Ph.D. Thesis

Molecular and structural organization of phospholipid membranes in livers of marine and fresh water fish adapted to radically different temperatures in their course of evolution

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Dedicated to my parents

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ABBREVIATIONS

BHT	Butylated hydroxytoluene
CAFF	cold-adapted fresh-water fish
CAMF	cold-adapted marine fish
CL	cardiolipin
DGDG	diglucosyldiglyceride
DMPC	dimyristoylphosphatidylcholine
DOPC	dioleoylphosphatidylcholine
DOPE	dioleoylphosphatidylethanolamine
DPH	1,6-diphenyl-1,3,5-hexatriene
DPH-PA	3-(p-(6-phenyl)-1,3,5-hexatrienyl) propionic acid
DPPC	dipalmitoylphosphatidylcholine
ESR	Electron Spin Resonance
FTIR	Fourier transform infrared
H_{II}	hexagonal phase
HPLC	high-performance liquid chromatography
$L\alpha$	liquid crystalline phase
LUV	large unilamellar vesicle
MGDG	monoglycosyldiglyceride

NMR	Nuclear Magenetic Resonance
n-AP	n-(9-anthroyloxy) palmitic acid
n-AS	n-(9-anthroyloxy) stearic acid
ODPE	1-Oleoyl-2-Docosahexaenoyl-Phosphatidylethanolamine
OEPE	1-Oleoyl-2-Eicosapentaenol-Phosphatidylethanolamine
OPPC	1-Oleoyl-2-Palmitoyl-Phosphatidylcholine
OSPC	1-Oleoyl-2-Stearoyl-Phosphatidylcholine
PA	phosphatidic acid
PC	phosphatidylcholine
PDPC	1-Palmitoyl-2-Docosahexaenoyl-Phosphatidylcholine
PE	phosphatidylethanolamine
PG	phosphatidylglycerol
PI	phosphatidylinositol
POPC	1-Palmitoyl-2-Oleoyl-Phosphatidylcholine
POPE	1-Palmitoyl-2-Oleoyl-Phosphatidylethanolamine
PS	phosphatidylserine
PUFA	polyunsaturated fatty acid
Q_I	cubic phase

R_{ss}	steady state anisotropy
R_O	spontaneous radius of curvature
SFA	saturated fatty acid
SOPC	1-Stearoyl-2-OleoylPhosphatidylcholine
sn-1	stereospecific numbering at the No.1 carbon atom in the glycerol backbone
sn-2	stereospecific numbering at the No.2 carbon atom in the glycerol backbone
T_h	temperature of the fluid/$H_{\|}$ transition
THP	thermal hysteresis protein
TLC	thin layer chromatography
T_m	temperature of the gel/fluid transition
UFA	unsaturated fatty acid
WAFF	warm-adapted fresh-water fish
WAMF	warm-adapted marine fish

Fatty acids (common name)	*Chain length:unsaturation*
Palmitic acid	**16:0**
Palmitoleic acid	**16:1 (9-*cis*)**
Stearic acid	**18:0**
Oleic acid	**18:1 (9-*cis*)**

Linoleic acid	**18:2 (9-,12-*cis*)**
γ-Linolenic acid	**18:3 (6-,9-,12-*cis*)**
α-Linolenic acid	**18:3 (9-,12-,15-*cis*)**
Arachidonic acid	**20:4 (5,8,11,14-(all)*cis*)**
Eicosapentaenoic acid	**20:5 (5,8,11,14,17-(all)*cis*)**
Docosahexaenoic acid	**22:6 (4,7,10.13,16,19-(all)*cis*)**

