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Effect of Docosahexaenoic Acid on Membrane Fluidity and Function in Intact Cultured Y-79 Retinoblastoma Cells[1]

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Considerable metabolic energy is expended in ensuring that membranes possess a characteristic fatty acid composition. The nature of the specific requirement of the retina for high levels of docosahexaenoic acid (DHA) is as yet undefined. Previous work has speculated that DHA is required to maintain the fluid nature and permeability necessary for optimal retinal function. Cultured Y-79 retinoblastoma cells were grown in serum-containing media with and without supplemental DHA. Resultant changes in membrane fluidity were assessed using fluorescent probes. No differences were observed in rotational probe mobility as assessed by fluorescence polarization despite a fourfold increase in cellular DHA content. Lateral probe mobility as assessed by pyrene eximer formation was significantly enhanced in DHA-supplemented cells. Both the DHA content and total fatty acid unsaturation index in retinoblastoma cells were directly correlated with membrane fluidity as reported by eximer formation (Pearson's rho = 0.96 and 0.92, respectively). DHA supplementation also resulted in a significant increase in cellular choline uptake. We speculate that the effect of DHA content on retinal function may be mediated by changes in membrane fluidity and associated enzyme and transport activities.

Recent studies provide clear evidence that long chain ω-3 fatty acids are essential for optimal function of the developing retina and brain. ω-3 fatty acid deficient diets have been shown to adversely affect learning capacity and electroretinograms in both rats (1) and nonhuman primates (2, 3). Additionally, we have recently confirmed in a controlled double-blind study the adverse effects on visual cortical and electroretinographic findings of available preterm infant formulas deficient in long chain ω-3 fatty acids fed to very-low-birth-weight newborns (4).

It appears that normal tissue levels of long chain ω-3 fatty acids are necessary during gestation or early infancy to support the development of proper retinal function. The recovery of the content of long chain ω-3 fatty acids in the brain and retina is extremely slow after reintroduction of these fatty acids following a dietary deficiency (5, 6). Moreover, in monkeys, the electroretinographic abnormalities induced by long chain ω-3 fatty acid deficiency were not reversible despite restoration of retinal ω-3 fatty acids to normal or supranormal levels (7). The brain and retina of human infants at birth are less well developed than those of nonhuman primates; thus, human infants may be even more vulnerable to postnatal dietary deprivation of ω-3 fatty acids (8). The ω-3 fatty acids present in mammalian tissues are primarily in the form of docosahexaenoic acid (DHA),[3] 22:6 ω-3 (9). DHA is the most unsaturated membrane fatty acid present in biological systems. It accounts for a small percentage of the fatty acid content of many tissues, but comprises 30–40% of the phospholipids of the cerebral cortex and retina (10). Within the retina, DHA is concentrated in highly specialized membranes that make up the photoreceptor outer segment. Rhodopsin, the photosensitive pigment of the rod photoreceptor, is tightly bound to the DHA-laden phospholipids that comprise the outer segment disk

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[3] Abbreviations used: DHA, docosahexaenoic acid; FBS, fetal bovine serum; BCS, bovine calf serum; BSA, bovine serum albumin; PBS, phosphate-buffered saline; DPH, 1,6-diphenyl-1,3,5-hexatriene; TMA–DPH, 1-(4-trimethylammoniumphenyl)-6-phenyl-1,3,5-hexatriene; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

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membranes (9). The specific role of DHA is not clearly understood, but its chain length and high degree of unsaturation suggest a role in maintaining a membrane with the fluid nature and permeability required for the dynamic behavior of the components of the visual cascade. The visual cascade begins with the absorption of a photon by rhodopsin which then interacts with numerous transducin molecules (13). In order for a single protein to mediate multiple interactions, a highly fluid membrane environment would be required. Indeed, the lateral diffusion coefficient for rhodopsin as determined by fluorescence photobleaching and recovery measurements has been reported to result in a diffusion rate an order of magnitude faster than that of most other cell surface proteins (14).

