Effects of omega-3 fatty acid and vitamin E supplementation on erythrocyte membrane fluidity, tocopherols, insulin binding, and lipid composition in adult men

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Dietary supplementation with an omega-3 fatty acid preparation (fish oil) together with pharmacologic doses of vitamin E increased both insulin binding and membrane fluidity in erythrocytes from human adult males. Supplementation with fish oil alone induced significant increases in the α- and γ-tocopherol contents of the red blood cell membranes. Forty healthy men were given controlled diets and supplements, which together provided 40% of energy from fat (polyunsaturated:monosaturated:saturated ratio of 0.8:1:1), 360 mg cholesterol/day, and a minimum of 22 mg α-tocopherol (αT)/day for three successive experimental periods of 10, 10, and 8 weeks, during which they were given capsules containing 15 g of a placebo oil/day, 15 g fish oil concentrate (FOC)/day, and 15 g fish oil + 200 IU αT (FOC + αT)/day, respectively. Erythrocyte ghost insulin binding (IB) and 1,6-diphenyl-1,3,5-hexatriene (DPH) fluorescence-determined fluidity were significantly increased following the FOC + αT period, however FOC alone had no effect. At the end of each experimental period, IB values, as percentage bound/100 μg ghost protein at 4° C, were 0.96, 0.91, and 1.35, and DPH steady state fluorescence anisotropies were 0.311, 0.303, and 0.296, at 4° C, respectively. Small but statistically significant decreases in fluorescence lifetimes further indicated increased fluidity. FOC supplementation resulted in significantly lower membrane cholesterol:phospholipid ratios and increased membrane tocopherols despite daily vitamin E consumption of only 22 mg as in the placebo period. Membrane incorporation of n-3 fatty acids was, however, limited. Thus, dietary polyunsaturated fatty acids exerted substantial effects on erythrocyte membranes by affecting membrane contents of lipid molecules other than the fatty acids.

Keywords: α-tocopherol; insulin receptor; n-3 fatty acid; n-6 fatty acid; fish oil; membrane fluidity

Introduction

Diets recommended for the general public to prevent cancer, heart disease, and diabetes involve reducing total fat and cholesterol and replacing saturated (S) fat with polyunsaturated (P) fats of both the n-3 and n-6 types. Implementing these recommendations may lead to changes in membrane lipid composition, membrane fluidity, and membrane physiological processes. Diet-induced modification of erythrocyte phospholipid fatty acyl composition has been reported for the rat,[1-3] the rabbit,[4] and women.[5] These changes in red cell membrane lipids often affect membrane fluidity by altering the membrane cholesterol:phospholipid ratio, the degree of unsaturation of membrane phospholipid acyl chains, and the membrane phosphatidylcholine:sphingomyelin ratio,[6,7] or by the intercalation of molecules into the membrane bilayer region. We have reported diet-induced modification of erythrocyte,[8] platelet,[9-11] and hepatic and cardiac[12] membrane phospholipids in rabbits. Reports[13-18] are available for

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several species indicating that fish oil feeding induces replacement of membrane n-6 fatty acids with n-3 fatty acids in several tissues including heart, aorta, liver, platelets, erythrocytes, and adipose tissue in pigs, rats, rabbits, and humans.

The various alterations in membrane fatty acyl composition, as well as induced changes in the concentrations of other membrane lipid components, are expected to impinge on membrane function such as enzyme catalyzed reactions and receptor activity. We have shown that diets favoring more fluid red cell membranes result in increased insulin binding and receptor number.[5,8,19,20] Omega-3 fatty acids improve insulin sensitivity[21] and affect hormones involved in carbohydrate and lipid metabolism.[22]

It is widely believed that administration of polyunsaturated fatty acids decreases the concentration of vitamin E in the body[23,24] as a result of its antioxidant function.[25] Fish oils are highly unsaturated and readily undergo peroxidation.[26,27] Vitamin E supplementation is often recommended as a necessary antioxidant when large amounts of unsaturated fats are consumed.[25,28] Vitamin E supplementation may affect membrane fluidity through intercalation of tocopherols between the fatty acyl chains in the hydrocarbon region of the membrane bilayer, through interactions involving the chroman ring of the tocopherol molecule, or both. Tocopherol-induced decreases in fluidity, as measured by 1,6-diphenyl-1,3,5-hexatriene (DPH) fluorescence polarization, have been reported for liposomes from dipalmitoyl phosphatidylcholine and egg yolk phospholipids,[29] and platelet membrane lipids.[30] We have reported losses in lipoprotein lipid phase fluidity following vitamin E supplementation in men and women.[31,32] We have therefore studied the effects of both fish oil and vitamin E supplementations on red cell membranes in a group of healthy men. This study was conducted to examine the effects of fish oil supplementation on red cell membrane composition, fluidity, and insulin binding and receptor number in a healthy adult male population under a protocol in which the total fat intake remained close to that of the typical USA dietary intake at 39% of energy. A major finding reported here is an accumulation of α-tocopherol in the red cell membrane when fish oil supplements were provided to the subjects without additional dietary or supplemental vitamin E.

Materials and methods

Subjects and experimental diets

Male non-smokers between the ages of 24–57 were recruited from the greater Beltsville, MD, USA area to participate in the study. Potential subjects were screened to exclude those with health problems such as metabolic disorders, history of any organic disease, regular use of prescription medications or alcohol, and dietary habits that were non-representative of the general population.

Those who met the initial selection criteria were examined by a physician from the Georgetown University School of Medicine after having given informed consent according to protocols approved by the Institutional Review boards of the university and the National Cancer Institute, United States Department of Health and Human Services. The pre-study medical evaluation included a medical history, hematologic profile, blood chemistry including plasma α-tocopherol levels, and a urine analysis. Those with body weights less than 90% or greater than 120% of the 1983 standards for desirable weights[33] of the Metropolitan Life Insurance Co. were excluded from this study. Similarly, those with plasma α-tocopherol levels of less than 13.9 μmol/L or greater than 27.86 μmol/L were excluded. Aspirin, aspirin-containing drugs, and other anti-inflammatory drugs were not permitted during the study. Tylenol (McNeil Consumer Products Co., Fort Washington, PA, USA) was the only analgesic approved for occasional use in case of an acute need. From the initial pool of eligible subjects, forty-one were selected for participation in the study, and 40 of these subjects completed the study.

A basal diet was designed from commonly available foods to be fed in conjunction with 15 g/day of either placebo oil (PO) or fish oil concentrate (FOC) (ROPUFA-50%, supplied by Hoffmann-La Roche, Inc. Nutley, NJ, USA). The combination of basal diet and oil supplement provided 40% of calories from fat. The cholesterol intake was adjusted to about 130 mg/4184 kJ. The nutrient composition of these diets was calculated using the USDA Lipid Nutrition Laboratory food database, derived mainly from data on food composition from USDA Handbook 8 and by analysis. The content of the long-chain (20 carbon atoms or longer) omega-3 fatty acids was minimized in the basal diet by excluding fish from the menus, and vitamin E intake was minimized by excluding highly fortified foods from the diet. No vitamins, minerals, other supplements, or alcohol were permitted during the study. A 14-day menu cycle was used to assure food variety and acceptability. Consumption of coffee, tea, and water was unrestricted. The meals were prepared in the Human Study Facility of the Beltsville Human Nutrition Research Center (BHNRC). Weekday morning and evening meals were eaten in the BHNRC dining facility, and a carry out lunch was provided. Meals for Saturdays, Sundays, and holidays were prepacked and distributed for home consumption. Subjects were initially assigned to an appropriate energy intake level based on their estimated need for weight maintenance. Subsequently, body weight was maintained by adjusting the menu intake in 1.7 kJ increments.

