Dietary Fish Oil Augments the Function and Fluidity of the Intestinal Brush-border Membrane of the Carp

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Two groups of carps were raised on a commercial nutritionally complete diet: one was the control group, the other was fed the same diet enriched with 7% fish oil. The experiment lasted seven months (July through February), during which time the environmental temperature dropped gradually from 25°C to 13°C. Intestinal microvillus membranes were isolated after four and seven month feeding and examined for fluidity by fluorescence polarization with 1,6-diphenyl-1,3,5-hexatriene. The functionality of the membrane was assessed by the activity of the intrinsic enzyme alkaline phosphatase.

The experimental group exhibited increased membrane fluidity and elevated enzyme activity only when the environmental temperature decreased to 13°C. These changes in the membrane properties seemed to correlate with alterations in the fatty acid profile of the membrane phospholipids. Whereas the control group showed some increase in the n-3 C20:5, C22:5, and C22:6 fatty acids most likely due to cold adaptation, the membranes isolated from the group fed fish oil showed a considerably higher level of these fatty acids reflecting the combined effect of the dietary manipulation and cold adaptation.
Lipids 24, 737-742 (1989).

It is now widely accepted that the function of membrane proteins depends on the membrane fluidity (1). The fluidity, in turn, is largely affected by the temperature. Poikilotherms such as fish may develop severe malfunction in winter, below their optimal existence temperature (2). Thus, it is known from common practice that fish often cease eating below a certain water temperature characteristic of the fish species. It is conceivable that rigidification of the membranes at low environmental temperatures contributes to the ill effects by impairing membrane permeability and function of membrane-bound proteins (2).

It was shown that fish can adapt to cold temperatures by preferentially synthesizing long-chain polyunsaturated fatty acids, especially docosahexaenoic acid (3, 4). Another study demonstrated the importance of dietary polyunsaturated fatty acids in maintaining the resistance of the carp to cold temperatures (5).

The intestinal brush-border membrane specializes in digestion and absorption of nutrients, and is rich in a large number of functional proteins, including bound enzymes and transport proteins (6). It was therefore of interest to evaluate at the membrane level the effect of dietary polyunsaturated fatty acids on the structure, dynamics and function of this membrane in the carp under decreasing environmental temperatures.

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Abbreviations: DPH, 1,6-diphenyl-1,3,5-hexatriene; PUFA, polyunsaturated fatty acids.

MATERIALS AND METHODS

Animals and diets. Two groups of carp *(Cyprinus carpio)* having individual initial weights of 80 g were grown in plastic cages of $1m^3$ suspended in a fish pond. One group was fed a commercial pelleted diet (control). The second group was fed the same pellets coated with 7% fish oil (experimental diet). The fatty acid profile of the fish oil is given in Table 2. The feeding started in July and continued until the end of February the following year.

Membrane isolation. Fish taken from each group in November and in February were killed by a blow to the head. Mucosa scrapings of the upper half of the small intestine of five fish were used for microvillus membrane preparation. Prior experiments (Behar, D., unpublished data) showed that the lower half of the small intestine of carp possesses poor alkaline phosphatase activity. Membranes were isolated essentially as described by Schmitz *et al.* (7) and Brasitus *et al.* (8), except that the initial homogenization step was longer, using a Waring Blender for 2.5 min at medium speed followed by 2 min at full speed. The purity of the isolated membranes relative to the crude mucosa homogenate was assessed by estimating the specific activity of alkaline phosphatase. The various preparations were purified 8-11-fold.

Fluorescence studies. The fludity of the membrane preparation was assessed by steady state fluorescence polarization measurements using 1,6-diphenyl-1,3,5-hexatriene (DPH) as the fluorescent probe (9). The polarization of fluorescence was expressed as the fluorescence anisotropy, r, and the anisotropy parameter, $[(r_o/r)-1]^{-1}$, was calculated using a limiting anisotropy value of DPH of $r_o=0.362$. The anisotropy parameter is inversely related to the membrane lipid fluidity (9) and was presented as Arrhenius plot of log $[(r_o/r)-1]^{-1}$ vs 1/T.