The effect of increasing fatty acid chain length and number of double bonds on membrane fluidity has been evaluated by many investigators using a variety of techniques. During the past decade, fluorescence methodologies have been among the most widely utilized approaches to characterizing the physical state of membranes. In particular, fluorescence probes which partition preferentially into membranes have been extensively used to monitor lipid fluidity (15, 16). The exact interpretation of fluidity data at the molecular level is still an area of active research, but it is clear that these methods do give valuable information regarding the physical state of membranes. The vast majority of fluorescent probe studies have been conducted in model membranes or membrane preparations.

It was our hypothesis that an increase in cellular DHA content would increase cellular fluidity, resulting in functional changes. The purpose of this study was to utilize current fluorescence technologies to evaluate the role of DHA in supporting the fluid nature of the membrane in a relevant intact retinal cell model and relate changes in membrane fluidity to membrane functional changes using cellular choline uptake as an example.

MATERIALS AND METHODS

Cell culture. Y-79 retinoblastoma is a human tissue cell line derived from the photoreceptor cells of the retina (17). Y-79 cells, obtained from American Type Culture Collection (Rockville, MD), were cultured as a suspension in RPMI 1640 media (GIBCO, Grand Island, NY) containing 10% heat-inactivated bovine serum, 100 units/ml penicillin, 100 μg/ml streptomycin, and 2 mM glutamine. The cells were grown in 25-cm^2 tissue culture flasks in an incubator maintained at 37°C with 5% CO_2 in humidified air. Some studies were conducted with cells grown in bovine calf serum (BCS) (Hyclone Laboratories, Logan, UT) containing media rather than fetal bovine serum (FBS) (GIBCO) due to the lower intrinsic DHA content of calf serum. Cells were harvested for fatty acid analysis and fluorescence spectrophotometry 72 h after subculture. DHA-supplemented cells (+DHA) were grown in media enriched with 20 μM DHA. "−DHA" is subsequently used to designate unsupplemented cells. The sodium salt of the fatty acid was obtained from NuCheck Prep (Elysian, MN), dissolved in distilled water, and complexed with bovine serum. All media containing supplemental DHA were sterilized by passage through a low-binding 22-μm Millipore filter. Loss of DHA by filtration of the media was less than 6%.

Fatty acid analysis. The cells were harvested by centrifugation and washed twice with a buffer containing 10 mM sodium phosphate, 0.15 M NaCl, and 11 mM glucose, adjusted to pH 7.4. The cells were resuspended in 100 μl distilled water and disrupted by a freeze–thaw technique with liquid nitrogen. Fatty acid methyl esters were prepared by direct methylation of a cell suspension utilizing the method described by Lepage and Roy (18). Separation and quantification of fatty acid methyl esters were accomplished using flame ionization detector capillary gas chromatography and comparing their retention times with standards as previously described for plasma and red blood cell fatty acids (4). The integrated chromatographic data are typically stored on a computer and then processed with customized software which allows for semiautomated identification of fatty acid peaks. Data are compiled on a spreadsheet for inspection and subsequent statistical analysis. The relative concentrations of individual fatty acids are expressed as percentages of total fatty acid equal to or greater than 14 carbons in chain length. To analyze DHA in the media, lipids were extracted from +DHA or −DHA media (5 ml) by the method of Bligh and Dyer (19) and concentrated by evaporation under nitrogen, and the fatty acids were methylated and quantitated by gas chromatography (4).

DPH fluorescence polarization photometry. Initial studies on the effect of supplemental DHA on membrane fluidity utilized steady-state fluorescence measurements of 1,6-diphenyl-1,3,5-hexatriene (DPH) and 1-(4-trimethylammoniumphenyl)-6-phenyl-1,3,5-hexatriene (TMA–DPH). These fluorescent probes (Molecular Probes, Eugene, OR) localize to different depths within the plasma membrane and are used to evaluate the rotational mobility of the probe within the membrane (20). Cells used in the fluidity analysis studies were collected and washed as described above. Each sample contained 1×10^6 cells in 1 ml of buffer. Preliminary studies indicated that this combination of buffer and cell number resulted in extremely low intrinsic fluorescence and minimal light scattering (results of this validation not shown). Probes were inserted into membranes of intact cells by incubation in the dark for 30 min at room temperature. The DPH and TMA–DPH incubation mixtures were made by addition of the probes in 2% tetrahydrofuran/93% ethanol/5% water to the intact cells in buffer to obtain DPH or TMA–DPH concentrations of 7 μM. The polarization studies were conducted at multiple temperatures. Representative results of the studies performed at 24°C are presented here.