All subjects were placed on the controlled basal diet for a total of 28 weeks divided into three periods. During period 1, lasting for 10 weeks, the subjects consumed 15 g PO provided in 1 g soft gelatin capsules fortified with 1 IU of dl-α-tocopherol/capsule. The subjects were given seven capsules at breakfast and eight capsules at dinner. During period 2, lasting for 10 weeks, the subjects consumed 15 g of FOC in soft gelatin capsules containing 1 g FOC and 1 IU dl-α-tocopherol. The PO and FOC capsules were indistinguishable in appearance. FOC capsules were also provided in two doses, seven capsules at breakfast and eight at dinner. During period 3, lasting 8 weeks, the subjects continued on the FOC capsules, but they were also given a capsule at breakfast containing 200 IU of dl-α-tocopherol (Hoffmann-La Roche, Inc.). Total α-tocopherol intake from the diet and the PO or FOC capsules was a minimum of 22 mg/day.

The PO was a blend of 48% stripped lard, 40% beef tallow (hormone-free), and 12% corn oil. The omega-3 fatty acid supplement was a 50% concentrate of refined anchovy oil (ROPUFA-50%). Subjects were informed of the sequence of administration of the capsules. The primary fatty acid compositions of the two supplements are presented in

Table 1. Estimated daily nutrient and fatty acid intakes of the subjects are given in *Table 2.* These data include both that which was provided in the controlled basal diet and in the supplementary capsules.

Blood sampling and erythrocyte membrane preparation

Morning fasting blood samples were collected at the end of each experimental period. Potassium EDTA was used as anticoagulant. After removal from plasma and platelets by differential centrifugation, the erythrocytes were dispersed in isotonic phosphate buffer (310 mOsm, pH 7.4) and washed by repeated centrifugations (20 min 1,000*g*). Erythrocyte ghosts were prepared by hypotonic lysis in 20 mOsm phosphate buffer (pH 7.4) according to the procedure of Dodge et al.[34] Ghosts were washed repeatedly in the 20 mOsm phosphate buffer to remove hemoglobin and other cytoplasmic components. Aliquots were removed for fluidity measurements and the remainder of the ghost preparations were stored at −70° C for assay of insulin binding and chemical analyses.

Fluidity measurements

Fluidity was assessed with freshly prepared membranes as a function of temperature between 5° C and 35° C by determining the anisotropy of fluorescence from the probe DPH using the methods of Shinitzky and Barenholz.[35] DPH (2 mmol/L in tetrahydrofuran) was diluted (1,000-fold) into the aqueous membrane suspension, which was then incubated with agitation at 35–37° C for 2 hours.

Steady-state fluorescence polarization was measured with an SLM Model 4800 spectrophotofluorometer (SLM-Aminco, Urbana, IL, USA) equipped with Glan-Thompson prism polarizers in the T-optical format. Excitation and emission wavelengths were 366 and 460 nm, respectively. Light scattering errors were minimized by assuring that the anisotropies were concentration-independent. DPH phase and modulation lifetimes were measured at 10° C and 30° C using oyster glycogen (Sigma Chemical Co., St. Louis, MO, USA) as a scattering reference. Lifetimes were measured at two frequencies by setting the Debye-Sears ultrasonic modulator at 18 and 30 Mhz, and lifetimes were resolved using Weber's[36] analysis.

Table 1 Fatty acid composition of the fish oil and placebo supplements

Acid	Placebo	Fish oil
	g/100g supplement	
12:0	0.16	0.13
14:0	2.08	4.87
16:0	21.81	9.29
16:1(n-7)	2.43	6.48
18:0	13.28	1.37
18:1(n-9)	36.39	5.43
18:2(n-6)	13.73	1.86
18:3(n-3)	0.44	1.04
18:4(n-3)	—	4.62
20:1(n-9)	0.44	0.38
20:4(n-6)	0.15	1.41
20:4(n-3)	—	1.11
20:5(n-3)	—	30.18
22:5(n-3)	—	2.51
22:6(n-3)	—	13.06

Table 2 Estimated daily intakes by subjects

Nutrient	Placebo period	Fish oil periods
Fat (% of energy)	39	39
Carbohydrate (% of energy)	45	45
Protein (% of energy)	16	16
Vitamin E (mg/d, minimum)	22	22
Cholesterol (mg/d)[a]	360	360
Fatty acid intake (g/d)[a]		
Saturates (total)	33	29
palmitate	20	18
stearate	9	7
Monounsaturates (total)	46	42
oleate	44	39
Polyunsaturates (total)	27	33
18:3(n-3)	2	2
20:5(n-3)	0	5
22:6(n-3)	0	2
18:2(n-6)	25	23

[a]At 11.72 MJ/d, or 2800 kcal/d.

Insulin binding

Erythrocyte membrane insulin receptor was assayed by measuring insulin binding to ghosts (100 μg protein/tube) incubated in 0.5 mL Tris-HEPES buffer[37] with 0.1 ng ^{125}I-insulin (specific activity 81.4 TBq/nmol) and 0–100 μg native porcine insulin/mL. ^{125}I-insulin was purchased from New England Nuclear (Boston, MA, USA) and native porcine insulin was a gift from Eli Lilly & Co (Indianapolis, IN, USA). After the incubation, 0.2 mL aliquots were layered over 0.2 mL chilled Tris-HEPES buffer and centrifuged (60 sec 7500*g*). The ghost pellet was washed once with 10% sucrose and radioactivity was determined in a gamma counter (Model A5550, Packard Instrument, Downers Grove, IL, USA). Insulin binding measurements were made at several temperatures and correlated with DPH anisotropy measurements at the same temperatures to correlate insulin binding with membrane fluidity. For this purpose, ghosts were incubated at 30° C for 2 hours, 10° C for 4 hours, and 4° C for 24 hours.

Binding data obtained at 4° C were analyzed by Scatchard plots[38] and competition-inhibition plots.[39] The number of receptors was assessed from the intercept on the abscissa of the Scatchard plots and the affinity was determined from the competition-inhibition plots as the amount of native insulin required to displace 50% of bound tracer.

Chemical analysis

Membrane cholesterol contents were determined enzymatically[40] using cholesterol esterase and cholesterol oxidase. Chemical methods were used for protein[41] and phosphorus[42] analyses. After protein analysis of the aqueous ghost suspensions, lipids were extracted with $CHCL_3/CH_3OH$ by an adaptation[43] of the method of Sperry and Brand,[44] and aliquots of the extracts were taken for cholesterol and phosphorus analyses. Vitamin E analyses were performed with intact cells and with aliquots of the aqueous membrane preparations. Tocopherols were separated and quantitated by HPLC[45] following ultraviolet absorption at 292 nm.

