)	0.5 (0.03)	0.4 (0.10)	0.5 (0.04)	0.01*	0.18	0.76
20:5	0.2	0.2 (0.03)	0.3 (0.06)	0.2 (0.07)	0.2 (0.05)	0.59	0.65	0.69
22:5	0.2	0.1 (0.02)	0.3 (0.05)	0.1 (0.04)	0.3 (0.03)	0.001*	0.47	0.87
22:6	2.0	1.7 (0.22)	1.8 (0.18)	2.0 (0.13)	1.5 (0.18)	0.22	0.80	0.06
UI[b]	64	68 (1)	75 (1)	77 (2)	73 (2)	0.10	0.49	0.08
MChL[c]	17.2	17.1 (0.01)	17.3 (0.04)	17.4 (0.06)	17.3 (0.07)	0.13	0.36	0.08

Mean values (± SEM) and *P* values for the effect of diet, age, and diet × age (d × a) interaction are provided: *$P < 0.05$; **FF and SMF values within age group are different by LSM comparison with Bonferroni correction.

[a]Sum of isomers.

[b]UI, unsaturation index.

[c]MChL, mean chain length.

TABLE 6. Synaptosomal phosphatidylserine fatty acid composition

Fatty Acid	Newborn (n = 1)	5 Days SMF (n = 3)	5 Days FF (n = 4)	25 Days SMF (n = 3)	25 Days FF (n = 4)	*P* Values Diet	*P* Values Age	*P* Values d × a
				% of total fatty acids				
Saturated								
16:0	6.9	7.1 (0.38)	6.1 (0.38)	7.6 (0.19)	6.6 (0.20)	0.02*	0.23	0.96
18:0	40.5	41.7 (0.90)	39.4 (0.14)	40.0 (0.78)	39.8 (0.20)	0.15	0.17	0.43
Monoene								
16:1[a]	0.7	1.3 (0.35)	0.9 (0.10)	1.5 (0.39)	0.8 (0.07)	0.10	0.32	0.32
18:1[a]	9.1	8.8 (0.004)	7.7 (0.21)	9.9 (0.24)	9.0 (0.17)	0.003*	0.03*	0.88
n-6 Series								
18:2	1.3	1.6 (0.30)	1.2 (0.10)	1.8 (0.25)	1.4 (0.10)	0.22	0.35	0.91
20:3	0.2	0.4 (0.05)	0.4 (0.03)	0.5 (0.04)	0.6 (0.05)	0.06	0.03*	0.14
20:4[b]	4.7	4.6 (0.31)	5.4 (0.40)	4.5 (0.05)	5.2 (0.40)	0.03	0.89	0.85
22:4	5.1	5.7 (0.23)	5.9 (0.37)	5.0 (0.31)	5.6 (0.10)	0.12	0.72	0.92
22:5	4.3	2.9 (0.49)	4.0 (0.28)	2.7 (0.56)	4.1 (0.41)	0.008*	0.33	0.95
n-3 Series								
18:3	0.1	0.2 (0.04)	0.3 (0.09)	0.2 (0.10)	0.2 (0.04)	0.19	0.57	0.47
20:5	0.1	0.4 (0.09)	0.3 (0.08)	0.3 (0.07)	0.2 (0.13)	0.36	0.88	0.99
22:5	0.2	0.3 (0.05)	0.4 (0.07)	0.4 (0.05)	0.1 (0.03)	0.23	0.22	0.09
22:6	17.5	16.9 (0.23)	18.8 (0.49)	18.1 (0.35)	14.1 (0.78)**	0.37	0.12	0.02*
UI[c]	180	178 (7)	198 (4)	182 (6)	165 (4)	0.22	0.06	0.08
MChL[d]	19.4	19.3 (0.05)	19.5 (0.04)	19.3 (0.06)	19.4 (0.01)	0.0001*	0.05*	0.13

Mean values (± SEM) and *P* values for the effect of diet, age, and diet × age (d × a) interaction are provided: *$P < 0.05$; **FF and SMF values within age group are different by LSM comparison with Bonferroni correction.

[a]Sum of isomers.

[b]Value given is the sum of the two fatty acids: 22:0 co-chromatographed with 20:4(n-6).