Analytical determinations. Protein was quantified by the method of Lowry *et al.* (10). Alkaline phosphatase was assayed according to the method described by Brasitus *et al.* (8), except for the elimination of zinc ions. Lipids were extracted from the microvillus membrane according to Folch *et al.* (11). The fatty acid profile of the membrane lipids and that of the fish oil was determined after methylation with boron trifluoride according to Miller (12), using a Model 3700 Varian gas chromatograph equipped with a flame ionization detector and Varian Model CDS 1112 integrator. The methyl esters were resolved on a column packed with GP 10% SP 2330 on chromosorb WAW (Supelco). Individual fatty acids were identified with a fatty acyl methyl ester standard from a marine source (PUFA-1 Supelco). The temperature of the injection port and detector was

TABLE 1

Fluoresence Anisotropy Parameter and Alkaline Phosphatase Activity in Brush-Border Membranes Isolated from Rats Fed Fish Oil and Control Diet at the End (February) of 7 Month Feeding[a]

	Anisotropy Parameter $[(r_0/r)-1]^{-1}$		Alkaline Phosphatase Activity (μmole•min^{-1}•mg protein^{-1})	
Diet type	10°C	25°C	10°C	25°C
Control	3.92±0.15	2.28±0.14	0.024±0.005	0.062±0.008
Fish-oil	2.84±0.18[c]	1.88±0.10[b]	0.056±0.007[c]	0.157±0.007[c]

[a]Values are mean ± SEM for 4 different preparations.

Significantly different from the respective control.
[b]$p<0.05$.
[c]$p<0.01$.

230°C and that of the column was 210°C. Unidentified peaks amounting to less than 0.5% of the total area were disregarded.

Statistical analysis was carried out using Student's t-test.

RESULTS

During the growth period of seven months, both the experimental and the control fish exhibited normal development and weight gain.

The temperature dependence of the fluorescence anisotropy parameter $[(r_0/r)-1]^{-1}$ of DPH in the intestinal brush-border membrane preparations of both carp groups, following four months of feeding, is illustrated by Arrhenius plots in Figure 1. At this stage of development with a relatively high environmental temperature of about 20°C, typical of the month of November, the dietary fish oil had no effect on the membrane fluidity. Similiarly, an essentially identical alkaline phosphatase activity was observed over the range of 1-38°C, as shown in Figure 2. The Arrhenius plots of the enzymatic activity at this high environmental temperature were characterized by a transition temperature of 18°C for both the control and the experimental groups. With the advancement of the season, the water temperature declined and reached 13°C in February. Typical Arrhenius plots (Fig. 3) as well as values at 10°C and 25°C (Table 1) of the fluorescence anisotropy parameter at this stage of the experiment show that the membranes of the carps fed fish oil became considerably more fluid as compared to the membranes of the control group. The effect of the polyunsaturated fatty acids was also manifested in the activity of the mem-