Fatty acyl compositions of the membrane phospholipid and neutral lipid fractions were determined by gas chromatography. Phospholipids and neutral lipids were separated

by silicic acid column chromatography with Unisil (Clarkson Chemical Co, Williamsport, PA, USA) and the fatty acid compositions of the fractions were determined by capillary gas chromatography of the corresponding methyl esters prepared by transesterification with methanolic HCl.[43] Chromatography was performed with a Hewlett-Packard Model 5890A gas chromatograph (Hewlett-Packard, Avondale, PA, USA). The instrument was equipped with dual flame ionization detectors, a Model 7673A automatic sampler, and a Model 3396A integrator. Chromatography was performed with a Supelco (Bellefonte, PA, USA) SP-2340 fused silica 30 m, 0.25 mm ID capillary column with a 0.20 μm thick film.

Statistical analysis

Data were subjected to ANOVA and linear regression analysis for the various measurements. The model included sources of variation due to diet changes in fatty acids and vitamin E. Duncan's multiple range test was used to determine differences in model classified composition, anisotropy, and insulin binding data.

Results

Dietary lipid modifications in this study resulted in alterations in red cell membranes via limited incorporations of n-3 fatty acids and significant changes in the contents of other membrane lipids, including cholesterol and tocopherols.

Table 3 RBC Membrane Phospholipid Fatty Acyl Composition (means ± SEM)

Diet	Placebo	Fish oil	Fish oil + vitamin E
Acid	*mole percent*		
12:0	0.27 ± 0.04[a]	0.08 ± 0.04[b]	0.11 ± 0.03[b]
14:0	1.82 ± 0.09[b]	1.40 ± 0.11[c]	2.25 ± 0.15[a]
15:0	0.55 ± 0.06[b]	0.61 ± 0.09[ab]	0.75 ± 0.00[a]
16:0	32.47 ± 0.68[a]	27.45 ± 0.46[c]	30.39 ± 0.35[b]
16:1(n-7)	0.87 ± 0.06[a]	1.04 ± 0.08[a]	0.94 ± 0.09[a]
18:0	33.50 ± 1.42[b]	36.22 ± 0.73[b]	41.30 ± 0.70[a]
18:1(n-9)	13.15 ± 0.91[a]	12.71 ± 0.44[a]	9.58 ± 0.31[b]
18:2(n-6)	7.74 ± 0.60[a]	8.91 ± 0.40[a]	5.91 ± 0.29[b]
18:3(n-3)	0.71 ± 0.16[a]	0.53 ± 0.05[ab]	0.27 ± 0.02[b]
20:0	0.86 ± 0.10[b]	1.31 ± 0.14[a]	0.82 ± 0.03[b]
20:3(n-6)	0.63 ± 0.05[a]	0.65 ± 0.14[a]	0.51 ± 0.04[a]
20:4(n-6)	2.47 ± 0.36[a]	2.44 ± 0.18[a]	2.30 ± 0.10[a]
20:5(n-3)	0.56 ± 0.08[a]	0.70 ± 0.13[a]	0.62 ± 0.07[a]
22:5(n-3)	0.40 ± 0.05[b]	0.59 ± 0.10[ab]	0.75 ± 0.08[a]
22:6(n-3)	0.40 ± 0.06[b]	0.80 ± 0.15[a]	1.03 ± 0.12[a]
24:0	2.19 ± 0.09[a]	1.61 ± 0.10[b]	1.54 ± 0.11[b]
24:1(n-9)	1.43 ± 0.15[b]	2.88 ± 0.39[a]	0.48 ± 0.02[c]
U/S*	0.42 ± 0.03[a]	0.46 ± 0.02[a]	0.30 ± 0.01[b]
P/S	0.19 ± 0.02[a]	0.22 ± 0.01[a]	0.15 ± 0.01[b]
N-6†	10.85 ± 0.22[a]	12.00 ± 0.34[a]	8.72 ± 0.23[b]
N-3‡	2.07 ± 0.86[a]	2.62 ± 0.52[a]	2.67 ± 0.32[a]

[a–c]Values for a fatty acid, ratio, or sum with different superscripts are significantly different ($P < 0.05$).
*U/S and P/S values were calculated by dividing the total moles of unsaturated (U) or polyunsaturated (P) fatty acids by the total moles of saturated (S) fatty acids.
†Σmoles (n-6) fatty acids = $n_{18:2} + n_{20:3} + n_{20:4}$
‡Σmoles (n-3) fatty acids = $n_{18:3} + n_{20:5} + n_{22:5} + n_{22:6}$

Table 4 RBC Membrane Neutral Lipid Fatty Acyl Composition (means ± SEM)

Diet	Placebo	Fish oil	Fish oil + vitamin E
Acid	*mole percent*		
12:0	0.74 ± 0.15[a]	0.60 ± 0.11[a]	0.14 ± 0.05[b]
14:0	3.62 ± 0.32[a]	3.17 ± 0.19[ab]	2.53 ± 0.20[b]
15:0	2.65 ± 0.63[b]	1.55 ± 0.41[b]	4.84 ± 0.50[a]
16:0	31.04 ± 0.62[b]	33.10 ± 0.54[a]	30.52 ± 0.27[b]
16:1(n-7)	0.88 ± 0.09[b]	1.26 ± 0.12[a]	0.70 ± 0.07[b]
18:0	51.26 ± 0.68[a]	51.02 ± 1.10[a]	52.18 ± 0.96[a]
18:1(n-9)	2.86 ± 0.31[a]	3.33 ± 0.22[a]	3.43 ± 0.21[a]
18:2(n-6)	1.37 ± 0.16[a]	0.79 ± 0.09[b]	0.87 ± 0.10[b]
20:0	1.46 ± 0.29[a]	0.75 ± 0.07[b]	0.90 ± 0.05[b]
20:4(n-6)	1.06 ± 0.15[b]	1.09 ± 0.05[b]	1.39 ± 0.05[a]
20:5(n-3)	0.79 ± 0.15[a]	1.10 ± 0.15[a]	0.75 ± 0.15[a]
22:6(n-3)	0.61 ± 0.23[a]	0.12 ± 0.03[b]	0.05 ± 0.02[b]
24:0	0.33 ± 0.09[a]	0.06 ± 0.02[b]	0.37 ± 0.09[a]
24:1(n-9)	0.53 ± 0.10[b]	0.82 ± 0.07[a]	0.38 ± 0.06[b]
U/S*	0.09 ± 0.008[a]	0.10 ± 0.005[a]	0.08 ± 0.005[a]
P/S	0.04 ± 0.004[a]	0.03 ± 0.002[a]	0.03 ± 0.003[a]
N-6†	3.03 ± 0.27[a]	1.99 ± 0.13[b]	2.31 ± 0.12[b]
N-3‡	1.39 ± 0.34[a]	1.22 ± 0.15[a]	0.81 ± 0.16[b]

[a–c]Values for a fatty acid, ratio, or sum with different superscripts are significantly different ($P < 0.05$).
*U/S and P/S values were calculated by dividing the total moles of unsaturated (U) or polyunsaturated (P) fatty acids by the total moles of saturated (S) fatty acids.
†Σmoles (n-6) fatty acids = $n_{18:2} + n_{20:3} + n_{20:4}$
‡Σmoles (n-3) fatty acids = $n_{18:3} + n_{20:5} + n_{22:5} + n_{22:6}$

Phospholipid fatty acyl composition data are given in *Table 3* and the neutral lipid fraction fatty acyl composition data are given in *Table 4*. Eicosapentaenoate, 20:5(n-3), levels were not affected in either fraction, but the 22-carbon, n-3, fatty acids, docosapentaenoate, 22:5, and docosahexaenoate, 22:6, were significantly increased during the second period, third period, or both in the phospholipid fraction alone. All phospholipid 18-carbon unsaturated fatty acids (18:1, 18:2, and 18:3) were significantly reduced by the end of the third period, i.e., following 18 weeks of FOC supplementation, while only linoleate was affected in the neutral lipid fraction. Phospholipid nervonate, 24:1(n-9), was also significantly reduced at that time. These effects noted at the end of the third period, i.e., FOC + αT, are probably in response to the continued FOC supplementation rather than the vitamin E supplementation. Saturated fatty acid effects probably do not reflect dietary incorporation as both palmitate and stearate were provided at reduced intakes during the fish oil supplementations (*Table 2*), but membrane phospholipid palmitate levels were significantly lowered and stearate levels were significantly elevated during the FOC supplementation (*Table 3*). Omega-3 fatty acid levels in the neutral lipid fraction, which is actually a minor component in membrane lipids, were not affected by the diets except for the unexpected decrease in docosahexaenoate following the FOC supplementations.