ABSTRACT

Composition and physical state of liver phospholipids of marine and fresh water fish adapted to relatively constant but radically different temperatures were compared. Fish adapted to low (5-10°C) temperature accumulated more unsaturated fatty acids than those from warm (25-27°C) environments. There were no measurable differences between marine and fresh water fish from identical thermal environments. Docosahexaenoic acid did not seem to participate in the process of adaptation. Cold adaptation was paralleled by oleic acid accumulation preferentially in phosphatidylethanolamine. Stereospecific distribution as well as molecular species composition of phosphatidylethanolamine demonstrated a 2-3 fold increase in level of ODPE and 10 fold increase in level of OEPE. ESR spectroscopy showed only 7-10% compensation in state of phospholipid vesicles of both warm and cold adapted fish. Combination of either PDPC or native phosphatidylcholines from both cold adapted fish with phosphatidylethanolamines from the same environment showed a drastic fluidisation in the vicinity of the C-2 segment of the bilayer but not in the deeper regions. A proper combination of such phosphatidylcholines and phosphatidylethanolamines (75:25%) mimicked a 100% homeoviscous efficacy in the C-2 segment when compared with phosphatidylethanolamines of warm adapted fish. The presence of ODPE and polar headgroup interaction was also detected by FTIR. A specific role of ODPE in controlling membrane structure and physical state in relation to thermal adaptation is proposed.

1. INTRODUCTION

Great advances have been made over the past 15 years in our understanding of the structure-function relationships of biological membranes. Yet little is known about the way in which the cells produce a diverse and highly differentiated collection of membranes or regulate their respective chemical compositions. One obvious way of revealing such control mechanism is to disturb the *Status quo* and look for the corrective responses. This strategy has recently been used to a great advantage in biological membranes, where the temperature has been used as the disturbing influence.

Historically, it has been known for many years that exposure of microorganisms, plants and animals to low temperature leads to the incorporation of increased proportions of unsaturated fatty acids in the storage lipids (i.e.triglycerides) and in the membrane phospholipids (Hazel,1984). On the basis of the physical studies this trend was interpreted as an adaptive response which offsets the direct effects of cooling upon the physical state of the lipid structures. Thus phospholipids containing unsaturated fatty acids displayed a greater cross sectional area (i.e. greater expansion of a monolayer), a greater molecular flexing motion and lower phase transition temperatures (i.e. melting temperature) than lipids containing saturated fatty acids.

A direct test of this hypothesis was only possible with the development of techniques for the direct investigation of membrane physical structure. This was first demonstrated in bacteria using electron spin resonance spectroscopy by Sinensky (1974), who showed that the "fluidity" and phase state of membranes from *E. coli*. was maintained constant at each of the several growth temperatures. He coined the term "homeoviscous adaptation" to emphasize

both the homeostatic nature of the response and its adaptive importance. Although the report of this "homeoviscous adaptation" describes a provocative phenomenon that merits more careful interpretation; otherwise it hypothesize, that the chemical composition of membranes is regulated to maintain the physical structure or "fluidity" of the bilayer within tolerable limits.

Fluidity is a term which describes the relative conformational freedom and mobility of membrane constituents, but membrane fluidity is difficult to define with rigour, due, in part, to the range of motions available to lipid molecules, which vary from rotamer formation within the acyl chain to wobbling and rotational motion of an entire molecule, to lateral diffusion within the plane of the membrane. No single physical technique is sensitive to this entire range of motions.

Biological chemists have often regarded the fluidity of a membrane as a property that may be adaptively altered to accommodate changes in environmental temperature. The implication that cells exhibit an intelligent (or at least, appropriate) response provides us with several questions to examine.

1) Is temperature or some aspects of the fluidity perceived by the responsive cellular elements?

2) How is the detected difference from "ideal" temperature or fluidity communicated to the synthetic enzyme(s)?

3) How can the selective actions of the enzyme(s) of phospholipids synthesis be changed to alter fatty acid content of the membrane lipids?

A primary intellectual challenge in examining these responses is to discern the degree to which the cells adapt "because of" signals from an inadequate membrane fluidity in contrast with an independent alteration of synthetic selectivity that "coincidentally" alters the

membrane fluidity. In a sense, one might ask whether the intracellular signalling is a 'dialogue' or a 'monologue', or in other term, whether the manner of membrane adaptation is purposefully successful ("Lamarckian") or fortuitously beneficial ("Darwinian"). Certainly cells containing either adaptive mechanism could exhibit better survival in a limiting environment than would those cells without.