Excitation of the fluorescently labeled sample in 3 × 3-mm quartz microcuvettes was accomplished with a xenon lamp beam passed through a monochrometer set to a 364-nm line. A Corning 7-60 broad band-pass filter placed in the excitation pathway was utilized to reduce parasitic light. The intensity of the lamp beam was attenuated with neutral density filters to avoid photodecomposition of the probe. The samples were placed in a thermostated cell holder of a custom-built T-format polarization photometer equipped with Glan–Taylor calcite polarizers. A circulating water bath kept the cell holder at a constant temperature. The emission was viewed through Schott KV399 and Corning 061 cutoff filters (λ_{em} > 430 nm) and detected with high-sensitivity photomultiplier tubes (EMI 9789QB) and photon-counting electronics from ISS (Champaign, IL). The output from the two photon counters corresponding to the parallel and perpendicular emissions relative to the plane of exciting polarized light was processed by an IBM XT equipped with customized software to calculate fluorescence polarization. Polarization was calculated according to the equation (21)

$$P = \frac{I_{\parallel} - I_{\perp}}{I_{\parallel} + I_{\perp}},$$

where $I_{\parallel}$ and $I_{\perp}$ are the intensities corresponding to the parallel and perpendicular polarized components of the emission. Typically, four readings were averaged for each determination and the averages and standard deviations calculated by on-line software. Polarization, as a measure of the structural ordering of the lipids of a membrane bilayer, is inversely proportional to membrane fluidity and is reported in mPolarization units (Polarization units × 10^3).

Pyrene spectrofluorometry. Pyrene eximer formation was used as an index of lateral mobility in Y-79 cell membranes. The pyrene incubation mixtures were made by adding 8 μl of a 0.7 mM solution of 1-pyrene dodecanoic acid (Molecular Probes) in 50% tetrahydrofuran/50% di-

TABLE I

Fatty Acid Composition of Serum-Containing RPMI 1640 Media with and without DHA Supplementation[a]

	Fetal bovine serum (n = 4)		Bovine calf serum (n = 3)	
	−DHA	+DHA	−DHA	+DHA
Saturated	37.4 ± 2.2	35.3 ± 3.0	26.9 ± 2.0	26.8 ± 1.3
Monounsaturated	27.9 ± 1.6	26.6 ± 0.9	18.4 ± 0.4	17.1 ± 0.3
18:2 ω-6	6.53 ± 1.11	5.94 ± 0.68	39.3 ± 1.1	38.9 ± 1.0
20:4 ω-6	8.81 ± 0.81	8.25 ± 0.65	4.13 ± 0.04	4.24 ± 0.29
22:5 ω-6	0.74 ± 0.45	0.56 ± 0.12	0.38 ± 0.06	0.35 ± 0.02
22:6 ω-3	2.79 ± 0.98	10.10 ± 0.98*	0.94 ± 0.29	3.21 ± 0.50*
Other ω-6	6.3	5.1	7.4	6.9
Other ω-3	1.9	2.5	1.1	1.8
20:3 ω-9	0.41	0.32	0.24	0.19
Unknown	7.22	5.33	1.2	0.5

[a] Fatty acid methyl esters were prepared, separated, and quantified by capillary gas chromotography. Relative concentrations of individual fatty acids are expressed as a percentage of total fatty acid equal to or greater than 14 carbons in chain length. Values are mean ± SD.

* The +DHA media value is significantly different from the −DHA media value, $P < 0.001$.

methyl sulfoxide to 1 ml of buffer containing 1×10^6 cells for a final probe concentration of 5.8 μM. The precise molarity was determined by absorption spectroscopy. The studies were conducted at 5 and 24°C. The eximer formation spectra were obtained using an SLM 8000 spectrofluorometer (SLM Instruments, Urbana, IL). Fluorescence of the sample was excited using a xenon lamp beam passed through a monochrometer set at 340 nm. Emission spectra (λ_{em} = 360–600 nm) were detected with a high-sensitivity photomultiplier tube (EMI 9635QA) which converted the light intensity to a proportional dc current that was amplified and processed by an IBM AT equipped with customized software to measure the peak heights of interest. Negligible interference of pyrene emission spectra due to intrinsic fluorescence of cellular macromolecules was observed by running spectra of +DHA and −DHA cell preparations without the addition of pyrene. By convention, the fluorescence intensity ratio of eximer to monomer is calculated from the ratio of fluorescence at 478 nm vs that at 398 nm. The higher the ratio the greater the lateral mobility of the probe within the membrane.

