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Essential fatty acids in early life: structural and functional role

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Essential fatty acids (EFA) are structural components of all tissues and are indispensable for cell membrane synthesis; the brain, retina and other neural tissues are particularly rich in long-chain polyunsaturated fatty acids (LCPUFA). These fatty acids serve as specific precursors for eicosanoids that regulate numerous cell and organ functions. Results from animal and recent human studies support the essential nature of *n*-3 EFA in addition to the well-established role of *n*-6 EFA for human subjects, particularly in early life. The most significant effects relate to neural development and maturation of sensory systems. Recent studies using stable-isotope-labelled tracers demonstrate that even preterm infants are able to form arachidonic acid (AA) and docosahexaenoic acid (DHA), but that synthesis is extremely low. Intracellular fatty acids or their metabolites regulate transcriptional activation of gene expression during adipocyte differentiation, and retinal and nervous system development. Regulation of gene expression by LCPUFA occurs at the transcriptional level and is mediated by nuclear transcription factors activated by fatty acids. These nuclear receptors are part of the steroid hormone receptor family. Two types of polyunsaturated fatty acid responsive transcription factors have been characterized, the peroxisome proliferator-activated receptor (PPAR) and the hepatic nuclear factor 4α. DHA also has significant effects on photoreceptor membranes involved in the signal transduction process, rhodopsin activation, and rod and cone development. Comprehensive clinical studies have shown that dietary supplementation with marine oil or single-cell oils, sources of LCPUFA, results in increased blood levels of DHA and AA, as well as an associated improvement in visual function in formula-fed premature infants to match that of human milk-fed infant. Recent clinical trials convincingly support LCPUFA supplementation of preterm infant formulations and possibly term formula to mimic human milk composition.

Essential fatty acids: Docosahexaenoic acid: Peroxisome proliferator-activated receptor: Gene expression: Retinal development

Essential fatty acids: basis for essentiality during early life

Over the past few decades, results from animal and recent human studies have strongly supported the essential nature of *n*-3 and *n*-6 essential fatty acids (EFA) for human subjects, and a particular need for long-chain polyunsaturated fatty acids (LCPUFA) in early life. The most significant effects relate to neural development and maturation of sensory systems. The essentiality of fatty acids is determined by the inability of animal cells to introduce double bonds before carbon *m*-9. Animal tissues, especially the liver, are capable of elongating and desaturating the parent EFA, generating a family of compounds for the respective families. Arachidonic acid (20 : 4*n*-6; AA) can be formed from linoleic acid (18 : 2*n*-6; LA) and docosahexaenoic acid (22 : 6*n*-3; DHA) from α-linolenic acid (18 : 3*n*-3; LNA). In the case of EFA deficit, eicosatrienoic acid (20 : 3*n*-9) will be formed from oleic acid (18 : 1*n*-9; Sprecher, 1981). The competitive desaturation of the *n*-3, *n*-6 and *n*-9 series by Δ^6-desaturase is of major significance, since this step is considered to be the controlling step of the pathway. Δ^6-Desaturase has the

Abbreviations: AA, arachidonic acid; DHA, docosahexaenoic acid; EFA, essential fatty acids; EPA, eicosapentaenoic acid; LA, linoleic acid; LCPUFA, long-chain polyunsaturated fatty acids; LNA, α-linolenic acid; PG, prostaglandin; PPAR, peroxisome proliferator-activated receptor; PUFA, polyunsaturated fatty acids.

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highest affinity for the *n*-3 series. Its activity is modulated by hormones and by interactions of substrates and metabolic products. Recently, the cloning of mammalian Δ^6-desaturase has permitted the evaluation of tissue-specific expression; the brain contains several-fold higher enzyme mRNA than the liver. Dietary *n*-6 polyunsaturated fatty acids (PUFA) significantly reduced the expression of liver Δ^6-desaturase (Cho *et al*. 1999). The endogenous synthesis of PUFA longer than C_{20} involves several elongation and desaturation steps and a partial peroxisomal β-oxidation (Voss *et al*. 1991). The triene : tetraene (docosatrienonic acid : AA) value traditionally used as an index of *n*-6 EFA deficiency does not serve to assess *n*-3 status. In *n*-3 fatty acid deficiency the *n*-6 LCPUFA docosapentaenoic acid (22 : 5*n*-6) accumulates while DHA decreases in plasma and tissue lipids (Sprecher, 1981).

Metabolic activity reflecting EFA elongation and desaturation in human subjects is found mainly in the liver, but also in the placenta, central nervous system, glial tissue and choroid plexus vasculature (Bourre *et al*. 1997; Cook, 1978). Whole-body elongase and desaturase activity can be assessed *in vivo* using either ^{2}H- or ^{13}C-labelled EFA substrates, enabling the evaluation of product formation in plasma and tissue compartments. We have studied endogenous DHA and AA synthesis using LA and LNA labelled with five ^{2}H atoms positioned on C-1 and C-2 (Salem *et al*. 1996). Concentrations of ^{2}H-labelled precursors and products were measured in plasma using negative-ion mass spectrometry of pentafluorobenzyl derivatives. Peak concentrations of labelled precursor in plasma were reached during the first day after dosing, ^{2}H-labelled product concentrations increased over time, peaking by 48 h in the term infants and closer to 96 h in the preterm infants. Based on product–precursor relationships of time-integrated ^{2}H enrichment, preterm infants appear to convert parent EFA to long-chain derivatives five to six times more actively than term infants. Unfortunately, neither these studies nor other published work permit a precise quantification of biosynthesis, since no account of precursor pool size can be made. In addition, it is apparent from studies in non-human primates that uptake kinetics are quite different across tissues, and that plasma does not reflect whole-body equilibrium (Sheaff Grenier *et al*. 1997). Studies using uniformly-labelled ^{13}C precursors confirm that preterm and term neonates are able to synthesize DHA and AA by elongating and desaturating parent EFA (Demmelmair *et al*. 1995; Carnielli *et al*. 1996; Sauerwald *et al*. 1997). These studies suggest greater biosynthesis of LCPUFA at younger gestational age relative to term infants. Alternatively, the results indicate a faster removal of products from the plasma pool in more immature infants and/or reduced turnover rate of LCPUFA with advancing age. This finding could be related to slower growth rate and lower metabolic rate with advancing postnatal age (Demmelmair *et al*. 1995).

Results from studies in several animal species and recent evidence from human subjects have established that tissue phospholipid-AA and -DHA decrease while *n*-9 and *n*-7 monounsaturated fatty acids and PUFA increase in EFA deficiency (Bourre *et al*. 1989*a*,*b*, Menon & Dhopeshwarkar, 1982). Typically, *n*-3 fatty acid-deficient cells have decreased DHA and increased levels of the endproduct of *n*-6 metabolism, docosapentaenoic acid. Within the subcellular organelles, synaptosomes and mitochondria seem to be more sensitive to a low dietary *n*-3 supply, as shown by the relative abundance of DHA and the changes in composition of these organelles in response to dietary deprivation (Bourre *et al*. 1989*a*,*b*).