The most striking membrane lipid effect was the increase in membrane tocopherols during the supplementations (*Table 5*). Both α- and γ-tocopherols were significantly increased during the second period when the subjects received the fish oil capsules with only 22 mg α-tocopherol/day, the same α-tocopherol intake as in the first period. Apparently some type of metabolic control mechanism is operating to modulate the membrane tocopherol levels, which do not reflect the total cellular tocopherol levels in the erythrocyte. During the second period, α-tocopherol in packed red cells was significantly reduced, compared to the placebo period, which likely relates to its antioxidant activity. Cellular α-tocopherol was elevated during the third period, which probably resulted from the increased daily supplementation with 200 IU vitamin E. Membrane tocopherols also reflected the latter supplementation, as membrane α-tocopherol was further elevated, but γ-tocopherol was not affected. Concentration of plasma[46] α-tocopherol significantly decreased after fish oil supplementation and significantly increased after supplementation with vitamin E at the high level of period 3. Plasma γ-tocopherol (*Table 5*) was significantly increased in the FOC period, as compared with the placebo period, and significantly decreased during the FOC + αT period, as compared with the FOC period. These changes in α- and γ-tocopherols are in opposite directions as is typical for these tocopherol forms.

Red cell membrane cholesterol concentrations (*Table 6*) were significantly reduced following the changes in dietary fatty acids (*Table 2*). The shifts in cholesterol levels and cholesterol:phospholipid ratios (*Table 6*) cannot be attributed to dietary cholesterol, which was not varied (*Table 2*).

DPH fluorescence polarization data (*Table 7*) show no effects of the dietary treatment on membrane fluidity at physiological temperature, 37° C, however at lower temperatures, 10° C and 4° C, r_s, the steady state fluorescence anisotropy, for DPH was reduced during the course of the supplementations indicating increased membrane fluidity. The increase in fluidity was most significant during the third period, resulting from either the additional 8 weeks of FOC supplementation or the addition of 200 IU vitamin E per day. The statistically significant decrease in DPH fluorescence lifetimes, measured at 10° C, also indicates an increase in membrane fluidity. The greater freedom of movement allowed to the probe molecule in the more fluid membrane results in a more rapid loss of energy and hence a shorter excited state period. The thermotropic fluorescence polarization data yielded linear Arrhenius plots on a logarithmic scale of the fluidity parameter, $(r_o/r_s - 1)^{-1}$, versus 1/T, thus indicating monophasic behavior in the temperature range 4–38° C. We used the published[47] value for the DPH limiting anisotropy $r_o = 0.362$, determined in propylene glycol at −50° C. Following the dietary manipulations, steeper slopes were observed in the Arrhenius plots for the more fluid membranes, resulting in the greater apparent fluidity differences at lower temperatures. It is unlikely that the fluidity changes resulted from the limited fatty acyl effects, but most likely follow the effects (*Table 6*) on the molar cholesterol:phospholipid ratios, N_{chol}/n_{pl}. Linear regression analysis yielded the statistically significant ($P < 0.0001$) relation $r_s = 0.296 + 0.0125 (n_{chol}/n_{pl})$ for the anisotropy data at 4° C. Similarly significant relationships exist for the anisotropy data at the higher temperatures as well. The low temperature anisotropy data were also significantly ($P < 0.0001$) related to the α-tocopherol content, αT in nmoles/g protein, according to the equation: $r_s = 0.304 - 2.1 \times 10^{-3}(\alpha T)$. The increased membrane fluidity following the third period is most likely due to the membrane tocopherol. Comparison of data reveal that phospholipid (*Table 3*) P/S and Unsaturated/S values after period 3 were actually lower than after period 2 and that cholesterol values (*Table 6*) were the same for both periods, whereas α-tocopherol values (*Table 5*) for periods 2 and 3 were significantly different.

Insulin binding by erythrocyte membranes (*Table 8*) was significantly increased during period 3 regardless of incubation temperature and appears to be the result of vitamin E supplementation. Incubation times were varied at the different temperatures to optimize the binding measurement at each temperature. Measurements of fluidity and binding were made at 4° C, 10°

Table 5 Plasma and erythrocyte, cellular and membrane, vitamin E contents (means ± SEM)

Diet	Placebo	Fish oil	Fish oil + vitamin E
	plasma: (μmol/L)		
α-tocopherol[a]	24 ± 0.94[c]	20 ± 0.7[d]	27 ± 0.94[b]
γ-tocopherol	1.91 ± 0.12[c]	3.59 ± 0.29[b]	2.00 ± 0.18[c]
	cell: (nmol/mL packed cells)		
α-tocopherol	4.89 ± 0.15[c]	2.74 ± 0.08[d]	8.09 ± 0.40[b]
	membrane: (nmole/g protein)		
α-tocopherol	185 ± 33[d]	948 ± 169[c]	1948 ± 176[b]
γ-tocopherol	128 ± 28[c]	405 ± 95[b]	449 ± 76[b]

[a]α-Tocopherol data from reference 46.
[b–d]Values in the same row with different superscripts are significantly different ($P < 0.05$).

Table 6 Erythrocyte Membrane Lipid Analysis (means ± SEM)

Diet	Placebo	Fish oil	Fish oil + vitamin E
Phospholipid (μmole/g protein)	0.33 ± 0.01[b]	0.46 ± 0.04[a]	0.35 ± 0.02[b]
Cholesterol (μmole/g protein)	0.24 ± 0.01[a]	0.16 ± 0.02[b]	0.15 ± 0.01[b]
Cholesterol/phospholipid	0.73 ± 0.03[a]	0.35 ± 0.01[c]	0.43 ± 0.03[b]

[a–c]Values in the same row with different superscripts are significantly different ($P < 0.05$).

Table 7 Erythrocyte membrane DPH fluorescence data (means ± SEM)

Diet	Placebo	Fish oil	Fish oil + vitamin E
Temperature ° C			
	r_s, *DPH steady state anisotropy*		
37	0.2122 ± 0.0008[a]	0.2114 ± 0.0007[a]	0.2100 ± 0.0008[a]
30	0.2348 ± 0.0008[a]	0.2324 ± 0.0008[b]	0.2303 ± 0.0009[b]
10	0.2927 ± 0.0008[a]	0.2876 ± 0.0010[b]	0.2841 ± 0.0010[c]
4	0.3066 ± 0.0008[a]	0.3013 ± 0.0009[b]	0.2978 ± 0.0010[c]
	τ, *DPH fluorescence lifetime, nsec*		
30	11.2 ± 0.3[a]	11.3 ± 0.8[a]	10.9 ± 0.6[a]
10	12.3 ± 0.7[a]	10.9 ± 0.5[ab]	10.5 ± 0.3[b]

[a–c]Values in the same row with different superscripts are significantly different ($P < 0.05$).