Cellular uptake of choline. Cells were collected by centrifugation and washed twice with Dulbecco's phosphate-buffered saline (PBS) containing 0.3% fatty acid-free bovine serum albumin (BSA) and resuspended in Dulbecco's PBS containing 20 mM 4-(2-hydroxyethyl)-1-hydroxyethyl)-1-piperazineethanesulfonic acid (Hepes). Each 200-μl cell sample contained 0.5×10^6 cells in 1.5-ml Eppendorf microcentrifuge tubes. The cells were incubated with 0.2 μCi/50 nmol [*methyl*-H^3]choline chloride (Amersham, Arlington Heights, IL) in a shaking water bath at 37°C for 30 min. The incubations were terminated by dilution with ice-cold PBS containing 0.3% BSA. Cell suspensions were centrifuged at 13,000*g* for 1 min and the supernatant was removed by aspiration. The cells were similarly washed and centrifuged with aspiration of the supernatant three more times. The resulting cell pellets were placed in scintillation vials, dispersed in 5 ml scintillant (Budget-Solve; Research Products International Corp., Mount Prospect, IL) by a bath sonicator for 5 min, and vortexed, and the radioactivity was measured with a Tracor Analytic Mark III liquid scintillation system. Cellular choline uptake is reported as pmol/10^6 cells/h.

Statistical analysis. Statistical significance between values for cells maintained in the presence or absence of supplemental DHA was evaluated by Student's t test. Pearson's rho correlation coefficients were determined using Kwikstat software.

RESULTS

Fatty Acid Analysis

The results of fatty acid analysis of the BCS- and FBS-supplemented media are given in Table I and indicate that the only significant variable between the Y-79 cells grown in +DHA vs −DHA media was the 22:6 ω-3 content. In order to obtain large differences in the cellular DHA content, sera with high (FBS) and low (BCS) intrinsic DHA levels were utilized. In all cases, the +DHA media were derived from an aliquot of bovine serum-containing media to which DHA was added to obtain a final concentration of 20 μM. The commercial preparation of BCS (Hyclone) had a sixfold higher level of linoleic acid compared to FBS. These differences are inherent to the source of the sera.

As estimated by trypan blue exclusion, there was no reduction or enhancement of viability in cells supplemented with 20 μM DHA. Table II summarizes the fatty acid composition of the Y-79 cells 72 h after subculture. Enrichment of the media with 20 μM DHA resulted in a four- to fivefold increase in cellular DHA. This large increase in DHA content was primarily responsible for the significant increase in the unsaturation index reported in the table. DHA supplementation also resulted in slight accumulation of saturated fatty acids but reduced amounts of monounsaturates and 22:5 ω-6 in cellular lipids. 20:3 ω-9, a typical marker for essential fatty acid deficiency, was slightly reduced in the DHA cell groups.

Fluidity Analysis

The results of the steady-state polarization analysis of DPH and TMA–DPH are depicted in Fig. 1. These studies were performed only on retinoblastoma cells grown in FBS-containing media. No significant differences in fluorescence polarization could be detected using either probe at any temperature despite a four- to fivefold cellular enrichment with DHA. In separate experiments at 15, 20, 25, 30, and 35°C, done in quadruplicate, neither DPH nor TMA–DPH was able to report membrane fluidity differences in Y-79 cells treated with 0 or 20 μM DHA for 72 h.

A fluorescence-excited pyrene molecule in close proximity to another pyrene molecule in its ground state may form a dimer in an excited state (eximer) (22). The fluorescence emitted by the eximer is broad, unstructured, and red-shifted with respect to the monomer emission and is thus readily distinguishable from the monomer. Since an eximer can form only if the excited pyrene molecule nears one in the ground state, the relative yield of eximeric and monomeric fluorescence is a measure of the lateral mobility of the pyrene probes within the membrane. The increase in the eximer/monomer ratio and thus, lateral mobility, is evident in Y-79 cells maintained in BCS + DHA compared to cells grown in BCS − DHA (Fig. 2).