A complicating factor in evaluating adaptive changes in the fatty acids composition of membrane phospholipids is that the synthetic enzyme(s) may act on only one single fatty acid molecule by recognizing its chemical structural features rather than fluidity, which is a cooperative aspect of the interaction of many molecules. Thus we must be sensitive to the language in which messages are translated and communicated within cells. The factors used by the synthetic enzymes in selecting preferred acyl chains, seem to be important elements in the "language" of membrane lipid adaptation. A battery of synthetic enzymes, the desaturases are thought to be involved in modifying the acyl chain, to lower the fluidity, but no clear concept indicates how desaturase activity may be enhanced by lower fluidity. Thus a high membrane fluidity does not appreciably restrain the entry of more fluidising fatty acids. Indeed, the possibility of a controlling response preventing too much fluidity has not been widely discussed or demonstrated, making a major void in our understanding of what may represent "too much" fluidity for cells. The embryonic fact that the cells continue to incorporate saturated fatty acids into the less fluid membranes suggests that little intelligent adaptive communication is acting in cells, lacking the enzyme(s) for esterification of membrane lipids were a suitable adaptive response (Cronan,1978), the proper occasion seemed present, but it did not trigger a response to alter the selectivity of the synthetic enzymes in time to save cells from a self-inflicted disaster (Henning et al, 1969). Thus we have no conclusive evidence that membrane fluidity alters the selectivity of biosynthesis or

the esterification of fatty acids.

1.1 Lipid as a backbone of Biological Membranes:

Lipid bilayers are the backbone of all biological membranes. Lipids act as a "solvent" for all the membrane components. Textbooks of biochemistry invariably illustrate the structure of biological membranes with diagrams of phospholipids aligned in a parallel and highly ordered manner. Although these membrane constituents are indeed organised as a bilayer they exhibit, at physiological temperatures, a considerable degree of disorder and molecular mobility exists. These motions range from a rapid flexing of the hydrocarbon chain by rotations about carbon-carbon bonds, to wobbling motion of the entire molecule and to the lateral displacement of the molecule along the plane of the membrane. Because of this motion biological membranes have liquid-like character and thus described as being fluid. Thus the accepted picture of biological membranes is the fluid-mosaic model of Singer and Nicholson (1972). It is thought that a liquid-crystalline phase is essential, which can be attributed to protein function of the membrane (Jost *et al*, 1973).

Although the membrane lipids may exist in a nonbilayer phase (Luzzati *et al*, 1968), the bulk of lipids are organized as a lipid bilayer and contain a central core of fatty acids surrounded by two phases of hydrated polar headgroups in an aqueous environment. Phospholipids are the major components of biological membranes and together with sterol and glycolipids form the matrix of the membrane.

The phospholipids of eukaryotic membranes consist basically of phosphatidylcholine (PC) and sphingomyelin (SM) and aminophospholipids, phosphatidylethanolamine (PE), phosphatidylserine (PS) and a small percent of phosphatidylinositol (PI). These lipids and the

protein constituents have an asymmetrical distribution between outer and inner leaflets of lipid bilayers (Rothman and Lenard, 1977).

Although the agreement between different laboratories at a quantitative level is often unsatisfactory, most biological membranes appear to have different phospholipid composition in their inner and outer leaflets. At least in plasma membranes, transverse lipid segregation is firmly established. In erythrocytes, the best documented system, PS and PE and probably PI are located mainly in the inner monolayer while PC and SM are essentially in the outer monolayer.