[c]UI, unsaturation index.

[d]MChL, mean chain length.

was not consistently separated from 20:4(n-6) of PS by GLC. Significant d × a interactions were obtained for 22:6(n-3) PS content. The level of 22:6(n-3) showed a large variation within each diet group at the different ages studied, but the values were significantly lower in 25-day-old FF compared to SMF piglets.

PI fatty acid composition

The PI fraction had a UI of 180 and contained ca. 26% fatty acid as n-6 LCP (**Table 7**). Age-specific reductions occurred in 20:0 and 22:6 (n-3). FF was associated with an age-independent decrease in 22:4 (n-6), 20:5(n-3), and 22:6(n-3), as well as an increase in 20:3(n-6) and 22:5(n-6). A d × a interaction was obtained for 18:3(n-3), a minor fatty acid in this phospholipid. A comparison of the group means suggested that the level of 18:3(n-3) was lower after 5 and 10 days but higher after 15 and 25 days feeding in FF compared to SMF piglets.

Phospholipid n-3/n-6 fatty acid content

In all the phospholipid classes studied, and in the synaptosomal membrane total lipid, the fatty acid compositional data indicated a reciprocal relationship between the content of n-3 and n-6 22-carbon (22C) fatty acids. We, therefore, examined the diet- and age-specific changes in the 22C n-3/n-6 ratio (**Fig. 1**). A significant d × a interaction was obtained for synaptosomal total lipid and EPG. A comparison of individual group means indicated a significant developmental increase of this ratio in the EPG of SMF pigs from 5 to 25 days post partum. In contrast, an age-related decrease occurred in the synaptosomal total lipid and EPG in FF animals. Thus, the 22C n-3/n-6 ratio was significantly lower in FF than SMF piglets at 10, 15 (data not shown) and 25 days post partum. A diet-specific decrease in the ratio was also found in PC, PS, and PI of FF compared to SMF pigs. Further, the ratio decreased with age independently of diet in PI.

Activity and thermotropic properties of synaptosomal acetylcholinesterase

The activity of synaptosomal acetylcholinesterase at 37°C and its A_e calculated above and below the TT in the Arrhenius plots were not altered by increasing age or the diet fed (data not shown). Values for the 25-day-old SMF piglets for acetylcholinesterase activity and A_e above and below TT were 106.8 ± 6.4 μmol/min per mg synaptosomal protein, 4.1 ± 0.5 kcal/mol, and 9.2 ± 0.6 kcal/mol, respectively. A significant diet effect, however, was found for the TT of this enzyme, with the higher TT of FF relative to SMF piglets inferring a less fluid microenvironment in the synaptosomal membranes due to FF (**Table 8**).

TABLE 7. Synaptosomal phosphatidylinositol fatty acid composition

Fatty Acid	Newborn (n = 1)	5 Days SMF (n = 3)	5 Days FF (n = 4)	25 Days SMF (n = 3)	25 Days FF (n = 3)	*P* Values Diet	*P* Values Age	*P* Values d × a
				% of total fatty acids				
Saturated								
16:0	15.5	14.8 (0.54)	13.9 (1.09)	14.9 (0.63)	14.0 (0.99)	0.56	0.81	0.91
18:0	22.0	25.2 (1.86)	26.2 (0.55)	26.0 (0.48)	27.6 (0.65)	0.82	0.59	0.63
Monoene								
16:1[a]	0.9	2.0 (0.66)	2.4 (0.18)	2.5 (0.44)	2.4 (0.39)	0.40	0.71	0.77
18:1[a]	10.0	10.0 (0.82)	10.9 (0.55)	10.0 (0.30)	9.3 (0.33)	0.62	0.67	0.26
n-6 Series								
18:2	1.7	2.0 (0.56)	2.5 (0.38)	1.8 (0.10)	2.4 (0.54)	0.14	0.68	0.31
20:3	1.7	0.4 (0.04)	1.2 (0.05)	1.1 (0.09)	1.08 (0.16)	0.02*	0.09	0.29
20:4	12.2	16.3 (1.49)	16.4 (0.37)	15.4 (1.27)	17.3 (1.38)	0.91	0.51	0.57
22:4	5.1	3.3 (0.72)	1.3 (0.14)**	3.0 (0.67)	1.2 (0.29)	0.0001*	0.97	0.75
22:5	9.3	6.0 (1.27)	9.6 (0.66)	7.6 (0.56)	8.7 (1.78)	0.03*	0.47	0.36
n-3 Series								
18:3	0.2	0.4 (0.15)	0.1 (0.05)	0.9 (0.73)	2.0 (0.26)	0.08	0.002*	0.05*
20:5	2.2	1.3 (0.29)	0.5 (0.11)	1.2 (0.50)	0.4 (0.10)	0.002*	0.94	0.99
22:5	0.4	0.1 (0.03)	tr[b]	tr	tr	0.37	0.26	0.27
22:6	2.4	3.6 (0.63)	3.2 (0.59)	3.0 (0.09)	1.7 (0.34)	0.02*	0.03*	0.48
UI[c]	193	180 (10)	185 (6)	180 (1)	178 (5)	0.86	0.55	0.67
MChL[d]	19.2	18.9 (0.10)	19.0 (0.08)	18.9 (0.03)	18.9 (0.11)	0.75	0.66	0.32