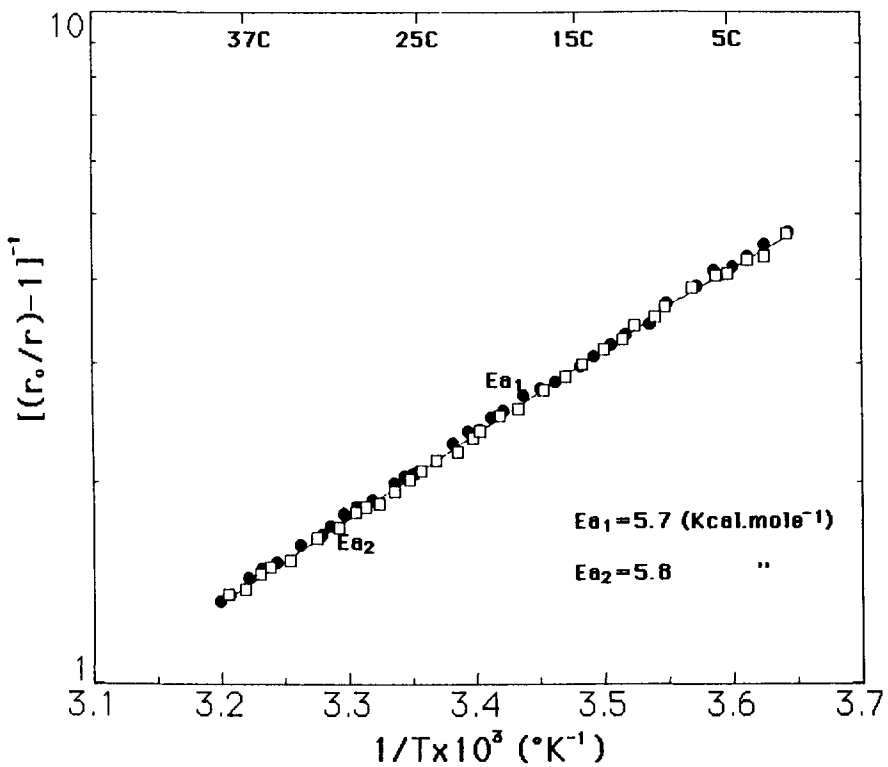

FIG. 1. Arrhenius plots of the temperature dependence of the diphenylhexatriene anisotropy parameter $[(r_0/r)-1]^{-1}$ of the intestinal microvillus membrane of carps fed a control diet (●) and a fish oil enriched diet (□) following 4 month feeding. Ea, energy of activation. Values are for one pair of preparations and are representative of four such comparisons.

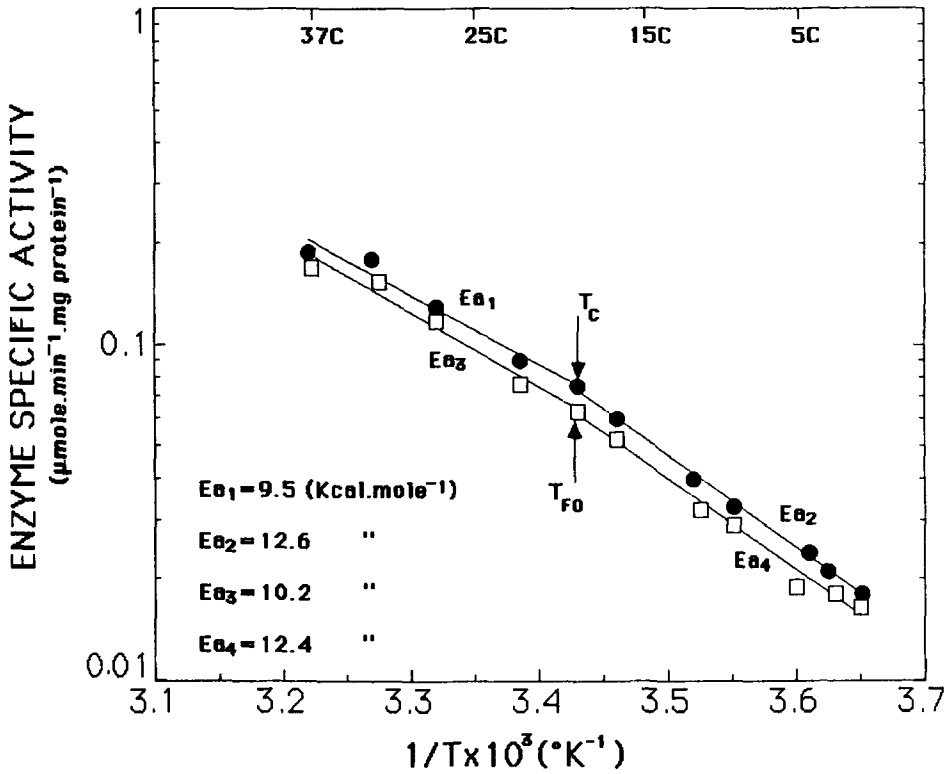

FIG. 2. Arrhenius plots of the temperature dependence of alkaline phosphatase activity in intestinal microvillus membranes of carps fed a control diet (●) and a fish oil enriched diet (□) following 4 month feeding. Ea, energy of activation; T_c and T_{fo} thermotropic transition temperatures of the membranes derived from fish fed control and experimental diets, respectively. Values are for one pair of preparations and are representative of four such comparisons.

brane bound alkaline phosphatase (Fig. 4 and Table 1). Thus, the observed specific activity of this enzyme was about 2-3-fold higher in the intestinal membranes prepared from the carp fed fish oil as compared to the respective activity in the membranes of the control group. No change was observed in the breakpoint of the Arrhenius plot of the enzymatic activity, which remained 18°C at a water temperature of 13°C. The energy of activation of the alkaline phosphatase activity and of the anisotropy parameter were not affected by the dietary lipids throughout the experiment.