Essential fatty acid supply affects molecular regulation of gene expression and function

Lipids, which serve as components of specialized cell membranes and organelles, may affect membrane fluidity and protein–lipid interactions that result in changes in overall cell function. These effects may modulate receptor activity, transport in and out of cells, hormonal and other signal transduction processes. By modulating gene expression, LCPUFA and derived eicosanoids are involved in the regulation of cell growth, differentiation and multiple other functions. We will focus our initial discussion on the effect of fatty acids on gene expression.

Fatty acids play an important role in modulating their own metabolism, synthesis and oxidation. This process is known as metacrine regulation and is mediated in part by allosteric control of enzymic activity, but it is also dependent on gene expression. In these two ways fatty acids regulate lipogenesis, mitochondrial oxidation, and gluconeogenic enzymes. This process is apparent very early in postnatal life since LCPUFA, in concert with pancreatic hormones, enhance the expression of neonatal rat liver genes coding for key mitochondrial β-oxidation enzymes necessary for ketogenesis (Pégorier *et al*. 1998). LCPUFA of both the *n*-3 and *n*-6 series reduce hepatic lipogenesis by decreasing the content and activity of enzymes involved in lipid synthesis (fatty acid synthetase, acetyl-CoA carboxylase, stearoyl-CoA carboxylase and malic enzyme). This reduction in lipogenesis is explained by down regulation of gene transcription (Clark & Jump, 1993, 1994).

Recent studies also indicate that fatty acids modulate adipogenesis. The development of white adipose tissue starts with the differentiation of fibroblasts into adipocytes. This process involves the induction of specific proteins, i.e. adipocyte fatty acid-binding protein, phospho*enol*pyruvate carboxykinase, acyl-CoA synthetase, and lipoprotein lipase. Fatty acids and/or their derived compounds induce the expression of these adipocyte-specific gene products and stimulate adipocyte differentiation (Amri *et al*. 1991*a*,*b*; Grimaldi *et al*. 1992). The evidence also indicates that *n*-3 supplementation in the form of perilla oil given to rats after weaning significantly reduces the growth of visceral adipose tissue, despite similar total food consumption. The expression of genes that serve as markers for late adipocyte differentiation (adipsin, adipocyte P2 and peroxisome proliferator-activated receptor (PPAR) α) were all down regulated in the perilla oil-fed group. This effect resulted in a lower weight for the epididymal fat pad in the *n*-3 supplemented group compared with olive oil-fed or beef tallow-fed groups (Okuno *et al*. 1997). A specific role for AA in the expression of fibroblast transcription factors (c-fos and Erg-1) which modulate cell growth and differentiation has been identified. This effect is mediated

by formation of prostaglandin (PG) E_2 and activation of protein kinase C (Danesch *et al*. 1994; Sellmayer *et al*. 1996).

The effect of DHA on photoreceptor differentiation in primary culture of retinal neuronal cells has also been studied. A recent study in rats demonstrated that DHA significantly increases rod outer segment apical process differentiation (Fig. 1(a)), the location for light transduction. This effect was paralleled by an increase in opsin expression and content in rod photoreceptor apical processes (Fig. 1(b)). The molecular mechanisms underlying these effects have not been fully elucidated, but recent studies from Bazan's group (Rodriguez de Turco *et al*. 1997; Rotstein *et al*. 1998) have demonstrated that opsin and rhodopsin transport to the apical process via post-Golgi membranes is coupled to DHA transport. The close molecular interaction between this key photoreceptor protein and DHA suggests a possible important mechanism by which DHA influences retinal photoreceptor structural development as well as function.

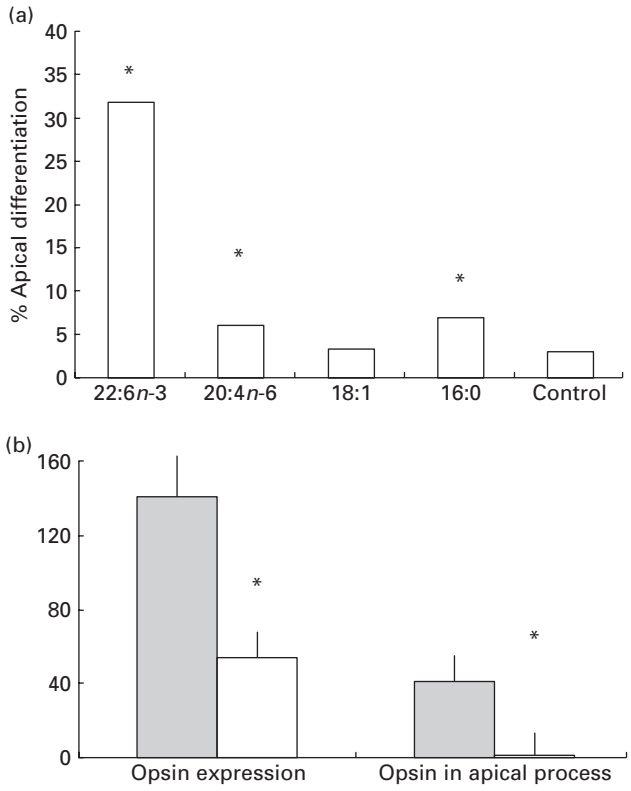

Fig. 1. Effect of docosahexaenoic acid (DHA) on photoreceptor differentiation. (a) The effect of DHA and other fatty acids on percentage of rat retinal neuronal cells showing apical differentiation after 7d of incubation in respective fatty acids. Values are means of three experiments; (b) opsin expression (no. of photoreceptors per dish$\times 10^{-3}$) in retinal cells incubated for 11d with (▨) or without (▭; control) 4–6 μM-DHA and localization of opsin in the apical process (no. of photoreceptors per dish$\times 10^{-3}$). Values are means and standard deviations for four to six samples from two experiments. Mean values were significantly different from control values: *$P < 0{\cdot}05$. (Adapted from data of Rotstein *et al.* 1998.)

Regulation of gene expression by LCPUFA occurs at the transcriptional level, and is mediated by transcription factors which bind *cis*-regulatory elements found in target genes. These transcription factors, which are activated by fatty acids, belong to the superfamily of nuclear receptors that includes the steroid hormone receptors, glucocorticoid receptor, vitamin D receptor, thyroxine receptor and the retinoic acid receptor (Clark & Jump, 1993, 1994). Two types of PUFA-responsive transcription factors have been characterized, the PPAR and the hepatic nuclear factor-4α. Fig. 2 provides a schematic representation of the regulation of the PPAR system. Ligands determine the dimerization of the receptors, specifying homodimer or heterodimer formation. Recent studies have identified a number of proteins, co-activators that interact with nuclear receptors playing a role in the regulation of transcriptional activity. The formation of the binding site for the co-activator in the nuclear receptor is ligand-dependent.

The PPAR family of nuclear receptors has received considerable attention due to its major role in the regulation of lipid and glucose metabolism, and adipocyte differentiation. PPAR isoforms α, β and γ are encoded by different genes and can be distinguished based on their metabolic effects, differential tissue specific expression and responsiveness to pharmacological agents. PPAR-α is involved predominantly in fatty acid metabolism in the liver, but is also expressed in other tissues such as kidney, heart, skeletal muscle and brown adipose tissue. The expression of PPAR-γ is predominantly observed in adipose tissue, where it normally acts by suppressing adipocyte differentiation. There are two PPAR-γ isoforms derived from the same gene by alternative promoter usage and splicing. Specific mutations for PPAR-γ-2 are associated with enhanced adipocyte differentiation, but have a negligible effect on insulin sensitivity. This genetic condition, presenting with massive obesity but minimal insulin resistance, has recently been identified in human subjects (Ristow *et al*. 1998). As previously shown in Fig. 2 the transcription activation process is mediated by a heterodimer formed by PPAR with retinoic acid receptor; drugs such as fibrates and thiazolidinediones also act by PPAR activation.