In contrast to fluorescence polarization analysis, the elevation of polyunsaturated fatty acids does appear to significantly increase membrane fluidity as measured by the eximer/monomer ratio. Significant differences were noted between enriched and unenriched cells grown in FBS- or BCS-containing media at both study temperatures (Fig. 3). Greater differences in membrane fluidities between DHA-supplemented and unsupplemented BCS-grown Y-79 cells compared to FBS-grown cells may reflect the greater differential content of cellular DHA (5.6-fold difference in +DHA vs −DHA BCS cells vs 4.0-fold in FBS cells). Furthermore, the DHA content in treated and untreated retinoblastoma cells directly correlated with pyrene eximer formation (Pearson's rho correlation coefficient = 0.96 at both 5 and 24°C). Similarly, the unsaturation index for total cellular fatty acids was highly correlated with the eximer/monomer ratio at both temperatures (Pearson's rho = 0.92). Also of interest is that the increase in membrane fluidity is of the same magnitude for a 4- to 5-fold increase in cellular DHA content as it is for a temperature increase from 5 to 24°C. This would suggest that increasing DHA content and its resultant enhancement of lateral mobility might have a significant impact on cellular function.

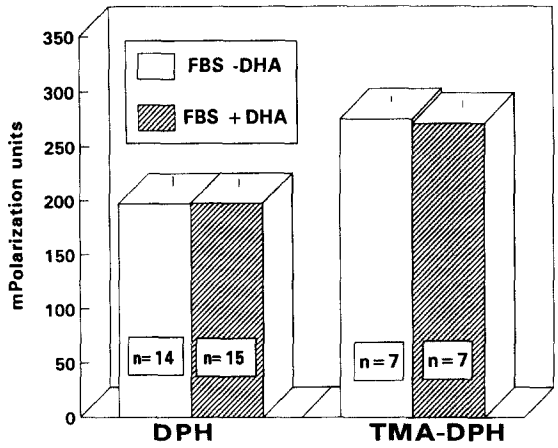

FIG. 1. Summary of fluorescence polarization studies in Y-79 cells using DPH and TMA–DPH. Fluorescence measurements were performed at 24°C. "Fluidities" of DHA-supplemented and unsupplemented cells, maintained in fetal bovine serum (FBS) containing media, were not statistically different. Bars and posts are means ± SD.

Choline Uptake

Choline uptake was measured in intact cells to confirm a functional change in DHA-enriched, fluidity-enhanced cells. These studies were performed only in cells grown in BCS-containing media. Figure 4 shows that a 5.6-fold cellular DHA enrichment significantly enhances [H^3]-choline uptake. Choline uptake was increased by 20% in those cells enriched with 20 μM DHA.

TABLE II

Fatty Acid Composition of Y-79 Cells Grown in Media With and Without DHA Supplementation[a]

	Fetal bovine serum (n = 3)		Bovine calf serum (n = 4)	
	−DHA	+DHA	−DHA	+DHA
Saturated	36.0 ± 1.0	39.9 ± 0.5**	36.4 ± 1.0	39.2 ± 0.8**
Monounsaturated	32.3 ± 0.6	25.4 ± 0.4*	29.9 ± 2.2	23.5 ± 1.5**
18:2 ω-6	0.88 ± 0.02	0.97 ± 0.04	5.90 ± 0.15	6.89 ± 1.32
20:4 ω-6	9.98 ± 0.30	11.43 ± 0.36	13.15 ± 0.88	14.76 ± 1.32
22:5 ω-6	2.41 ± 0.21	0.91 ± 0.07*	2.99 ± 0.15	0.64 ± 0.37*
22:6 ω-3	2.92 ± 0.19	11.61 ± 0.41*	1.18 ± 0.18	6.64 ± 0.83*
Unsaturation index	126.6 ± 4.7	169.9 ± 7.1*	129.7 ± 8.1	151.4 ± 3.4**
Other ω-6	4.8	3.6	4.5	2.0
Other ω-3	2.3	2.8	1.3	1.4
20:3 ω-9	1.1	0.6	0.8	0
Unidentified	6.7	2.7	3.8	4.9

[a] Fatty acid methyl esters were prepared, separated, and quantified using capillary gas chromatography. The relative concentrations of individual fatty acids are expressed as a percentage of total fatty acid equal to or greater than 14 carbons in chain length. The unsaturation index was calculated as the sum of (number of double bonds for each fatty acid × % of each fatty acid). The values represent mean ± SD.