Table 8 Erythrocyte Membrane Insulin Binding and Related Parameters (means ± SEM)

Diet	Placebo	Fish oil	Fish oil + vitamin E
	% binding/100μg protein		
incubated 2 hr at 30° C	0.678 ± 0.05[b]	0.646 ± 0.08[b]	0.881 ± 0.06[a]
incubated 4 hr at 10° C	0.538 ± 0.04[b]	0.646 ± 0.06[ab]	0.747 ± 0.06[a]
incubated 24 hr at 4° C	0.965 ± 0.06[b]	0.911 ± 0.07[b]	1.350 ± 0.10[a]
	Receptor number (fmol/100μg protein)		
incubated 24 hr at 4° C	31.3 ± 2.5[a]	26.1 ± 3.4[a]	29.9 ± 2.5[a]
	Receptor affinity (nmol/L)		
incubated 24 hr at 4° C	1.03 ± 0.05[a]	0.52 ± 0.03[b]	0.47 ± 0.02[b]

[a–c]Values in the same row with different superscripts are significantly different ($P < 0.05$).

C, and 30° C to study the correlation between insulin binding (IB) and fluidity at each of these temperatures. Linear regression analyses yielded the following set of equations, with greater statistical significance for the low temperature data, relating insulin binding and membrane α-tocopherol: IB = 0.648 + 0.082(αT); $P < 0.02$ at 30° C; IB = 0.555 + 0.081(αT); $P < 0.007$ at 10° C; and IB = 0.930 + 0.136(αT); $P < 0.0009$ at 4° C.

Scatchard analysis of the insulin binding data at 4° C showed no significant differences in the numbers of receptors at the three experimental periods. Analysis of the binding data by competition-inhibition plots revealed significantly lower receptor affinities following both fish oil feeding periods.

Discussion

Incorporation of dietary n-3 fatty acids into cell membrane phospholipids has been reported in several human and animal studies. Nelson et al.[48] fed a salmon oil diet to men and observed eicosapentaenoate (EPA) and docosahexaenoate (DHA) incorporation into platelet, erythrocyte, and plasma fatty acid fractions. The increases in n-3 fatty acids reported for the erythrocyte were significantly higher than our current observations despite similar n-3 fatty acid intakes. They fed 2 g EPA and 3 g DHA daily, and we fed our subjects 2 g EPA and 5 g DHA daily. Flaten et al.[49] observed increases in red-cell EPA and DHA when they fed 64 human volunteers 14 g fish oil concentrate daily in place of olive oil. Von Shacky et al.[50] reported dietary EPA and DHA incorporation into human erythrocytes and platelets. Gibney and Bolton-Smith[51] reported n-3 fatty acid incorporation into erythrocyte phospholipids of eight human volunteers receiving 15 g MaxEPA (Solgar Vitamin Co., Lynbrook, NY, USA) daily, but they saw no effects on membrane fluidity, as determined by DPH polarization. Bordoni et al[52] observed dietary lipid effects on both n-3 and n-6 fatty acids and fluidity in red blood cells in the rat. Croset and Kinsella[53] observed fatty acid changes in cardiac organelles in DHA-fed mice but observed no effects on membrane enzyme activities or membrane fluidity. De Schrÿver et al.[54] reported fish oil and beef tallow effects on rat liver membranes, and Bourre et al.[55] observed fish oil fatty acid incorporation into rat brain and liver. Membrane inclusion of dietary fatty acids is apparently species and tissue dependent. We have noted that feeding menhaden oil to rabbits[12] resulted in significant effects on fatty acyl composition of plasma and tissue phospholipids, however there were substantial differences in n-3 fatty acid incorporation into plasma lipoproteins on the one hand and liver and heart membranes on the other.

Increasing membrane content of n-3 polyunsaturated fatty acids does not necessarily result in more fluid membranes. Bordoni et al.[52] observed fatty acyl effects on rat red-cell membrane fluidity in contrast with Croset and Kinsella[53] who found no effects on n-3 fatty acids on fluidity in cardiac organelles in mice. Neuringer and Connor[56] noted that although retinal rod outer segment disk membranes are very fluid, DHA does not specifically enhance fluidity in these membranes. Phosphatidylcholine vesicles were less fluid when DHA replaced linoleate or arachidonate. Neuringer and Connor therefore concluded that fluidity does not depend on the total number of double bonds, and the additional double bonds in EPA and DHA do not contribute to fluidity. Sanders[57] claimed that attempts to show differences in membrane fluidity by dietary manipulation of the n-3/n-6 fatty acid balance in membranes have been unsuccessful, but EPA incorporation into red blood cells increased whole blood viscosity and deformability. Cartwright et al.[18] inferred from their data pertaining to red cell membrane phospholipid fatty acid composition, whole blood viscosity,

and red cell deformability that n-3 fatty acid replacement of n-6 acids altered membrane fluidity in human subjects fed fish oils. Conroy et al.[58] fed olive oil and fish oil to rats and reported that DPH-determined fluidity was not affected in intact liver microsomes, however phosphatidylcholine vesicles were more fluid when n-3 fatty acyl levels were increased. They concluded that although lipid fluidity/order can be modified by n-3 levels, alterations in membrane domain organization, phospholipid species composition, or both effectively compensate for the changes. The effects of fish oil and vitamin E supplementation on fluidity reported here cannot be associated with total fatty acyl unsaturation or specifically n-3 fatty acids (*Tables 3 and 4*). Significant linear relationships were noted between DPH fluorescence anisotropy and the molar cholesterol:phospholipid ratio, suggesting that the increase in fluidity is related to fish oil-induced hypocholesterolemia. Hypolipidemia induced by fish oil feeding is usually associated with triacylglycerols, however the effects on blood cholesterol are still uncertain.[59] In the present study[60] fish oil supplementation significantly lowered plasma mean triacylglycerols from 1.62 to 1.17 mmol/L, but plasma cholesterol was not significantly affected. No further change occurred in plasma triacylglycerol upon the additional supplementation with 200 mg α-tocopherol/day.

Vitamin E supplementation may induce effects on membrane fluidity through intercalation of tocopherols between the fatty acyl chains in the hydrocarbon region of the membrane bilayer as well as interactions involving the chroman ring of the tocopherol molecule.[29] Tocopherol-induced fluidity decreases, as measured by DPH fluorescence polarization, have been reported for liposomes from dipalmitoyl phosphatidylcholine,[29] egg yolk phospholipids,[61] and platelet membrane lipids.[30] Srivastava et al.[62] studied the relative effects of α-tocopherol and α-tocopheryl acetate on lipid fluidity in dipalmitoyl phosphatidylcholine vesicles by magnetic resonance techniques. α-Tocopherol became strongly bound to the phospholipid with a loss in mobility of the tocopherol and the phospholipids. Our data for the lower temperatures indicate that fluidity increases as a function of membrane α-tocopherol. Urano et al.[63] demonstrated increased DPH and trimethylammonium DPH-determined fluidities when α-tocopherol is added to phospholipid vesicles, suggesting increased fluidity both deep in the bilayer and near the surface as well. Aranda et al.[62] reported fluorescence data for probes in α-tocopherol–containing liposomes demonstrating a high "mobility" for α-tocopherol in fluid regions of the membrane. They concluded that the α-tocopherol has a particularly high lateral diffusion coefficient that results in the availability of a small amount of vitamin E in a membrane to function adequately as an antioxidant.