PPAR activation involves the conversion of the receptor to a transcriptionally-active form. Assays to measure PPAR activation are based on the transfection of cell lines with chimeric constructs. Typically, these constructs encode the ligand-binding domain of the PPAR gene fused to the DNA-binding domain of a well-known receptor, such as the bacterial tetracycline repressor, yeast GAL 4, or the mammalian glucocorticoid receptor. Binding of the ligand-activated chimeric receptor to the DNA response element results in the transcription of the reporter genes. Transcriptional activation assays do not represent unequivocal evidence of direct binding of activators to the PPAR molecule. In fact, since evidence for physical association of fatty acid activators with PPAR was lacking, their function as actual ligands was questioned for some time. However, binding has recently been demonstrated using labelled peroxisome proliferators as well as naturally-occurring fatty acids or their metabolic products, 15-deoxyprostaglandin J_2, hydroxyeicosatetraenoic acid and leukotriene B_4 (Kliewer *et al*. 1995, 1997; Brown, 1997).

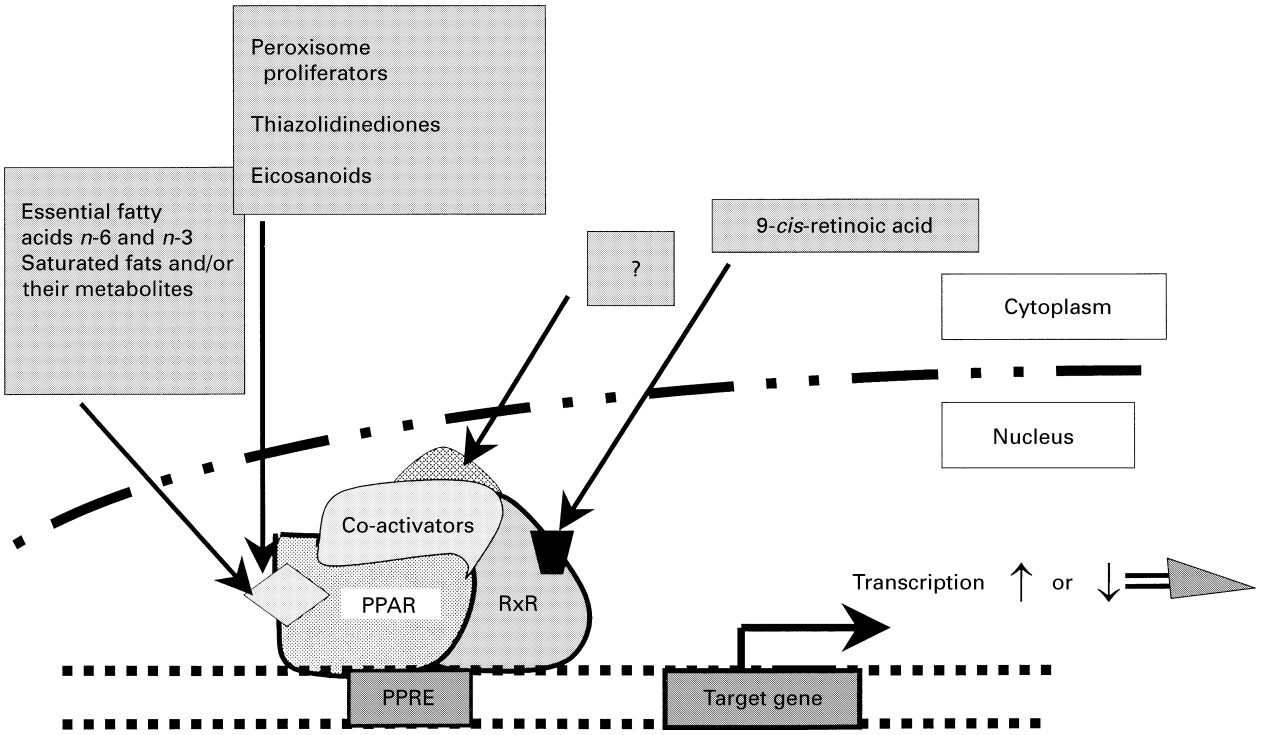

Fig. 2. The mechanism for transcriptional regulation of the peroxisomal proliferator-activated receptor (PPAR) system by fatty acids. PPAR is a nuclear protein. PPRE, peroxisomal proliferator responsive element in the DNA; RxR, retinoic acid receptor. In addition to fibrates and thiazolidinediones, fatty acids and eicosanoids can bind and activate PPAR expression, which acts as a transcription regulator. ?, Unknown potential activators or inhibitors.

The activation of PPAR by fatty acids was first characterized in *Xenopus laevis*; α, β and γ isoforms are able to respond to fatty acids with overlapping specificity (Dreyer *et al*. 1993). However, few studies have systematically explored the differential activation of PPAR isoforms by fatty acids of different chain length, and degree and type of unsaturation. Yu *et al.* (1995) compared the ability of fatty acids to activate the different PPAR isoforms using chimeric constructs. Whereas the tetracycline repressor–PPAR-α chimeric receptor was activated to almost the same extent by LA and by DHA, the tetracycline repressor–PPAR-γ receptor was activated by DHA but not by LA, and the tetracycline repressor–PPAR-β receptor was responsive to DHA and to a lesser degree to LA. PPAR-α is apparently also activated by medium-chain PUFA and LCPUFA. This evidence has been used to support the notion that dietary *n*-3 and *n*-6 PUFA-induced reduction of hepatic expression of lipogenic enzymes is mediated by PUFA-activated PPAR-α (Yu *et al*. 1995). At high concentrations PUFA have a significant effect on PPAR expression, while at lower concentrations the effects are marginal (Gottlicher *et al*. 1993). In this chimeric PPAR expression model, DHA appears to be most active, while saturated myristic acid induces the lowest activation. The presence of a carboxylic acid group and a hydrophobic domain of varying size characterize all the fatty acids and drugs which activate PPAR expression (Gottlicher *et al*. 1993; Kliewer *et al*. 1995, 1997). The effects of selected fatty acids in relation to the potent peroxisomal proliferator drug WY-14,643 (Chemsyn Science Labs, Lenexa, KS, USA) at two molar concentrations is presented in Fig. 3.