* The + DHA value is significantly different from the −DHA control, *$P < 0.001$.

** $P < 0.005$.

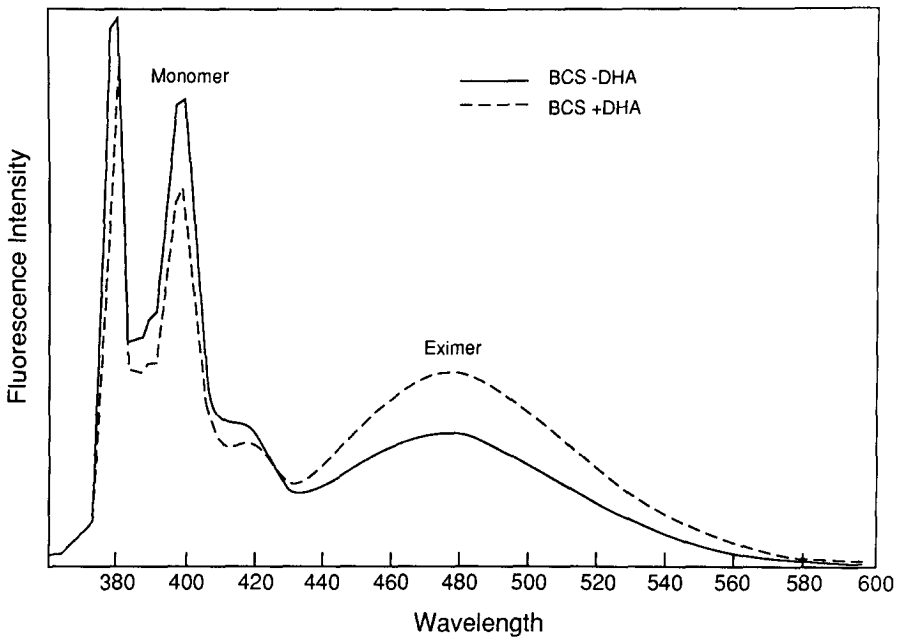

FIG. 2. Typical emission spectra for DHA-supplemented and unsupplemented cells maintained in bovine calf serum (BCS). The eximeric emission is broad, unstructured, and red-shifted relative to the monomeric emission. The fluorescence intensity ratio is calculated from the ratio of the peak height of the eximeric emission to the peak height of the monomeric emission.

DISCUSSION

Modification of the fatty acid composition of cultured cells by altering the lipid contained in the growth medium has been achieved by numerous investigators (23, 24). This study confirms previous observations that cultured retinoblastoma cells readily incorporate preformed DHA (25). The level of DHA enrichment of these cells is quite similar to that reported by other investigators (26). The accumulation of saturated fatty acids in the +DHA cells suggests that cellular enrichment with DHA inhibits the metabolic pathways for fatty acid elongation and desaturation. This concept is further supported by a 60–80% reduction in the 22:5 ω-6 content in +DHA cells compared to that in −DHA cells. Evidence for active elongation and desaturation was found in −DHA cells, where the 22:5 ω-