The major finding of the present work is the increase in membrane tocopherol induced by fish oil feeding (*Table 5*) without supplemental vitamin E. Intact erythrocyte (*Table 5*) and plasma[46] α-tocopherols decreased during the fish oil period and increased significantly during the fish oil + vitamin E period, reflecting the vitamin E supplementation. Shapiro et al.[64] reported that supplementation with MaxEPA resulted in elevated plasma α-tocopherol, which they attributed to the 1.35 mg α-tocopherol in the MaxEPA capsules. They provided male subjects with 19 g fish oil and 24 mg α-tocopherol daily and observed increases in plasma α-tocopherol from 15.8 ± 3.9 to 19.1 ± 2.8 μmol/L. Meydani et al.[65] observed no effects on plasma vitamin E in women after 3 months of supplementation with 1.6 g EPA and 0.72 g DHA daily in fish oil capsules with 6 IU vitamin E daily. Dimitrov et al.[66] provided 64 human subjects with daily doses of 440, 880, or 1320 mg α-tocopherol and observed corresponding increases in plasma α-tocopherol that were enhanced when the subjects consumed a high-fat diet. Our findings with respect to the ghosts are probably unrelated to plasma α-tocopherol increases but are more likely related to a specific binding. It is suggested that there may be a specific vitamin E-binding protein functioning in the membrane. Apparently some kind of homeostatic mechanism is operating to insure membrane integrity with the high membrane polyunsaturated fatty acid (PUFA) content that would make the membrane subject to peroxidative degradation. Chautan et al.[67] recently reported a similar effect in heart, but not liver, membranes of rats fed diets with n-3 fatty acids. The heart membranes displayed a four-fold increase in α-tocopherol in response to even low amounts of dietary fish oil. Indeed, Chautan et al.[67] speculated on the potential for extrapolating their results to humans, particularly with respect to homeostasis in membranes of vital organs. Clément and Bourre[68] studied vitamin E in developing nervous tissue. They reported a close correlation between α-tocopherol content and n-6 PUFA content of the sciatic nerve during development and aging. These reports are similar to ours in that membrane homeostasis is operating by increasing the membrane α-tocopherol content in the presence of PUFA.

The data from this and several other studies in our laboratory demonstrated variable relationships between PUFA feeding, insulin receptor binding, and membrane fluidity. Feeding diets higher in saturated fats lowered insulin binding to minipig erythrocytes[19] and rabbit erythrocyte ghosts.[8] Insulin binding was lower in erythrocytes from monkeys fed diets higher in *trans* fatty acids[20] in keeping with the concept that the *trans* acids behave like saturated fatty acids because many of their physical properties are similar. Feeding premenopausal women high-fat diets resulted in significantly higher insulin binding,[69] which was directly related to membrane fluidity.[5] The rabbit study showed a similar correlation between insulin binding and fluidity, however insulin binding in the monkeys fed the *cis* and *trans* diets was not related to membrane fluidity as determined by DPH fluorescence polarization.[20] It is likely that these differences are related to the significance of various membrane domains with respect to insulin receptor activity. The present study,[22] as well as the earlier study with premenopausal women,[69]

showed an inverse relationship between insulin binding and plasma insulin level, which contrasts with some other reports.[70–72] It is apparent from the present work that the lipid environment of the erythrocyte membrane influences insulin receptor activity, however α-tocopherol exerted a more significant effect than dietary n-3 polyunsaturates on both membrane fluidity and insulin receptor.

References

1 Bloj, B., Morero, R., Farras, R.N., and Truco, R.E. (1973). Membrane lipid fatty acids and regulation of membrane bound enzymes. Allosteric behavior of erythrocyte Mg^{2+}-ATPase, (Na^+ + K^+) ATPase and acetylcholinesterase from rats fed different fat-supplemented diets. *Biochim. Biophys. Acta* **311,** 67–79

2 Rao, G.A., Siler, K., and Larkin, E.C. (1979). Diet induced alterations in the discoid shape and phospholipid fatty acid composition of rat erythrocytes. *Lipids* **14,** 30–38

3 Schouten, J.A., Beynen, A.C., Popp-Snijders, C., and Mulder, C. (1985). Effect of a hypercholesterolemic diet on the fatty acid composition of rat erythrocyte phospholipids. *Nutr. Repts. Intl.* **31,** 229–236

4 Schouten, J.A., Beynen, A.C., Popp-Snijders, C., and Van der Veen, E.A. (1984). Lipid composition of erythrocytes isolated from cholesterol-fed rabbits. *Nutr. Repts. Intl.* **29,** 1223–1232

5 Berlin, E., Bhathena, S.J., Judd, J.T., Nair, P.P., Jones, D.Y., and Taylor, P.R. (1987). Dietary fat and hormonal effects on erythrocyte membrane fluidity and lipid composition in adult women. *Metabolism* **38,** 790–796

6 Smith, J.E. (1987). Erythrocyte membrane: Structure, function and pathophysiology. *Vet. Pathol.* **24,** 471–476

7 Borochov, H., Zahler, P., Wilbrandt, W, and Shinitzky, M. (1977). The effect of phosphatidylcholine to sphingomyelin mole ratio on the dynamic properties of sheep erythrocyte membrane. *Biochim. Biophys. Acta* **470,** 382–388

8 Berlin, E., Bhathena, S.J., Kliman, P.G., and Revett, K., (1989). Effect of saturation of dietary lipids on insulin receptors and membrane fluidity in rabbit erythrocytes. *Nutr. Repts. Intl.* **39,** 367–381

9 Berlin, E., Matusik, E.J., and Young, C. (1980). Effect of dietary fat on the fluidity of platelet membranes. *Lipids* **15,** 604–608

10 Berlin, E., Shapiro, S.G., and Friedland, M. (1984). Platelet membrane fluidity and aggregation of rabbit platelets. *Atherosclerosis* **51,** 223–239

11 Berlin, E., Shapiro, S.G., and Kliman, P.G. (1987). Influence of saturated and unsaturated fats on platelet fatty acids in cholesterol-fed rabbits. *Atherosclerosis* **63,** 85–96

12 Loo, G., Berlin, E., Peters, R.C., Kliman, P.G., and Wong, H.Y.C. (1991). Effect of dietary corn, coconut, and menhaden oils on lipoproteins, liver, and heart membrane composition in the hypercholesterolemic rabbit. *J. Nutr. Biochem.* **2,** 594–603

13 Hartog, J.M., Lamers, J.M.J., Achterberg, P.W., Van Heuven, D., Nijkump, F.P., and Verdouw, P.D. (1987). The effects of dietary mackerel oil on the recovery of cardiac function after acute ischemic events in the pig. *Basic Res. in Cardiol.* **82,** 223–234

14 Charnock, J.S., Abeywardena, M.Y., and McLennan, P.L. (1986). Comparative changes in the fatty acid composition of cardiac phospholipids after long-term feeding of sun seed oil- or tuna oil-supplemented diets. *Ann. Nutr. Metab.* **30,** 393–406