The fatty acid profile of the intestinal brush-border membrane lipids of both groups of fish following four and seven month feeding is presented in Table 2. Feeding the fish oil for four months resulted in an increase in the content of the fatty acids which are typical for this oil, namely, the C20:5 and C22:6 acids. Thus, the amount of these n-3 fatty acids found in the membranes derived from the animals fed fish oil rose to 14.8%, and was significantly higher than the respective value of 10.1% observed for the membranes of the control group. Throughout this first phase of the experiment the environmental temperatures were 25-20°C. During the following three month feeding, in a period in which the environmental temperature dropped to 13°C, the n-3 fatty acid content of the membranes separated from the experimental carps increased to 21.4%, which was significantly higher than the amount of 16.0% observed for the control group.

DISCUSSION

Membrane dynamics which controls various membrane functions is determined by membrane composition and structure (1). In the present study membrane dynamics was assessed in terms of membrane fluidity, and alkaline phosphatase activity was used to evaluate membrane function. Alkaline phosphatase is known to be intimately associated with the hydrophobic core of the membrane (8). Such an intrinsic enzyme may be more sensitive to minor changes in the membrane ultrastructure when compared to extrinsic enzymes.

As long as the environmental temperature remained high, above or in the vicinity of 20°C, feeding of fish oil for a period of four months had no effect on membrane fluidity and on the enzymatic activity of the intrinsic protein alkaline phosphatase. Continuation of the feeding for an additional three months during which the temperature declined to 13°C resulted in marked changes in the properties of the brush-border membranes, namely elevated fluidity and enzyme activity in the membranes of the experimental group relative to the control.

Brasitus *et al.* (13) showed that membrane fluidity in rats can be manipulated by dietary lipids. Cossins *et al.* (14) showed that the fluidity of goldfish synaptosomal membranes increased as the water temperature decreased and attributed it to cold adaptation. In experiments with carps, Farkas *et al.* (3) observed that fish exposed to cold temperatures synthesized unsatu-