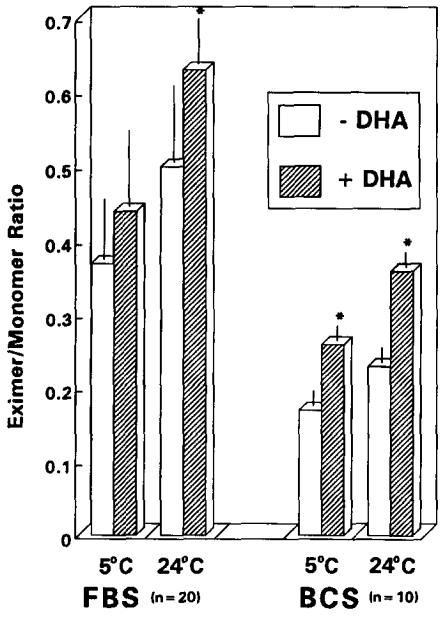

FIG. 3. Summary of pyrene eximer formation studies in Y-79 cells grown in DHA-supplemented and unsupplemented FBS (A) or BCS (B) containing media. Fluorescence measurements were performed at 5 and 24°C. Significant differences in eximer formation were noted between DHA-supplemented and unsupplemented cells at both temperatures by t test, (*) $P < 0.001$. Bars and posts are means ± SD.

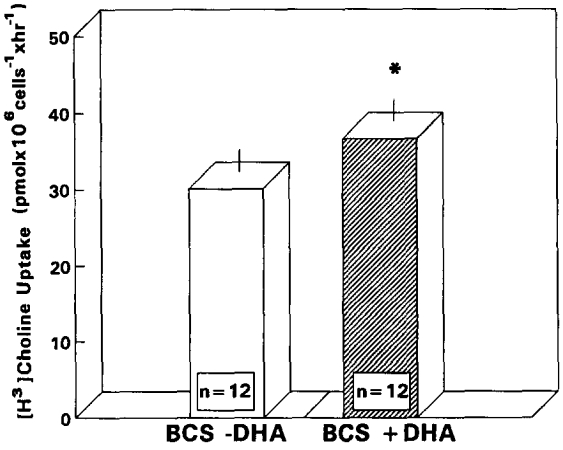

FIG. 4. [*methyl*-H³]Choline uptake in Y-79 cells grown in DHA-supplemented and unsupplemented BCS-containing media. Significant differences in choline uptake were noted between supplemented and unsupplemented cells by t test, (*) $P < 0.001$. Bars and posts are means ± SD.

6 level was several-fold higher than that of the media. This finding suggests that Y-79 cells maintained in the presence of limited DHA are preserving unsaturation by accumulating the end product of ω-6 fatty acid elongation and desaturation. This line of thought is supported by the rat studies of Tinoco *et al.* (27). They demonstrated that there was normally little 22:5 ω-6 in the retina, but when dietary 18:3 ω-3 restriction lowered the DHA content of the retina, it was found that DHA was replaced one for one with 22:5 ω-6. The replacement of long chain ω-3 fatty acids in the retina with 22:5 ω-6 has been demonstrated to have adverse visual functional implications in at least two different animal models (28, 29). The mechanism for these functional changes is not clearly understood. Thus the present studies address the hypothesis that higher cellular levels of DHA would support a higher degree of cellular fluidity and result in significant functional changes.

Phospholipid metabolism in Y-79 retinoblastoma cells has been extensively studied by Spector and co-workers (23, 25, 26, 30, 31). Yorek *et al.* (25) report that greater than 90% of radiolabeled DHA accumulates in cellular phospholipids after a 48-h incubation and that about 80% of the label accumulated in ethanolamine phosphoglycerides. It was further noted that only 3–4% of the DHA was retroconverted to eicosapentaenoic acid and this occurred primarily in phosphatidylinositol/phosphatidylserine classes. This is consistent with the apparent unique characteristic of Y-79 cells of readily elongating and desaturating parent essential fatty acids to long chain polyunsaturated derivatives (26). Due to the localization of highly unsaturated species of ethanolamine, inositol, and serine phosphoglycerides in the inner leaflet of the plasma membrane of numerous cell types (32), DHA is likely to be similarly localized in Y-79 retinoblastoma cells. The juxtaposition of hexaenoic phospholipid species with enzymes and proteins constitutive to nutrient (Fig. 4) and ion (32) transport systems supports a vital role for DHA as a modifier of the visual cascade.

Steady-state fluorescence polarization has been a useful tool in membrane fluidity assessment. Despite a fourfold cellular enrichment with DHA, no significant differences in rotational mobility of the DPH probes could be ascertained. These results are in agreement with the findings of Stubbs *et al.* (33) who reported no change in steady-state DPH polarization of phosphatidylcholine bilayers with different numbers of double bonds in the phospholipid sn-2 chain. In contrast, these findings differ from fluorescence polarization reports on the effect of polyunsaturated fatty acid membrane modification on fluidity of intact rabbit sarcoplasmic reticulum (34) and rat synaptic membranes (35).