15 Vas Dias, F.W., Gibney, M.J., and Taylor, T.G. (1982). The effect of polyunsaturated fatty acids of the n-3 and n-6 series on platelet aggregation and platelet and aortic fatty acid composition in rabbits. *Atherosclerosis* **43,** 245–257

16 Goodnight, S.H., Harris, W.S., and Connor, W.E. (1981). The effects of dietary omega-3 fatty acids on platelet composition and function in man: A prospective controlled study. *Blood* **58,** 880–885

17 Thorngren, M. and Gustafson, A. (1981). Effects of 11-week increase in dietary eicosapentaenoic acid on bleeding time, lipids and platelet aggregation. *Lancet* **2,** 1190–1193

18 Cartwright, I.J., Pockley, A.G., Galloway, J.H., Greaves, M., and Preston, F.E. (1985). The effects of dietary omega-3 polyunsaturated fatty acids on erythrocyte membrane phospholipids, erythrocyte deformability and blood viscosity in healthy volunteers. *Atherosclerosis* **55,** 267–281

19 Bhathena, S.J., Berlin, E., Revett, K., and Ommaya, A.E.K. (1986). Modulation of erythrocyte insulin receptors by dietary lipid. *Ann. NY Acad. Sci.* **463,** 165–167

20 Barnard, D.E., Sampugna, J., Berlin, E., Bhathena, S.J., and Knapka, J.J. (1990). Dietary *trans* fatty acids modulate erythrocyte membrane fatty acyl composition and insulin binding in monkeys. *J. Nutr. Biochem.* **1,** 190–195

21 Popp-Snijders, C., Schouten, J.A., Heine, R.J., and Van der Veen, E.A. (1987). Dietary supplementation of omega-3 polyunsaturated fatty acids improves insulin sensitivity in non-insulin-dependent diabetes. *Diabetes Res.* **4,** 141–147

22 Bhathena, S.J., Berlin, E., Judd, J.T., Kim, Y.C., Law, J.S., Bhagavan, H.N., Ballard-Barbash, R., and Nair, P.P. (1991). The effects of omega-3 fatty acids and vitamin E on hormones involved in carbohydrate and lipid metabolism in men. *Am. J. Clin. Nutr.* **54,** 684–688

23 Bjorneboe, A., Soyland, E., and Bjorneboe, G-E. (1989). Effect of n-3 fatty acid supplement to patients with atopic dermatitis. *J. Int. Med.* **225,** 233–236

24 Stam, H., Hülsmann, W.C., and Jongkind, J.F. (1989). Endothelial lesions, dietary composition and lipid peroxidation. *Eicosanoids* **2,** 1–14

25 Diplock, A.T. (1983). The role of vitamin E in biological membranes, In *Biology of vitamin E,* (R. Porter and J. Whelan, eds.) Ciba Foundation Symposium 101, p. 45–55, Pitman Books, London, UK

26 Herbert, K.E. and Wills, E.D. (1987). Platelet function and tissue peroxidation in rats fed polyunsaturated fatty acids. *Biochem. Soc. Trans.* **51,** 410–411

27 Piché, L.A., Draper, H.H., and Cole, P.D. (1988). Malondialdehyde excretion by subjects consuming cod liver oil vs a concentrate of n-3 fatty acids. *Lipids* **23,** 370–371

28 Hu, M-L., Frankel, E.N., Leibovitz, B.E., and Tappel, A.L. (1989). Effect of dietary lipids and vitamin E on in vitro lipid peroxidation in rat liver and kidney homogenates. *J. Nutr.* **119,** 1574–1582

29 Fukuzawa, K., Chida, H., and Suzuki, A. (1980). Fluorescence depolarization studies of phase transition and fluidity in lecithin liposomes containing α-tocopherol. *J. Nutr. Sci. Vitaminol.* **26,** 427–434

30 Steiner, M. (1981). Vitamin E changes the membrane fluidity of human platelets. *Biochim. Biophys. Acta* **640,** 100–105

31 Berlin, E., Bui, T.B., Sainz, E., Sundaram, S.G., Manimekalai, S., and Goldstein, P.J. (1986). Vitamin E modulation of lipoprotein fluidity. *Ann. NY Acad. Sci.* **463,** 80–82

32 Berlin, E., Lehmann, J., and Judd, J.T. (1988). Vitamin E supplementation and lipoprotein fluidity in normal human subjects, In *Advances in Diet and Nutrition:2,* (S. Leff, ed), p. 193–198, John Libbey & Co., London, UK

33 Metropolitan Life Insurance Company. (1983). 1983 Metropolitan height and weight tables. *Stat. Bull.* **64,** 1–9

34 Dodge, J.T., Mitchell, C., and Hanahan, D.J. (1963). The preparation and chemical characteristics of hemoglobin-free ghosts of human erythrocytes. *Arch. Biochem. Biophys.* **100,** 119–130

35 Shinitzky, M. and Barenholz, Y. (1978). Fluidity parameters of lipid regions determined by fluorescence. *Biochim. Biophys. Acta* **515,** 367–394

36 Weber, G. (1981). Resolution of the fluorescence lifetimes in a heterogeneous system by phase and modulation measurements. *J. Phys. Chem.* **85,** 949–953

37 Gambhir, K., Archer, J.A., and Bradley, C.J. (1978). Characteristics of human erythrocyte insulin receptors. *Diabetes* **27,** 701–708

38 Scatchard, G. (1949). The attraction of proteins for small molecules and ions. *Ann. NY Acad. Sci.* **51,** 660–672

39 Bhathena, S.J. (1987). Insulin receptor, In *Peptide hormone receptors,* (M.Y. Kalimi and J.R. Hubbard, eds), p. 179–285, Walter deGruyter and Co, Berlin, Germany

40 Allain, C.C., Poon, L.S., Chan, C.S.G., Richmond, W., and Fu, P.C. (1974). Enzymatic determination of total serum cholesterol. *Clin. Chem.* **20,** 470–475

41 Lowry, O.H., Rosebrough, N.J., Farr, A.L., and Randall, R.J. (1951). Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193,** 265–275

42 Bartlett, G.R. (1959). Phosphorus assay in column chromatography. *J. Biol. Chem.* **234,** 466–468

43 Matusik, E.J., Reeves, V.B., and Flanagan, V.P. (1984). Determination of fatty acid methyl esters. Elimination of tissue-derived contamination and artefacts and preparation of a support-coated acid modified polyester liquid phase. *Anal. Chim. Acta* **166,** 179–188

44 Sperry, W.M. and Brand, F.C. (1955). The determination of total lipids in blood serum. *J. Biol. Chem.* **213,** 69–76

45 Craft, N.E., Brown, E.D., and Smith, J.C. (1988). Effects of storage and handling conditions on concentrations of individual carotenoids, retinol and tocopherol in plasma. *Clin. Chem.* **34,** 44–48

46 Kramer, T.R., Schoene, N., Douglas, L.W., Judd, J.T., Ballard-Barbash, R., Taylor, P.R., Bhagavan, H.N., and Nair, P.P. (1991). Increased vitamin E intake restores fish-oil–induced suppressed blastogenesis of mitogen-stimulated T lymphocytes. *Am. J. Clin. Nutr.* **54,** 896–902