Finally, PPAR-α is also activated by AA and by several eicosanoids such as leukotriene B_4 and 8-hydroxyeicosatetraenoic acid. PGA, PGD and PGJ series are activated by all PPAR isoforms, including PPAR-γ. However, PPAR-γ responds to the lowest PG concentrations, suggesting that PGJ_2 metabolites (Δ^{12}-PGJ_2 and 15-deoxy-$\Delta^{12,14}PGJ_2$ are

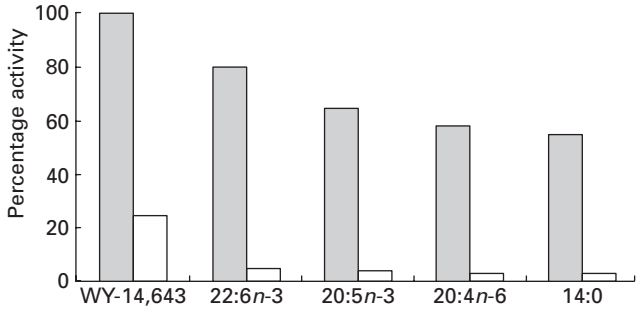

Fig. 3. Effect of selected fatty acids at two molar concentrations ((▬), log [–4]; (▭) log [–5]) on relative peroxisomal proliferator-activated receptor (PPAR) expression using a chimeric model; response to the potent peroxisomal proliferator drug WY-14,643 is taken as 100 % activity. (Adapted from data of Gottlicher *et al.* 1993.)

highly specific PPAR-γ activators. In addition, direct binding of 15-deoxy-$\Delta^{12,14}PGJ_2$ to PPAR-γ has been demonstrated.

Transcription-activation assays and binding studies clearly demonstrate that PPAR transcription factors are activated by PUFA. In addition, PPAR-α null mice display defective mitochondrial fatty acid oxidation when compared with wild-type mice (Aoyama *et al*. 1998). However, it is not possible to rule out the possibility that the observed transcriptional regulation may also be mediated directly by fatty acid metabolites, or indirectly by modifying PPAR activity. For example, in the case of the transcription factor hepatic nuclear factor-4α, the identified ligand is the acyl-CoA thioester derivative of the long-chain fatty acid and not the fatty acid itself. Binding of acyl-CoA thioesters of long-chain fatty acids to the ligand-binding domain of hepatic nuclear factor-4α modulates its transcriptional activity. Agonistic ligands include saturated acyl-CoA with C_{14}-C_{16} chain length. While antagonistic ligands include *n*-3 and *n*-6 PUFA-CoA (Hertz *et al*. 1998).

The net effects of PPAR on cellular processes and metabolism include enhanced peroxisomal proliferation, increased fatty acid oxidation, lower plasma triacylglycerol levels and improved glucose tolerance (Ibrahimi *et al*. 1994; Tontonoz *et al*. 1994; Hertz *et al*. 1998). More recently, PPAR-α-induced macrophage activation of oxidized LDL uptake has been demonstrated, this process was mediated by a scavenger CD36 protein. Increases in the content of oxidized LDL in the macrophage leads to increased PPAR activity, which activates the transcription of CD36, which in turn increases oxidized LDL uptake. Thus, a vicious cycle is generated, leading to the accumulation of oxidized LDL and formation of lipid-laden foam cells (Nagy *et al*. 1998).

The regulation of the genes encoding fatty acid metabolism during early life represents an excellent model to evaluate nutrient–gene interactions, since these genes are affected by the major changes in substrate availability which occur after birth. The shift in perinatal fuel metabolism from glucose predominant to mixed fat–glucose oxidation and other nutritional changes such as enteral nutrition have profound effects on lipid metabolism. The role of LCPUFA in adipocyte differentiation and in central nervous system development adds further interest to this emerging field. Additional work will be necessary to better characterize the intracellular fatty acid metabolites that regulate transcriptional activation mediated by LCPUFA and other factors that may interact to determine the responsiveness of target genes.

Essential fatty acid supply affects lipid membrane structure and functional properties

Fatty acid composition of structural membrane lipids can affect membrane function by modifying overall membrane fluidity, membrane thickness, lipid-phase properties, membrane microenvironment, or by interaction of fatty acids with membrane proteins (Wheeler *et al*. 1975; Stubbs & Smith, 1984; Lee *et al*. 1986). Most dietary *n*-3 fatty acid-induced membrane changes are not reflected by an overall change in membrane fluidity, but result in selective changes in membrane microenvironment affecting specific domains. The replacement of DHA by docosapentaenoic acid observed in *n*-3 deficiency results in a very similar overall lipid unsaturation level, since only one double bond has been lost. Thus, membrane fluidity on average remains unchanged. Furthermore, the major changes in the physical state induced by changes in the fatty acid composition of lipid bilayers occur after the first and second double bonds are introduced, i.e. when a saturated fatty acid such as stearic acid (18 : 0) is replaced by oleic acid (18 : 1*n*-9) or by LA (18 : 2*n*-6; Yorek *et al*. 1984; Treen *et al*. 1992). Other researchers have suggested that DHA supply modifies the phospholipid molecular species present in neural tissues, thus possibly affecting overall function (Lin *et al*. 1990).

One of the most significant membrane effects of DHA is its role in the photoreceptor signal transduction process. Recently, Litman & Mitchell (1996) reported that LCPUFA present in membrane phospholipid molecular species have profound effects on rhodopsin activation and related structural modifications. Rhodopsin is a membrane protein present in rod outer segment disc membranes, accounting for 90 % of the protein content. It functions as a photon receptor coupled to a G protein. The light-induced conformational change in rhodopsin triggers a biochemical cascade, finally leading to an increase in phosphodiesterase activity and a decrease in cGMP which closes Na^+ channels in the photoreceptor disc membrane. The result is a hyperpolarization, increasing the negative charge of the plasma membrane, which is followed by a depolarization. The resulting signals correspond to the 'a' and 'b' waves of the electroretinogram. Membrane fatty acid composition affects the ability of photons to transform rhodopsin to the activated state (Weidmann *et al*. 1988; Mitchell *et al*. 1992). The rhodopsin activation in response to light involves a transformation of the metarhodopsin I form to the metarhodopsin II form.

Fig. 4(a) depicts the effect of lipid unsaturation on membrane microenvironment; proteins such as rhodopsin have greater mobility in the photoreceptor if surrounded by DHA. Fig. 4(b) depicts the results of Litman & Mitchell (1996) that demonstrate the effect of lipid microenvironment (studied in artificially-reconstituted membranes) on the metarhodopsin I–metarhodopsin II equilibrium. The equilibrium constant is six times higher with di DHA-acylated phosphatidyl choline than with di myristic (saturated 14 : 0) phosphatidyl choline. The di DHA phosphatidyl choline has an equilibrium constant that is almost identical to that of native rod discs. The effect is mostly explained by the increase in membrane free volume. This greater mobility of rhodopsin within the lipid microenvironment most probably explains the change in G protein activation and the corresponding enhanced signal transduction to photon stimuli (Litman & Mitchell, 1996). The corresponding physiological phenomenon is the increase in retinal sensitivity to light associated with DHA supply in the diet.