Mean values (± SEM) and *P* values for the effect of diet, age, and diet × age (d × a) interaction are provided: *$P < 0.05$; **FF and SMF values within age group are different by LSM comparison with Bonferroni correction.

[a]Sum of isomers.

[b]Trace.

[c]UI, unsaturation index.

[d]MChL, mean chain length.

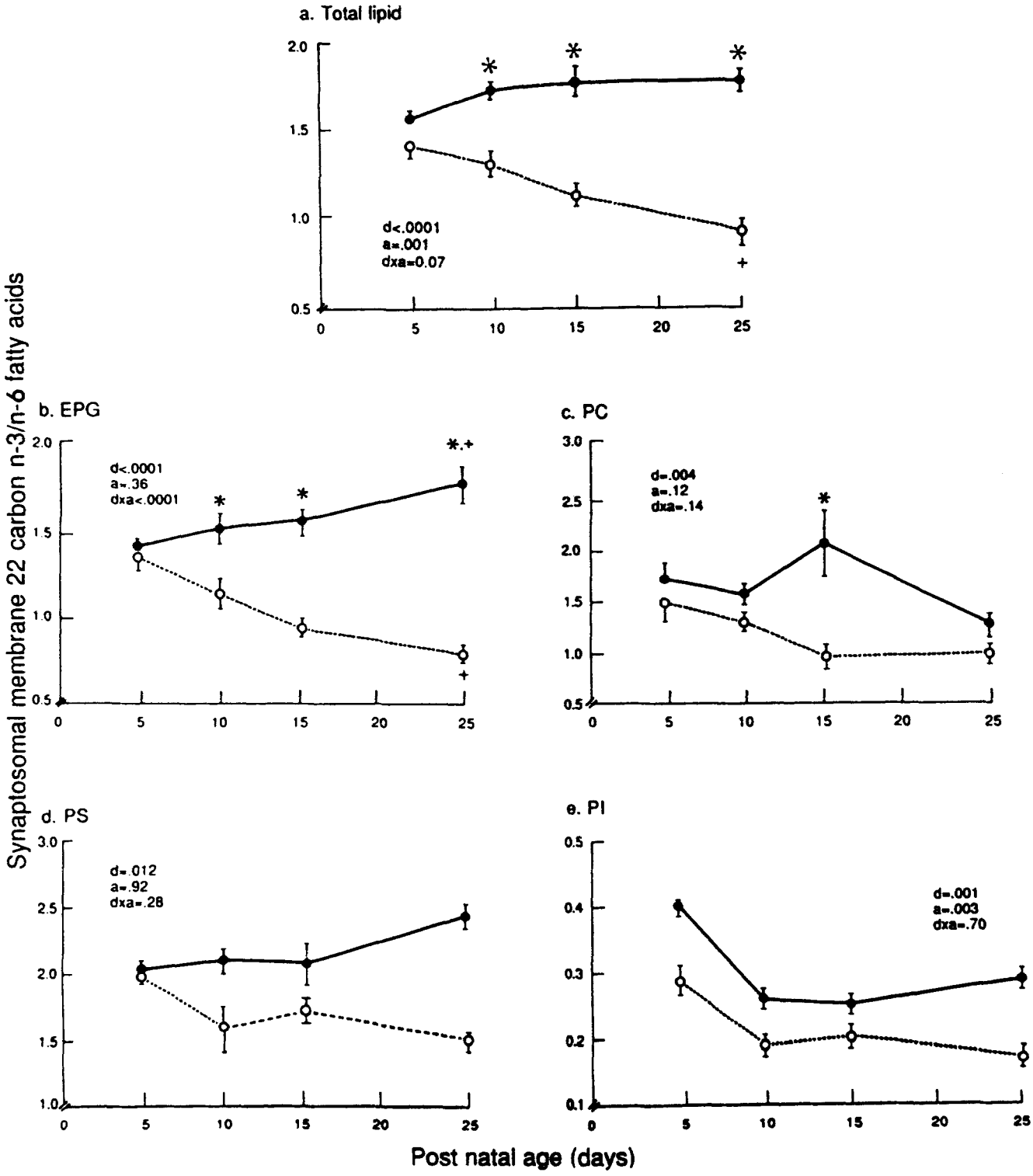

Fig. 1. Ratio of 22-carbon n-3/n-6 fatty acids in synaptosomal membrane a) total lipid, b) EPG, c) PC, d) PS, and e) PI of piglets fed sow milk (●) or formula (○) from birth to 5, 10, 15, or 25 days post partum. Values are means ± SEM. *P* values for the effect of diet (d), age (a), and diet-age interaction (d × a) are provided. *Means within age group are different by LSM comparison with Bonferroni correction. +means with diet group at 5 versus 25 days are different $P = 0.003$.

TABLE 8. Activity and thermotropic properties of synaptosomal acetylcholinesterase

		5 Days		25 Days		*P* Values		
	Newborn (n = 1)	SMF (n = 4)	FF (n = 4)	SMF (n = 5)	FF (n = 4)	Diet	Age	d × a
TT (°C)	21.4	22.2 (0.9)	21.9 (0.9)	18.4 (1.6)	21.3 (2.2)	0.05*	0.63	0.60

Piglets were fed sow milk (SMF) or formula (FF) from birth for 5, 10, or 15 (data not shown) or 25 days. Synaptosomal acetylcholinesterase activity was measured over a temperature range of 8–37°C. Arrhenius plots of enzyme activity were constructed and transition temperatures (TT) were determined. Data are means (± SEM). *P* values for the effect of diet, age, and diet × age (d × a) interaction are provided; $*P < 0.05$.

DISCUSSION