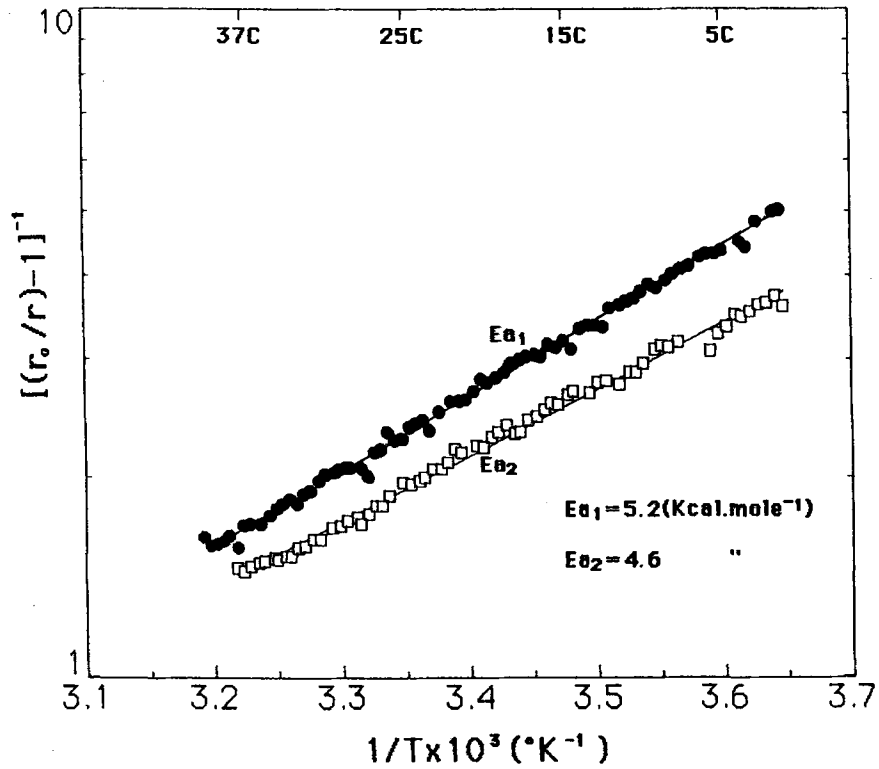

FIG. 3. Arrhenius plots of the temperature dependence of the diphenylhexatriene anisotropy parameter $[(r_o/r)-1]^{-1}$ of the intestinal microvillus membrane of carps fed a control diet (●) and a fish oil enriched diet (□) following 7 month feeding. Ea, energy of activation. Values are for one pair of preparations and are representative of four such comparisons.

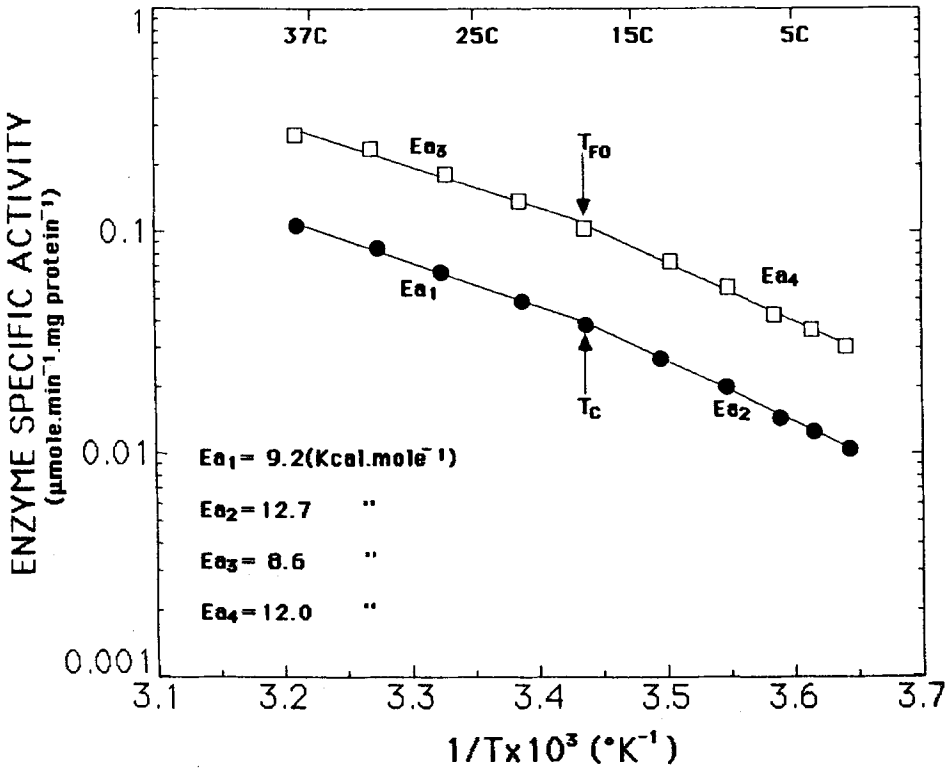

FIG. 4. Arrhenius plots of the temperature dependence of alkaline phosphatase activity in intestinal microvillus membranes of carps fed a control diet (●) and a fish oil enriched diet (□) following 7 month feeding. Ea, energy of activation; T_c and T_{fo} thermotropic transition temperatures of the membranes derived from fish fed control and experimental diets, respectively. Values are for one pair of preparations and are representative of four such comparisons.