Diet-induced changes in structural lipids affect the functional characteristics of other excitable membranes (Wheeler *et al*. 1975; Neuringer *et al*. 1984; Love *et al*. 1985; Holh & Rosen, 1987; Bourre *et al*. 1989*b*). Electrocardiographic abnormalities, such as a notching in the QRS

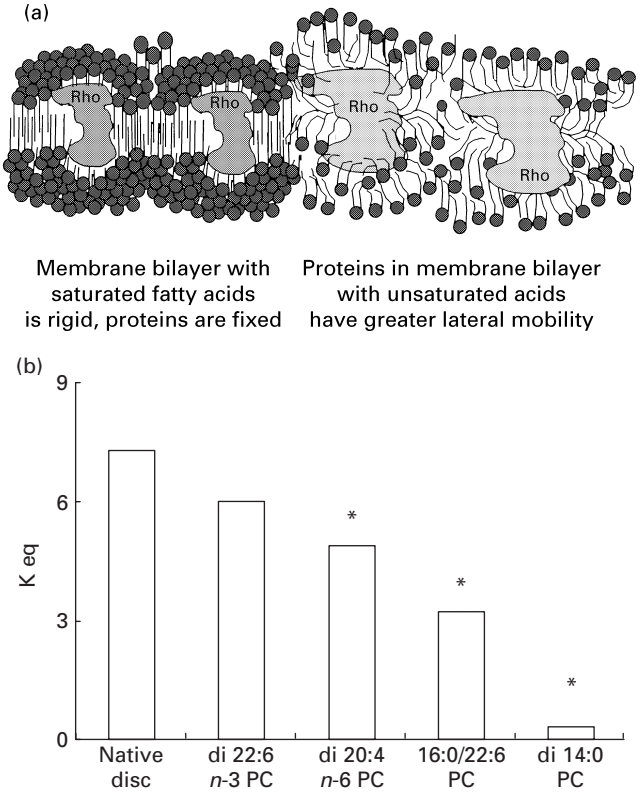

Fig. 4. (a) Schematic representation of the effect of lipid unsaturation on membrane (➷), microenvironment and on lipid–protein interaction. Solid elements (●) represent protein within the lipid bilayer, mobility of proteins, such as rhodopsin (Rho), and membrane packing are affected by fatty acid composition. (b) The effect of fatty acid composition of reconstituted phospholipid membranes on the 1nsequilibrium constant (K eq) for metarhodopsin activation. Di DHA phosphatidyl choline (PC) has the highest K eq and is similar to reconstituted native disc membranes. Mean values were significantly different from those of the native disc membranes: $*P<0{\cdot}05$. (Adapted from data of Litman & Mitchell, 1996.)

(wave of the electrocardiogram) complex, indicating impaired electrical conduction, occur in LA and LNA deficiency before clinical arrhythmia appears (Caster & Ahn, 1963). More recently, the effect of dietary fatty acids in decreasing the incidence and severity of cardiac arrhythmia has been demonstrated (Charnok, 1991). Furthermore, studies with myocardial preparations have indicated that the vulnerability to catecholamine-induced arrhythmia is reduced in animals fed on either *n*-6 or *n*-3 PUFA-enriched diets (Kang & Leaf, 1995). Feeding fish oil from bluefin tuna (*Thunnus maccoyii*) rather than sunflower oil and saturated fat resulted in a marked reduction in induced arrhythmia in two animal species and in isolated papillary muscle (Charnok, 1991). Changes in cardiac electrophysiological responses to β-mimetics, and reduced excitability and susceptibility to arrhythmia of cardiac myocytes have also been noted (Hallaq *et al*. 1992; Kang & Leaf, 1995). Myocytes form minimal amounts of cyclo-oxygenase products and no lipoxygenase products, thus the changes in excitability and conduction are probably related to structural lipid composition and not to eicosanoid release (Holh & Rosen, 1987).

The role of membrane lipid composition in determining the electrical properties of cultured neuronal cells exposed to exogenous fatty acids has also been investigated (Love *et al*. 1985; Lin *et al*. 1990). Both *n*-3 and *n*-6 fatty acids induced slower rates of rise, and to a lesser extent, lower amplitude of Na^+ action potentials. The opposite effects were observed when saturated or *trans*-monoenoic fatty acids were added. These effects are probably mediated by a change in the number of active Na^+ channels. A change in membrane composition or altered fatty acid availability to the cells may explain this effect (Love *et al*. 1985). Free LCPUFA modulate the inactivation of Ca and Na channels (Leaf & Kang, 1997). In addition to the anti-arrhythmic consequences for cardiac myocytes, there are also changes in cation currents in hippocampal neurons (Vreugdenhil *et al*. 1996) and a higher seizure threshold in rat cortex (Voskuyl *et al*. 1998). These effects appear to depend on free extracellular LCPUFA concentration and not on membrane phospholipid composition (Weylandt *et al*. 1996). The responsiveness of free LCPUFA to dietary interventions which alter tissue composition remains unclear. The release of free LCPUFA from membranes could have widespread effects on neurosensory organ function.

The significance of these experimental studies is hard to determine, but clearly these findings are most relevant to the function of critical organs, such as the brain, lung, kidney and intestine and vascular responses. Dietary supply during development may condition, for example, membrane function and integrity, transport functions, electro-physiological responses, neuroendocrine function and vasomotor tone. The possibility of metabolic programming of physiological responses can also be related to early regulation of membrane receptor and second messenger responses.

Production of various eicosanoids is another mechanism by which the effect of LCPUFA supplementation on different physiological functions can be explained. Phospholipases liberate AA and eicosapentaenoic acid (20 : 5*n*-3; EPA) from membrane lipids, and through the action of cyclooxygenase or lipoxygenase eicosanoid products are formed. Prostaglandins, prostacyclins, thromboxanes and leukotrienes derived from LCPUFA play a key role in modulating inflammation, cytokine release, immune response, platelet aggregation, vascular reactivity, thrombosis, and allergic phenomenon. The balance between AA (*n*-6) and EPA (*n*-3) in biological membranes is regulated based on dietary supply. The *n*-6 : *n*-3 value in phospholipids modulates the balance between prostanoids of the 2 and 3 series derived from AA and EPA respectively. Series 3 prostanoids are weak agonists, or in some cases antagonize the activity of series 2 prostanoids. Eicosanoids of the 2 series promote inflammation, platelet aggregation and activate the immune response. In contrast, series 3 prostanoids tend to ameliorate these effects. We will not expand on this theme and suggest recent reviews for interested readers (Whelan, 1996; Calder, 1997).

Essential fatty acid supply affects retinal and brain cortical functional development in infants

The short-term effects of combined *n*-6 and *n*-3 EFA deficiency have been well characterized in the past. Over the past decade the separate effects of *n*-3 and *n*-6 deficiencies have been described, principally affecting growth and central nervous system development during early life. In the late 1960s clinical signs of combined EFA deficiency became apparent in infants fed on skimmed-milk-based diets in the 1950s and in those given lipid-free parenteral nutrition. These infants presented with dryness, desquamation and thickening of the skin, and growth faltering as manifestations of LA deficiency (Hansen *et al*. 1963; Caldwell *et al*. 1972). In LNA deficiency abnormal visual function and peripheral neuropathy have been noted (Holman *et al*. 1982).