Concern has been raised over the extent to which the vegetable oil blends used in current infant formula may perturb normal tissue structural lipid fatty acid accretion, and hence the integrity and lipid-dependent functions of biomembranes, in the developing infant. This study investigated the effect of feeding a corn–coconut oil formula, devoid of n-6 and n-3 LCP but high in 18:2 (n-6) and 18:2(n-6)/18:3(n-3) ratio, from birth throughout normal suckling on the developmental changes in lipid composition and thermotropic properties of acetylcholinesterase of brain synaptosomal membranes in piglets.

The developmental changes in piglet synaptosomal membrane lipid composition during the first 25 days of life were small. In contrast, studies in the rat have documented more pronounced postnatal developmental changes in synaptosomal lipid composition (6,10,39,40). Conceivably, this difference may be related to species differences in the perinatal timing of the brain major growth spurt and lipid deposition, which is perinatal in pig and human (3,14,23,24), but postnatal in the rat (23,41). The lack of developmental change in the piglet synaptosomal cholesterol, total lipid phosphorus, and phospholipid class distribution during suckling (birth to 25 days post partum) is, however, similar to data on the synaptosomal membrane cholesterol and phospholipid of weanling rats (6). In contrast, a developmental increase in the sterol to PL ratio of rat cortical synaptosomes from 7 to 14 days post partum, together with a continued rise in this ratio, SPH and PE, and a decrease in PC over a longer timespan, has also been reported (40).