Our study differs from those mentioned above in its use of intact cultured cells. DPH polarization values of intact cells represent a measure of the average fluidity of all lipids present in the cell (36). Additionally, intracytoplasmic lipid droplets, as encountered in cells grown in serum-containing media, may mask true changes in membrane fluidity induced by changes in membrane phospholipid fatty acid composition. Intracytoplasmic neutral lipid pools decline rapidly during the first 48 h after subculture (37); thus we attempted to counter their effect by studying the cells at the 72-h time point. Similar polarization analyses were performed at 24- and 48-h time points with no significant difference in polariziation values between supplemented and unsupplemented cells.

A remaining difficulty in interpretation of DPH fluorescence polarization data is that it is not certain where in the membrane DPH is located or if it is located in several places (33). Selective distribution in highly fluid regions of the membrane would tend to minimize any increases in fluidity induced by DHA enrichment. In addition, TMA–DPH is a fluorescence probe considered to localize primarily at the cell surface, yet no effect on TMA–DPH rotational mobility was observed in DHA-enriched cells. Our results with the TMA–DPH fluoroprobe (Fig. 1) at first appear to conflict with those of Yorek *et al.* (38) who found an increase in membrane fluidity in DHA-enriched Y-79 cells compared to controls. However, by using serum-free media, these investigators were able to obtain a 15-fold increase in the DHA content and a 2-fold increase in fatty acid unsaturation in treated retinoblastoma cells vs controls. Discrepancies between the two studies in Y-79 membrane rotational mobility may be based on the sensitivity of the TMA–DPH response to the absolute cellular content of DHA, the total change in the DHA level compared to controls, or the unsaturation of cell membrane lipids.

Several studies have been published using pyrene probes to evaluate membrane fluidity, but pyrene fluidity data for intact cells are rare (39–41). Early studies suggested that the lateral mobility of the probe molecule in an artificial lipid bilayer did not depend on the hydrocarbon chain length or the number of double bonds in fatty acids of the bilayer (22). These studies, however, did not include phospholipids with a fatty acid chain length greater than 18 carbons or more than two double bonds. Additionally, these studies were performed at temperatures at or greater than 50°C, at which differences in fluidity would be minimized by starting with a highly fluid environment.

The elevation in cellular fluidity resulting from increases in temperature as reported here is similar to the findings with pyrene probes published by Dix *et al.* (41). They documented an increase in eximer formation in Swiss 3T3 fibroblasts over a temperature range from 16 to 35°C. No other published study is currently available using pyrene probes to evaluate cellular fluidity after fatty acid modification. These results suggest that this methodology may be a useful tool to address membrane properties after cellular lipid modification. All cell culture systems may not lend themselves as readily to this application. For example, Y-79 cells readily incorporated

the pyrene probe, but those cells studied by Dix *et al.* (41) required the addition of Pluronic F127 for labeling.

The effects of membrane fatty acid composition on membrane transport and enzyme activity have been studied in numerous cell culture and animal models (23, 24). The enhancement of choline uptake by DHA supplementation has been reported previously for Y-79 microsomal membranes by Hyman and Spector (31). They additionally reported that the high-affinity choline uptake in Y-79 retinoblastoma cells is energy and sodium independent; thus any changes produced by membrane lipid modification can be directly attributed to the transport process. These authors speculated that the high degree of unsaturation in neural cell membranes was to facilitate the capacity of the high-affinity choline uptake system to transport low concentrations of choline. Additional studies have shown that dietary deficiency of long chain ω-3 fatty acids results in alterations in multiple membrane-associated enzyme activities (23, 24).

The weight of current evidence clearly supports the essential nature of long chain ω-3 fatty acids for visual function. Additionally, there is now a multitude of literature to support a role for DHA in membrane function. In view of the results presented here, we speculate that the effect of DHA content on retinal function may be mediated by changes in membrane fluidity and associated enzyme and transport activities.

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