The specific effects of LCPUFA deficit in the presence of an adequate supply of LA and LNA have proved more difficult to elucidate, given that biosynthesis of AA and DHA from the respective precursors occurs even in premature infants. Whether endogenous biosynthesis from dietary precursors is sufficient to meet the needs for growth and development remains the object of present research efforts. The subtle effects observed with LCPUFA supplementation are most probably related to discrete changes in membrane properties or possibly to changes in the developmental programme related to the expression of specific genes. As previously noted, the lipid composition of the diet can affect the structure and function of membrane lipids, modifying overall membrane properties, including fluidity and volume. In addition, specific changes in membrane microenvironment and/or interaction of fatty acids with membrane proteins may affect critical specific membrane functional domains. The changes in neural membranes of greatest potential significance to the human infant are those related to changes in physical properties and to changes that affect membrane excitability (Salem *et al*. 1988). The functional response of the retina and the occipital cortex can be assessed by electrophysiological methods (electroretinogram and pattern-reversal visual-evoked potential), or by behavioural methods (forced-choice preferential looking acuity). Additional studies have included measures of novelty-choice preferential looking (Fagan, 1984), and mental and motor development at 12 and 18 months.

Studies of term infants fed on essential fatty acid-controlled diets

Most recent studies in term infants have shown the effects of LCPUFA supplementation on visual function and mental development. Differences in the level of supplementation and the sensitivity of outcomes measured most probably explain why some studies have not demonstrated benefits.

The first controlled randomized study compared infants fed on a formula supplemented with 0·36 g DHA/100 g total fat, a formula providing ample LNA but no DHA, and a breast-fed reference group. This study revealed delayed visual acuity at 4 and 6 months of age in the group on the formula lacking DHA (Makrides *et al*. 1995*a*). In contrast, a multicentre trial using formula supplemented with 0·2 g DHA and 0·4 g AA/100 g total fat did not show an effect of this level of supplementation on visual acuity (Auestad *et al*. 1997). A beneficial effect of DHA, AA and γ-linolenic acid (18 : 3*n*–6) supplementation on psychomotor development at 4 and 12 months but not at 24 months of age has been reported (Agostoni *et al*. 1995*a*,*b*, 1997). These authors demonstrated a strong association between the AA : LA in erythrocyte phosphatidylcholine and developmental quotient at 24 months. However, there was no relationship with the dietary intervention in the first 4 months of age (Agostoni *et al*. 1997). In an interesting model to study the effects of the amount of DHA present in human breast milk, Gibson *et al*. (1997) supplemented mothers to produce breast milk with a DHA concentration ranging from 0·1–1·7 g/100 g total fatty acids. Infants' plasma and erythrocyte phospholipid contents were related to breast-milk DHA in a saturable manner. Specifically, there was no further increase in erythrocyte DHA content when breast-milk DHA was > 0·8 g/100 g total fatty acids. At 12 months of age the developmental quotient had a low but significant ($P < 0·05$) correlation with erythrocyte DHA. This correlation was not evident at 24 months of age.

Recently, we have shown a persistent effect of DHA on visual acuity development during the first year of life in breast-fed infants and in DHA-supplemented formula-fed infants, as compared with infants randomized to formula with and without LCPUFA during the first 17 weeks of life (Birch *et al*. 1998). The formula was supplemented with 0·35 g DHA/100 g total fat with or without 0·72 g added AA/100 g total fat, from single-cell oil sources. The dietary effects on visual acuity development were evident during the first year of life using the more accurate sweep visual-evoked potential acuity, but were not demonstrable using the behavioural testing of acuity. We demonstrated changes in visual acuity and in the fatty acid composition of plasma and erythrocytes. Moreover, the differences in visual acuity were significantly ($P < 0·05$) correlated with the DHA content of erythrocyte phospholipids. The differences in visual function were most significant during periods of rapid changes in development of visual-evoked potential acuity (Birch *et al*. 1998). Fig. 5 (a,b and c) summarizes the results of this study. Willats *et al*. (1998) studied forty-four term infants fed on a DHA and AA-supplemented formula or a non-LCPUFA formula during the first 4 months. Infant cognitive behaviour was assessed at 10 months of age by a means-end problem-solving test. The LCPUFA-supplemented group had significantly more intentional solutions and scored higher than infants fed on the non-LCPUFA-containing formula. Problem-solving scores in infancy are significantly ($P < 0·05$) related to higher childhood IQ scores (Slater, 1995).

Small effects of a low LA : LNA value in formulas on weight-for-height have been reported in some studies; the relevance of these findings remains to be determined (Jensen *et al*. 1997). As previously discussed LCPUFA supplementation may have potential long-term effects on weight by regulating the expression of genes responsible for adipocyte development. Evidence of potential beneficial long-term effects of LCPUFA supplementation on brain development of term infants is highly suggestive. However,