The high levels of LCP in piglet synaptosomal membrane phospholipid in this report are similar to data published for the rat (6,11,39). The developmental changes in PC 16:0 and 18:0 from normally suckled neonatal piglets and the increase in n-3 and decrease in n-6 fatty acids, which were most pronounced in EPG, are similar to data for rodent brain synaptosomal membranes (6,10,39,40) and human infant cortex (42). No developmental changes were found in the fatty acid UI of the piglet total membrane lipid, PC, PS, or PI, but changes were found in EPG as a decreased UI at 15 days post partum. In contrast, a developmental increase in synaptosomal membrane UI has been reported in the weanling rat (6), although as with the piglet (Table 4), this may be transient (39).

Formula feeding had a major effect on the synaptosomal membrane fatty acids. It did not, however, influence the membrane cholesterol, total lipid phosphorus, or phospholipid class distribution. In contrast, diets varying in fatty acid content have been reported to alter rat synaptosomal membrane phospholipid and cholesterol (6). More similar to the present findings in piglets, Yamamoto et al. (13), found no effect of feeding diets with high or low 18:2(n-6)/18:3(n-3) ratios for two generations on the phospholipid distribution of rat brain.

The major effect of FF on the piglet synaptosomal membrane was depletion of n-3 LCP, a reciprocal enrichment of n-6 LCP, and consequently a decreased n-3/n-6 LCP ratio. Similar changes have been reported for synaptosomes (4,6,10,11), whole brain (5,7,9,12,13), and other brain subcellular fractions (6,8) of rodents fed diets containing high 18:2(n-6)/18:3(n-3). Conceivably, they may be explained by competition for microsomal desaturation between dietary n-3 and n-6 fatty acids (43–46), although differences in acylation may also be involved. Since 18:1 is a major component of myelin (12,47), the finding of reduced 18:1 in the synaptosomal lipid of FF piglets in these studies is potentially important. Whether or not this was due to the low content 18:1 of the formula compared to sow milk should be clarified.

The membrane association of acetylcholinesterase (48–51) and dependency of its activity and thermotropic behavior (16,18,19) on the membrane lipid microenvironment have been well described. As documented for other species (15,16,18,19,49,52), the Arrhenius plots of piglet synaptosomal acetylcholinesterase activity were discontinuous (37). A developmental increase in rat synaptosomal (15,16) and murine whole brain acetylcholinesterase activity (53) and in rat synaptosomal acetylcholinesterase TT (15,16) as well as the microviscosity and lipid order in rat cortical synaptic membranes and isolated lipid bilayers (38,54,55) have been documented. In apparent contrast, no significant developmental changes in piglet synaptosomal acetylcholinesterase activity or TT were found in the present studies. This, however, appears consistent with the small developmental changes in the piglet synaptosomal membrane lipid composition. The major increase in the physiologically active (G_4) acetylcholinesterase (48,50), maximal brain growth velocity (23), and synaptosomal maturation (56) occur after birth in the rat. Although the characteristics of pig brain acetylcholinesterase maturation are unknown, human brain, which has a perinatal growth pattern similar to the pig (23), shows

maximal G_4 activity at birth (48). Thus, possible species differences in neural acetylcholinesterase and brain development may at least partly explain the difference in the results of this study in piglets from studies in rats.

The similar activity of acetylcholinesterase between SMF and FF piglets, despite differences in their synaptosomal membrane fatty acid composition, is in contrast to the increased maximal velocity of synaptosomal acetylcholinesterase of rats fed high versus low 18:2/18:3 diets (16). The extent to which acetylcholinesterase, which is a peripheral extrinsic membrane enzyme, is sensitive to alterations in the inner hydrophobic membrane lipid is uncertain (15). Unlike the present findings for the piglet, studies in the rat also found diet-induced changes in the synaptosomal cholesterol and phospholipid (16). Thus, it seems possible that the modulation of rat acetylcholinesterase activity may be related to changes in the membrane hydrophilic or sterol components rather than its acyl composition. The physiological significance of the higher acetylcholinesterase TT in FF compared to SMF piglets is unknown. The TT of acetylcholinesterase is below physiological temperatures, and when assayed at 37°C no difference in enzyme activity was evident among the FF and SMF piglets.