Any change in the lipid composition of the membranes (cholesterol or phospholipids) results in changes in the local environment of integral proteins (receptor molecules, enzymes, transport etc.)(Brasitus and Dudeja,1986;Rathiers and Chen,1986; Zabrecky and Raftery,19-85; Kolena and Blazucek,1986). Moreover, membrane bound enzymes and receptors require specific phospholipids or acyl chains for optimal activity (Tung and Mc.Namee,1986). There are many approaches to demonstrate the specificity of the lipid requirements of membrane-bound enzymes (Sanderman,1978; Roelofsen and Schatzmann,1977). The specificity for a particular species of phospholipids could depend on the nature of the head groups, the nature of the backbone (glycerol or sphingosine), the length of the acyl chains and the insertions of the double bonds in the acyl chains. All the above stated factors in a more general way affect the membrane by influencing so called "membrane fluidity".

Anyway, there are abundant studies to show the relationship between the lipid composition of biological membranes and its physio-chemical properties with enzymatic activities (Carruthers and Melchor,1986; Carriere and Le Grimellec,1986; Worman *et al*, 1986; Kreutler *et al*, 1984; Harper *et al*, 1990; Baldwin and Hochacka,1970; Arindam Sen *et al*, 1991).

1.2 The Bilayer Paradigm :

It is basic assumption that phospholipids in biomembranes are organised in a continuous bilayer. If that is the case, clearly, has not been demonstrated in all membranes. It is however, a reasonable assumption. Inspite of very extensive work undertaken notably by Cullis, de Kruijff and co-workers (Cullis and de Kruijff, 1979), to date there is no example of permanent nonbilayer structure in a real biological membrane, unambiguously demonstrated by X-ray, ^{31}P-NMR, or electron microscopy. Lipids extracted from biomembranes frequently form hexagonal or cubic phases at physiological temperatures, but this by no means demonstrates the occurrence of such structures in the presence of proteins. Thus, it can be said comfortably, that the phospholipids that surround membrane proteins indeed form a bilayer and that nonbilayer structures, if any, are only transient non-equilibrium configurations : topologically, inner and outer leaflets are well defined.

The bilayer paradigm is not restricted to topological considerations. This description includes important considerations of dynamics. In the early seventies it was shown in Mc. Connell's laboratory that in the liquid crystalline state (Luzzati's L_α phase) i) the rate of lateral diffusion is fast, within the plane exchange rates are of order of $10^7 S^{-1}$ (Devaux and Mc.Connell, 1972) and ii) the rate of transverse diffusion, or flipflop is slow (half time of exchange of several hours) (Korenberg and Mc.Connell, 1971). Values obtained with spin labelled phospholipids in egg lecithin liposomes, were confirmed and refined by many techniques. Thus, one important feature of a pure lipid bilayer is absence of phospholipid communication between the two opposite leaflets. It is to be noted that the rapid lateral diffusion implies that a few transmembrane defects or even very localised nonbilayer structures would suffice to allow the flip-flop of a large fraction of phospholipids. In pure lipid systems, these defects are unavoidable, a priori, must have short life times since the

transmembrane diffusion is slow. It may not be true in protein-containing bilayers where a few permanent "defects" can modify the transmembrane diffusion of membrane components, extending to small hydrophobic molecules, if uncharged, is fast (halftime of exchange of a second or less). Thus, the membrane impermeability is not true for neutral lipids (diacylglycerols, fatty esters, and probably cholesterol) as well as for weak acids and weak bases (Zachowski and Devaux,1989).

The third dogma of the bilayer paradigm is that unlike lateral segregation spontaneous transmembrane segregation is exceptional. It only happens with vesicles of small radius obtained by sonication (Op den Kamp,1979). As a consequence, asymmetrical large unilamellar vesicles (LUV) (Sen *et al.*, 1991) which can be obtained for instance by selective chemical modifications of the lipids or by the addition of charged amphiphilic molecules on one layer, are out of equilibrium and relax towards a random distribution. LUV's that are made asymmetrical by the effect of a pH gradient (Hope and Cullis,1987) can be considered also as out of equilibrium, since the release of this external constrain results in the bilayer symmetrization.