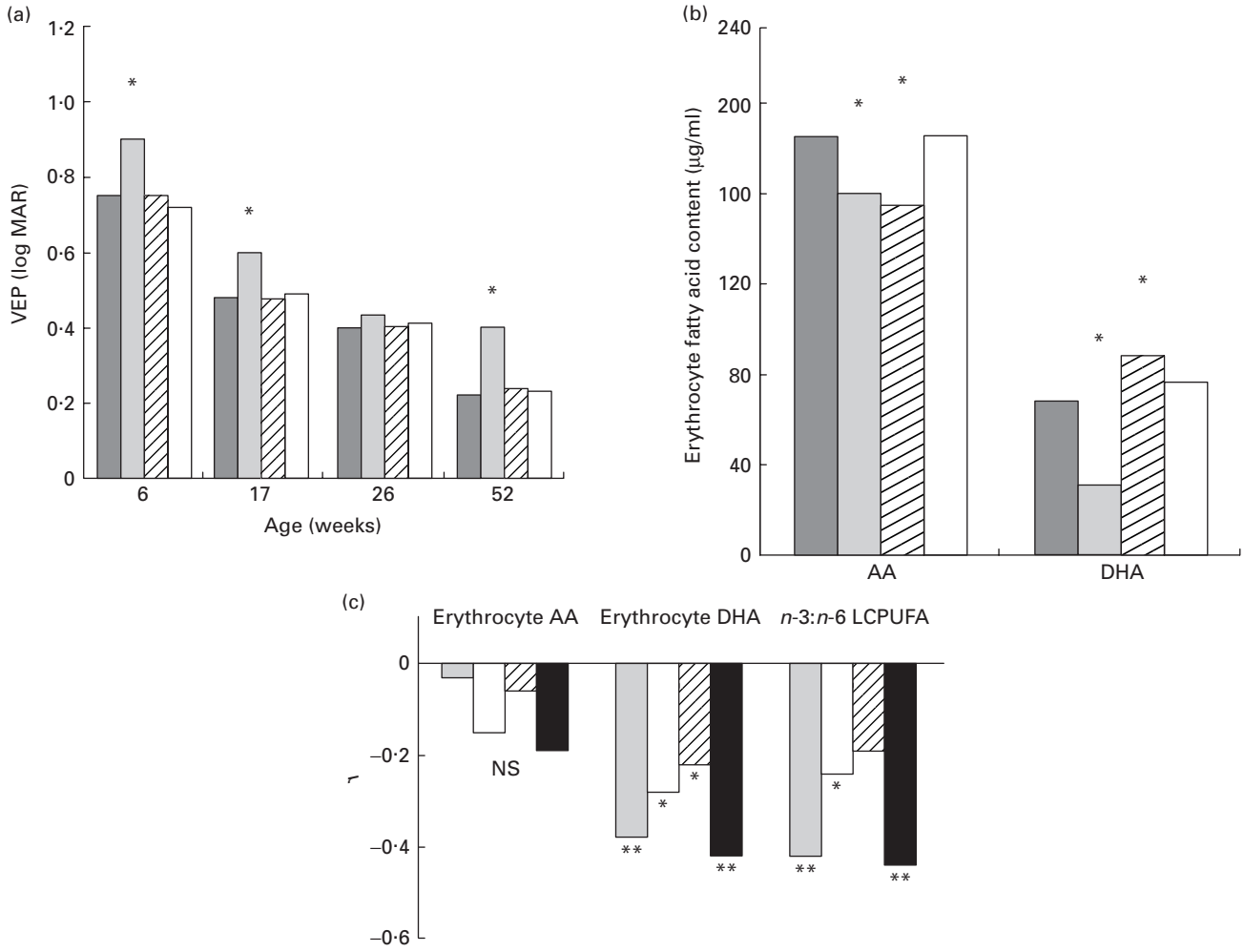

Fig. 5. (a) Visual acuities of term study infants measured using sweep visual-evoked potential (VEP) during the first year of life in relation to type of feeding (breast-fed (▒), or fed on formula alone (▒) or supplemented with docosahexaenoic acid (DHA; ▨), or DHA and arachidonic acid (AA; ▭)) for the first 4 months of life. Larger log minimum angle of resolution (log MAR) visual acuity values are associated with poorer visual acuity. The unsupplemented formula had sufficient α-linolenic acid but no long-chain polyunsaturated fatty acids (LCPUFA). (b) Fatty acid composition of erythrocytes in term infants at 4 months of age (measured as g/ml packed cells) in relation to type of feeding for the first 4 months of life. Mean values within age-groups, or for individual fatty acids, were significantly different from those for breast-fed infants (ANOVA): *$P < 0{\cdot}05$. (c) Simple correlations of sweep VEP at various ages ((▒), 6 weeks; (▭), 17 weeks; (▨), 26 weeks; (▬), 52 weeks) and erythrocyte fatty acid composition, where *n*-3:*n*-6 LCPUFA refers to LCPUFA with chain lengths longer than C_{18}. *$P < 0{\cdot}05$, **$P < 0{\cdot}01$. (Adapted from Birch *et al.* 1998.)

absolute proof is lacking. The resolution of this issue should be forthcoming when the follow-up of term infants included in the controlled randomized clinical trials of LCPUFA supplementation into the school age period are completed.

Controlled studies in preterm infants

We have also studied preterm infants receiving breast milk or randomly assigned to formulas with different EFA contents from 30 weeks gestation to early infancy. The functional impact of *n*-3 LCPUFA supplementation included enhanced maturation of the rod photoreceptor responses in LCPUFA-supplemented infants, mimicking matched breast-fed preterm infants of similar post-conceptional age (36 weeks). By 57 weeks, a time when retinal development is nearly complete, the difference in photoreceptor function was not apparent, except for changes in oscillatory potentials, which reflect inner retinal signal processing. Visual acuity tests throughout the 6-month study were also less mature in infants receiving formula devoid of *n*-3 LCPUFA, despite ample provision of LNA. The LCPUFA-supplemented group had significantly better visual acuity as measured by visual-evoked potential and forced-choice preferential looking acuity than the control-formula group. Highly significant ($P < 0{\cdot}05$) correlations were found for both visual-evoked potential and forced-choice preferential looking acuity when compared with the level of DHA in multiple lipid fractions from study infants (Birch *et al*. 1992; Uauy *et al*. 1990, 1996).

Carlson's (Carlson *et al*. 1993, 1996*a*,*b*; Werkman & Carlson, 1996) randomized clinical studies in preterm infants supplemented with LCPUFA also demonstrated better visual acuity in infants up to 4 months of age. After this time, control infants 'caught-up' in visual function measures. These investigators also reported evidence of

more rapid visual processing, as measured by the Fagan (1984) test of visual recognition, at 6–12 months of age in LCPUFA-supplemented infants. When fish oil was provided as a source of *n*-3 LCPUFA, the reduction in AA was associated with reduced weight and length gain. In a second preterm infant study using low-EPA marine oil for up to 2 months corrected age, Werkman & Carlson (1996) demonstrated improved visual development at the 2 months follow-up and a ten-point intelligence quotient difference favouring the DHA-supplemented group at 12 months. No significant drop in AA or deleterious effects on growth were observed when low-EPA marine oil was used. The DHA-supplemented group had shorter look times in the novelty preference test at 9 months, suggesting better visual processing. Present efforts are centred on evaluating the long-term effects of DHA supplementation on central nervous system development and other relevant outcomes such as body adiposity and risk for development of diet-related chronic disease. The role of DHA in myelin formation has been demonstrated in patients with generalized peroxisomal disorders, who have greatly diminished brain DHA. This factor could also be of potential importance in the white matter disease of micro-premies (Martinez & Vasquez, 1998).

Recent studies in young infants indicate that having a higher LCPUFA content in membrane phospholipids from skeletal muscle phospholipids is associated with lower fasting plasma glucose. Changes in muscle membrane phospholipid-fatty acid saturation may influence the subsequent development of insulin resistance (Baur *et al*. 1998). The prevalence of several diet-related chronic diseases, e.g. type II diabetes, Alzheimer's dementia, age-related macular degeneration, cancer and several autoimmune diseases, may be influenced by the balance of dietary *n*-6 and *n*-3 LCPUFA during the first two-thirds of lifespan as well as during old age (Fernandez & Venkatraman, 1993; Folsom *et al*. 1996). Changes in membrane composition induced by dietary changes can be measured by GC fatty acid analysis of plasma or erythrocyte total lipid, or lipid sub-fractions, or lipids extracted from scraped buccal mucosal cells (Hoffmann *et al*. 1999). The study of infants who died suddenly of an unexplained cause has served to document a strong correlation between the composition of the brain cortex and erythrocyte total lipids. In addition, the fatty acid composition of brain phospholipids is clearly affected by early dietary LCPUFA supply (Farquharson *et al*. 1992, 1995; Byard *et al*. 1995).

Essential fatty acid supply in early life to promote optimal growth and development

Essential fatty acid supply in fetal life

The fetus and the placenta are fully dependent on maternal EFA supply for their growth and development. The major fat deposition in the human fetus occurs during the third trimester, but key phospholipids in placental vessels and uterine vasculature are dependent on EFA supplied by the mother for eicosanoid formation from the moment of conception (Ongary *et al*. 1984; Honstra *et al*. 1996).

Maternal dietary LA and LNA supply serve as precursors for *n*-3 and *n*-6 LCPUFA synthesis by the maternal liver. The placental transfer of fatty acids is regulated in part by the transplacental fatty acid gradient. Serum albumin concentration and α-fetoprotein have a high binding affinity for free fatty acids, thus may be important for placental fatty acid transfer (Dutta Roy, 1997; Kimura, 1998). Mammalian fetuin is a placental protein with a 50-fold greater efficiency in binding fatty acids relative to albumin. Lipoprotein lipase on the maternal surface of the sincytiotrophoblast hydrolyses maternal triacylgycerol releasing free fatty acids. Fetal erythrocytes also appear to perform a significant role in the placental DHA transfer (Kimura, 1998). There is a progressive enrichment in the concentration of AA and DHA in circulating lipids in the fetus during the third trimester, at a time when fetal demands for vascular and especially neural growth are greatest (Van Houwelingen *et al*. 1996). Significant increases in the AA and DHA content of fetal brain tissue during the last trimester of gestation and initial postnatal months have also been observed (Clandinin *et al*. 1980). A total of 600 g of EFA are transferred from mother to fetus during a full-term gestation. The average net uptake is approximately 2·2 g/d. AA and DHA are supplied to the fetus from the maternal diet and by endogenous fetal biosynthesis (liver desaturation and elongation).