The possible association of diet-related changes between acetylcholinesterase TT and membrane lipid composition was studied by univariate regressions between TT and lipid components known to influence membrane fluidity (57) using the combined data for all piglets and for each diet group. No relationship was found between TT and membrane cholesterol, total phospholipid, phospholipid head group, MChL, or UI (data not shown). The total lipid 22C LCP n-3/n-6 ratio and monoene content, however, showed a significant inverse relationship to TT in the data from all piglets, and the SMF group (**Fig. 2**), but not the FF group. The apparent relationship of TT to fatty acid components but not UI may be explained by recently described limitations of the UI calculation (58,59). Potential relationships suggested by these statistical associations cannot be further addressed by our study.

In summary, this study demonstrates that normal postnatal changes in synaptosomal fatty acids are less extensive in the pig than in rodents. Despite apparent maturity at birth, the synaptosomal fatty acid composition of newborn piglets was readily changed by postnatal FF. The concomitant changes in acetylcholinesterase TT suggests decreased membrane fluidity due to FF, and may be related to the associated changes in membrane fatty acid composition. The results suggest that exclusive feeding of formula containing minimal n-6 and n-3 LCP, and high 18:2(n-6) and 18:2(n-6)/18:3(n-3) ratio alters brain synaptosomal membrane fatty acid accretion and physical properties. Since membrane fluidity is known to influence a variety of synaptic functions including receptor binding,

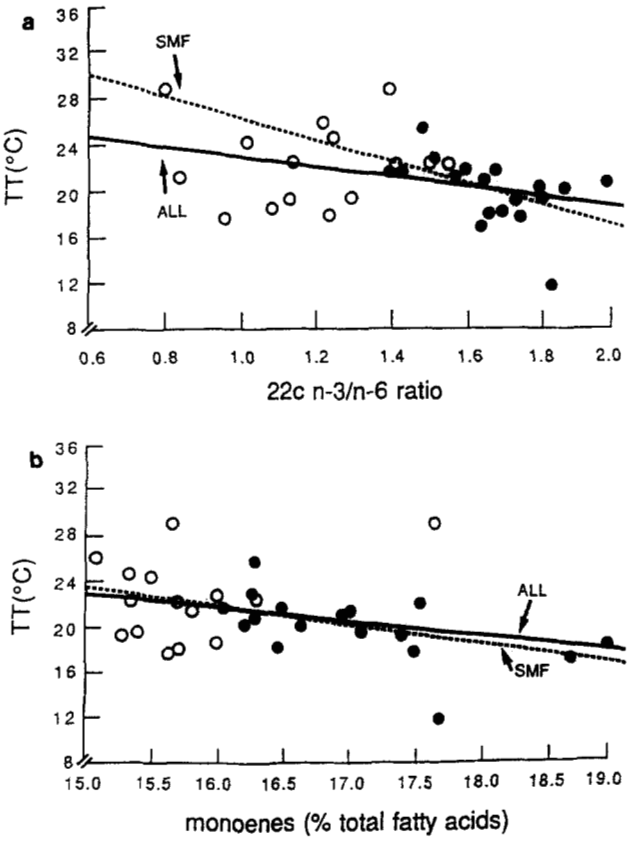

Fig. 2. Univariate regression of a) 22-carbon n-3/n-6 fatty acid ratio and b) monoene content of synaptosomal total lipid, versus acetylcholinesterase transition temperature (TT), of all piglets (ALL: n = 32), sow milk-fed (SMF: n = 17) and formula-fed (FF: n = 15) sub-groups. a: Equations for ALL and SMF are $y = 4.4x + 27.2$, $r = -0.403$, $P = 0.02$ and $y = -9.5 + 35.61$, $r = -0.514$, $P = 0.03$, respectively. Regression for FF was not significant, $P = 0.99$. b: Equations for ALL and SMF are: $y = -1.2x + 40.8$, $r = -0.354$, $P = 0.04$ and $y = 1.8x \pm 51.1$, $r = -0.538$, $P = 0.02$, respectively. Regression of FF was not significant, $P = 0.23$.

neurotransmitter release and reuptake, and ion transport (20,60,61), further consideration of the effects of formula fat blends on membrane structure and function is warranted.

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