TABLE 2

Fatty Acid Composition of the Dietary Fish Oil (Experimental) and of the Intestinal Microvillus Membranes after 4 (November) and 7 (February) Month Feeding (% of total fatty acids)[a]

Fatty acids	Fish oil	November		February	
		Control	Experimental	Control	Experimental
14:0	6.4	1.8±0.3	1.7±0.5	1.0±0.3	1.4±0.3
15:0	—	5.7±0.5	5.2±0.2	3.9±0.7	3.6±0.7
16:0	10.8	19.6±1.3	21.8±0.6	22.2±1.1	21.7±0.6
16:1	10.4	7.4±0.8	8.1±0.7	5.3±0.6	5.6±0.9
18:0	1.4	5.8±0.6	5.4±0.6	4.0±0.5	3.2±0.6
18:1	12.7	19.6±0.2	19.9±1.0	18.7±1.0	17.1±0.6
18:2	1.8	8.1±1.2	5.4±0.8	10.0±0.5	8.5±1.4
18:3	1.0	4.7±0.5	4.5±0.2	7.9±0.4	3.0±0.6
20:1	14.4	4.5±0.7	6.4±0.7	3.5±0.5	6.2±1.0
18:4	4.1	—	—	—	—
20:3(n3)	—	3.5±0.4	1.9±0.4	2.5±0.3	1.9±0.5
22:1	15.2	2.6±0.6	2.3±0.4	1.0±0.4	3.4±0.2
20:4	—	1.2±0.3	0.9±0.3	0.8±0.2	0.9±0.1
20:5	8.8	0.9±0.2	3.5±0.6	1.5±0.2	4.1±0.3
22:5	1.5	0.2±0.1	0.7±0.3	0.7±0.2	1.0±0.3
22:6	6.1	9.2±0.7	11.3±0.2	14.5±0.8	17.3±1.2
Σ(20:5,22:6)		10.1±0.9	14.8±0.8[b]	16.0±1.0	21.4±1.5[c]

[a]Values are means ± SEM for 4 different preparations.

Significantly different from the respective control.
[b]$p<0.01$.
[c]$p<0.05$.

rated fatty acids in the liver more than those exposed to warm water. These investigators related the increase in unsaturated fatty acid synthesis in the cold to the mechanism by which the organism regulated its membrane fluidity. Wodtke (15) who worked with mitochondrial membranes of carp also suggested that such fatty acid substitution in the membrane phospholipids represents a temperature induced fluidity adaptation. Nonetheless, direct fluidity measurements were not performed in these last two studies.

In our study the fatty acyl composition of the brush border membrane phospholipids of the experimental as well as the control group changed throughout the experiment. Such a change can conceivably result from reduction in the environmental temperatures (16-18) and from effects of dietary lipids (3). The changes in the fatty acid pattern of the control group following the seven month feeding period reflect cold adaptation, whereas the changes in the fatty acid pattern of the experimental group reflects both cold adaptation as well as dietary manipulation.

As far as the membrane lipid fluidity and the function of the intrinsic enzyme alkaline phosphatase are concerned, the dietary manipulation of the membrane phospholipid fatty acids was effective only at reduced environmental temperatures. It is conceivable that as long as the environmental temperature was high (above 20°C), the manipulation of the membrane fatty acids by dietary means was accompanied by changes in other membrane lipids so as to maintain homeoviscosity (17).

It is well known that marine fish oil is particularly rich in n-3 20:5 and 22:6 fatty acids. Some investigators (19, 20) have shown that one of the major changes in fatty acid composition of fish lipids at low temperatures is an increase in the level of the 22:6 fatty acid. A similar effect was observed in our study with the intestinal microvillus membrane. With regard to 20:5, the level of this acid in the membrane phospholipids also increased throughout the experiment, but to a lesser extent. Thus, the content of these two fatty acids in the membranes rose upon cold adaptation to 16.0% and increased more markedly upon cold adaptation and dietary manipulation to 21.4%.

It was suggested that the 22:6 acid plays an important role in raising membrane fluidity (20). It is also worth noting that the 20:5 acid is known to have the lowest melting point of any PUFA (2). Smith *et al.* (21) offered an explanation for the possible role of long chain polyunsaturated fatty acids, with 20 or more carbon atoms, in membrane fluidization. They suggested that these fatty acids are likely to introduce a disordering effect also in the region near the membrane phospholipid headgroups, since double bonds in these acids are located close to this region.

In conclusion, the present study suggests that the carp possesses only a limited capability to incorporate the long chain n-3 fatty acids into the membrane lipids during cold adaptation; the cold adaptation can be enhanced by the inclusion of these fatty acids in the diet, as evidenced by the assessment of the dynamics and function of the intestinal microvillus membrane.

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