Studies conducted in different populations using similar methodology have shown that the pattern of change in fatty acid composition during gestation and pregnancy is similar across different ethnic and diet groups (Otto *et al*. 1997). Populations with higher maternal plasma concentrations of *n*-6 fatty acids have a lower *n*-3 fatty acid content and *vice versa*. DHA concentrations decrease during late gestation, and cord levels of *n*-6 fatty acids in term infants are less dependent on maternal levels than cord DHA. Most *n*-3 fatty acids which come into the fetal circulation will be accrued by the fetus despite low maternal *n*-3 concentrations. A need for LCPUFA supplementation during pregnancy is therefore suggested by these results. This type of supplement may be particularly important for populations with a low *n*-3 EFA intake, for multiparous women, or during multiple pregnancies (Foreman-van Drogelen *et al*. 1995; Honstra *et al*. 1996). The changes in maternal plasma and erythrocyte fatty acid content are paralleled by changes in fetal levels, measured by cordocentesis. The latter are similar to values obtained at birth, except for a significantly higher cord LA level measured during centesis (Van Houwelingen *et al*. 1996). The results confirm that for fetal DHA, relative blood content and absolute concentration increase with gestational age. In the case of AA, levels increase or decrease with gestation depending on the study. It should be noted that there is a significant positive correlation between AA and birth weight (Al *et al*. 1990, 1995). Differences in fatty acid profiles at 34 weeks gestation have been demonstrated in women delivering preterm babies relative to those delivering term babies. Erythrocyte and plasma AA are higher in mothers delivering preterm babies. The comparison suggests that maternal AA mobilization and availability may be altered in women delivering preterm infants. Indicators of *n*-3 EFA deficiency in preterm maternal erythrocytes and amniotic membranes are depressed. Preterm maternal *n*-3 : *n*-6 fatty acid values

suggests that perinatal *n*-3 EFA metabolism plays an important role in the pathogenesis of pre-term birth (Reece *et al.* 1997). This factor is of special significance since AA-derived PG play an important role in the onset of labour, they are also used to induce abortion and labour. In contrast, *n*-3 fatty acids may retard the onset of labour, as shown by a randomized controlled trial of fish oil supplementation in human subjects. This trial demonstrated longer gestation with higher birth weight in the *n*-3-supplemented group as compared with a control group receiving olive oil (Olsen *et al.* 1992).

Breast milk as a reference for the fatty acid composition of infant formulas

A good starting point to define the optimal fatty acid composition of infant formulas is to mimic the composition of human breast milk. Unfortunately, the lipid composition of breast milk varies greatly depending on the mother's diet during pregnancy and lactation, postpartum age, preterm or term delivery and maternal diseases affecting lipid metabolism (diabetes, cystic fibrosis and abetalipoproteinaemia). AA is the predominant *n*-6 LCPUFA and DHA is the most important of the *n*-3 LCPUFA in breast milk. Total *n*-6 : *n*-3 is 5 : 1–10 : 1, ranging up to 18 : 1 if maize, sunflower or safflower oils are consumed. AA : DHA is most commonly 1 : 1–2 : 1. EPA is found in minimal amounts, except in populations having high fish intakes (Jensen, 1996). DHA levels range from approximately 0·1 g/100 g total fat reported from Germany to 1·4 g/100 g total fat in Inuits of North America; however, typical values range from 0·3 to 0·4 g/100 g total fat (Innis, 1992; Jensen, 1996). Makrides *et al.* (1995*b*) reported a longitudinal reduction in breast-milk DHA of Australian women on Western diets from 0·32 g/100 g total fat in 1981 to 0·21 g/100 g total fat in 1995.

The definition of what to feed is not simply answered by deciding to follow the breast-milk model. Deciding how much to feed based on the observed range of LCPUFA content, the lower range, or the mid-point must be based on biological responses. If the effort is primarily focused on demonstrating efficacy, selecting a value in the upper range is preferable. On the other hand, if serious safety concerns were an issue, selecting a value in the lower range would be more appropriate. Supplementation of formula-fed preterm infants with graded levels of AA (0–1·1 g/100 g total fat) and DHA (0–0·76 g/100 g total fat) compared with breast-fed babies demonstrated that plasma and erythrocyte LCPUFA levels can be mimicked by supplementing formula at similar levels to those present in breast milk. These levels translated into 0·54 g AA and 0·3 g DHA/100 g total fat for omnivorous women (Gibson *et al.* 1997). Most LCPUFA-containing formulas presently used have 0·2–0·4 g DHA and 0·6–0·8 g AA/100 g total fat.

Sources of essential fatty acids for use in the infant formulas

The main source for the *de novo* synthesis of *n*-3 fatty acids in the aquatic environment are marine autotrophic bacteria, microalgae and protozoa which constitute the zooplankton and phytoplankton (Iwamoto & Sato, 1986; Cohen *et al.* 1995). Fish, which are higher in the food chain, incorporate the *n*-3 PUFA and further elongate them to form EPA and DHA. Thus, fish will concentrate EPA and DHA as triacylglycerols, mainly in the adipose tissue and in the fat of muscle and visceral organs. The higher the fat content of fish, the higher its content of *n*-3 fatty acids (Ackman, 1964; Akimoto *et al.* 1990). Another important source of LCPUFA used is egg-yolk phospholipids. The concentrations of PUFA are different depending on the feed given to animals, the ample use of fish meal in chicken feed has increased egg-yolk DHA (Simopoulus & Salem, 1992; Sawatzki *et al.* 1994). LCPUFA products for use in infant formulas can be successfully produced from egg if chicken feed is carefully monitored and refined lipid-extraction procedures are used. Egg-yolk phospholipids are presently an important LCPUFA source used in some infant formulas. Bacterial strains and microalgae isolated from the intestinal content of some fish show a remarkably high content of EPA and DHA (Akimoto *et al.* 1990; Cohen *et al.* 1995). Efforts to grow these micro-organisms in natural or artificial sea water to obtain DHA for nutritional or pharmacological use have been successful. In addition, selected fungal strains produce concentrated AA which is suitable for human consumption. The industrial production of AA, EPA and DHA from strains of single-cell organisms has lead to an expanded use of this source. Single-cell oils offer a promising new source of LCPUFA, provided sustained mass production becomes commercially feasible (Iwamoto & Sato, 1986). Rigorous purity and toxicological testing should be conducted on fatty acid sources intended for use in commercial infant formulas. Initial studies used a mixture of vegetable oils to supply LA and LNA, and marine oil as a source of *n*-3 LCPUFA. More recent studies, including our own, have used nearly pure DHA from marine oil fractions or DHA and AA from single-cell oils. Most published work to date is based on marine oil, marine-oil fractions, egg phospholipids and single-cell oils as sources.

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