47 Shinitzky, M. and Barenholz, Y. (1974). Dynamics of the hydrocarbon layer in liposomes of lecithin and sphingomyelin containing dicetylphosphate. *J. Biol. Chem.* **249,** 2652–2657

48 Nelson, G.J., Schmidt, P.C., and Corash, L. (1991). The effect of a salmon diet on blood clotting, platelet aggregation and fatty acids in normal adult men. *Lipids* **26,** 87–96

49 Flaten, H., Høstmark, A.T., and Kierulf, P. (1990). Fish oil concentrate: effects on variables related to cardiovascular disease. *Am. J. Clin. Nutr.* **52,** 300–306

50 von Shacky, C., Fischer, S., and Weber, P.C. (1985). Long term effects of dietary marine omega-3 fatty acids upon plasma and cellular lipids, platelet function, and eicosanoid formation in humans. *J. Clin. Invest.* **76,** 1626–1631

51 Gibney, M.J. and Bolton-Smith, C. (1988). The effect of a dietary supplement of n-3 polyunsaturated fat on platelet lipid composition, platelet function and platelet plasma membrane fluidity in healthy volunteers. *Br. J. Nutr.* **60,** 5–12

52 Bordoni, A., Biagi, P.L., Parenti Castelli, G, Hrelia, S., Rossi, C.A., Lercker, G., Izpisua, J.C., Barber, T., Cabo, J., and Lenaz, G. (1989). Effect of a hyperlipidic diet on lipid composition, fluidity, and (Na^+-K^+) ATPase activity of rat erythrocyte membranes. *Membrane Biochemistry* **8,** 11–18

53 Croset, M and Kinsella, J.E. (1989). Changes in phospholipid fatty composition of mouse organelles after feeding graded amounts of docosahexaenoate in presence of high levels of linoleate: Effect on cardiac ATPase activity. *Ann. Nutr. Metab.* **33,** 125–142

54 De Schrÿver, R., Vermeulen, D., and Viane, E. (1991). Lipid metabolism responses in rats fed beef tallow, native or randomized fish oil and native or randomized peanut oil. *J. Nutr.* **121,** 948–955

55 Bourre, J-M., Bonneil, M., Dumont, O., Piciotti, M., Calaf, R., Portugal, H., Nalbone, G., and Lafont, H. (1990). Effect of increasing amounts of dietary fish oil on brain and liver fatty composition. *Biochim. Biophys. Acta* **1043,** 149–152

56 Neuringer, M. and Connor, W.E. (1989). Omega-3 fatty acids in the retina, In *Dietary ω-3 and ω-6 fatty acids: Biological effects and nutritional essentiality,* (C. Galli and A.P. Simopoulis, eds) p. 177–190, Plenum Press, New York, NY, USA

57 Sanders, T.A.B. (1986). Nutritional and physiological implications of fish oils. *J. Nutr.* **116,** 1857–1859

58 Conroy, D.M., Stubbs, C.D., Belin, J., Pryor, C.L., and Smith, A.D. (1986). The effects of dietary (n-3) fatty acid supplementation on lipid dynamics and composition in rat lymphocytes and liver microsomes. *Biochim. Biophys. Acta* **861,** 457–462

59 Kinsella, J.E., Lokesh, B., and Stone, R.A. (1990). Dietary n-3 polyunsaturated fatty acids and amelioration of cardiovascular disease: possible mechanisms. *Am. J. Clin. Nutr.* **52,** 1–27

60 Bhathena, S.J., Berlin, E., Judd, J.T., Kim, Y.C., Law, J.S., Bhagavan, H.N., Ballard-Barbash, R., and Nair, P.P. (1991). Effects of ω3 fatty acids and vitamin E on hormones involved in carbohydrate and lipid metabolism in men. *Am. J. Clin. Nutr.* **54,** 684–688

61 Aranda, F.J., Coutinho, A., Berberan-Santos, M.N., Prieto, M.J.E., and Gómez-Fernández, J.C. (1989). Fluorescence study of the location and dynamics of α-tocopherol in phospholipid vesicles. *Biochim. Biophys. Acta* **985,** 26–32

62 Srivastava, S., Phadke, R.S., Govil, G., and Rao, C.N.R. (1983). Fluidity, permeability and antioxidant behaviour of model membranes incorporated with α-tocopherol and vitamin E acetate. *Biochim. Biophys. Acta* **734,** 353–362

63 Urano, S., Kitahara, M., Kato, Y., Hasegawa, Y., and Matsuo, M. (1990). Membrane stabilizing effect of vitamin E: Existence of a hydrogen bond between α-tocopherol and phospholipids in bilayer liposomes. *J. Nutr. Sci. Vitaminol.* **36,** 513–519

64 Shapiro, A.C., Meydani, S.N., Meydani, M., Morrow, F., McNamara, J.R., Schaefer, E.J., Endres, S., and Dinarello, C.A. (1991). The effect of fish oil supplementation on plasma α-tocopherol, retinol, lipid and lipoprotein levels in normolipidemic subjects. *Nutr. Res.* **11,** 539–548

65 Meydani, M., Natiello, F., Goldin, B., Free, N., Woods, M., Schaefer, E., Blumberg, J.B., and Gorbach, S.L. (1991). Effect of long-term fish oil supplementation on vitamin E status and lipid peroxidation in women. *J. Nutr.* **121,** 484–491

66 Dimitrov, N.V., Meyer, C., Gilliland, D., Ruppenthal, M., Chenoweth, W., and Malone, W. (1991). Plasma tocopherol concentrations in response to supplemental vitamin E. *Am. J. Clin. Nutr.* **53,** 723–729

67 Chautan, M., Calaf, R., Léonardi, J., Charbonnier, M., Andre, M., Portugal, H., Pauli, A-M, Lafont, H., and Nalbone, G. (1990). Inverse modifications of heart and liver α-tocopherol status by various n-6/n-3 polyunsaturated fatty acid ratios. *J. Lipid Res.* **31,** 2201–2208

68 Clément, M. and Bourre, J.M. (1990). Alteration of α-tocopherol content in the developing and aging peripheral nervous system: persistence of high correlations with total and specific (n-6) polyunsaturated fatty acids. *J. Neurochem.* **54,** 2110–2117

69 Bhathena, S.J., Berlin, E., Judd, J.T., Jones, D.Y., Taylor, P.R., Campbell, W.S., Blanchard, S., and Nair, P.P. (1989). Dietary fat and menstrual-cycle effects on the erythrocyte ghost insulin receptor in premenopausal women. *Am. J. Clin. Nutr.* **50,** 460–464

70 Beck-Nielsen, H, Pedersen, O., and Sørensen, N.S. (1978). Effect of diet on the cellular insulin binding and the insulin sensitivity in healthy young subjects. *Diabetologia* **15,** 289–296

71 Ip, C., Tepperman, H.M., Holohan, P., and Tepperman, J. (1976). Insulin binding and insulin response of adipocytes from rats adapted to fat feeding. *J. Lipid Res.* **17,** 588–599

72 Hjøllund, E., Pedersen, O., Richelsen, B., Beck-Nielsen, H., and Sørensen, N.S. (1983). Increased insulin binding to adipocytes and monocytes and increased insulin sensitivity of glucose transport and metabolism in adipocytes from non-insulin-dependent diabetics after a low-fat/high-starch/high-fiber diet *Metabolism* **32,** 1067–1075