Clear departure from the above behaviour will demonstrate the influence of proteins and generally imply non-equilibrium situations. It rapidly becomes apparent that transmembrane distribution in biomembranes has to be viewed in the context of lipid traffic within cells; i.e. dynamic considerations are necessary.

1.3 Lipid asymmetry in biomembranes:

The techniques used to determine lipid asymmetry in biomembranes were reviewed by several authors (Op den Kamp,1979; Etemadi,1980;Krebs,1982). These techniques

comprise chemical labelling with non-penetrating agents, for example, TNBS or fluorescamine, immunological methods, phospholipase digestion, use of phospholipase-exchange proteins and physio-chemical methods such as X-ray diffraction (Luzatti *et al.*,1968, Rand *et al.*,1971) and NMR (Gally *et al.*,1980, Taylor and Smith,1981, Cullis and deKruiff, 1976, 1978). When carrying out these experiments, an implicit and sometimes unjustified assumption is that, the lipid topology is stable over reaction time and can resist membrane perturbations, such as attack by exogenous phospholipases. Clearly, in the case of fast flip-flop some of these techniques are no longer valid. Also, if lipid asymmetry is the result of a subtle balance between various lipid fluxes, membrane purification by differential centrifugation and sucrose gradient, as well as the analysis of virus membranes that are formed by budding through a plasma membrane has allowed one to identify the underlying pattern obtained with human erythrocytes, which is the most extensively studied system. But the situation is more complex with other eukaryotic cells like fish erythrocytes and other nucleated cells, where plasma membrane is only a small fraction of the total cell membranes.

Many attempts to investigate lipid asymmetry in internal membranes from eukaryotic cells can be found in the literature. Unfortunately, when the same system have been studied by different laboratories, the results often differ considerably. The difficulty in finding conclusive evidence on lipid topology in organelles, which may reflect a very rapid lipid redistribution within each membrane and from membrane to membrane within each eukaryotic cell. Massive transfer of phospholipids from the ER to Golgi and to the mitochondria and back journey to the plasma membrane following the same route takes place continuously (Bishop and Bell, 1988). According to Wieland *et al* (1987) about half of the total ER phospholipids is transferred out of the Golgi every 10 min^2. On the other hand, it is conceivable that specialised organelles in differentiated cells, such as granules in

chromaffin cells, synaptic vesicles in axons, and disks in retinal rods have a well defined phospholipid asymmetry, because the turnover of the lipids in such system is not so important. In the above mentioned organelles the aminophospholipids were generally reported to be exposed on the outer monolayer, which corresponds to the cytosolic surface (Buckland *et al*, 1978; Michaelson *et al*, 1983; Litman, 1982). Thus very little is known about the stability of lipid asymmetry in biomembranes.

1.4 Membrane fluidity and membrane order and phase structure :

The properties and physical structure of the hydrocarbon chain interior of phospholipid bilayers is quite distinct from that of bulk hydrocarbon fluids,such as paraffin, and concept of bulk viscosity and fluidity (i.e. reciprocal of viscosity) can not have same precise meaning. For one thing the bilayer is highly anisotropic and semi-ordered, and for another, the ΔH values associated with gel-to-liquid crystalline phase transition are significantly lower than for the melting of hydrocarbon fluids (Phillips *et al*, 1969). Finally, biological membranes are heterogenous systems in which a diverse chemical composition may lead to microdomains in the plane of the membranes as well as different physical properties in each monolayer.

In practice, membrane fluidity is operationally defined and quantified using one of several biophysical techniques which measure the rotational characteristics of spectroscopic nuclei or molecules (probes) which by chemical synthesis or by partitioning can be intercalated within the bilayer interior. The basic assumptions in this approach are first, that the measured motional characteristics of the probe are sensitive to the dynamical motion or order of the surrounding hydrocarbon chain and second that normal physical structure of the bilayer is not greatly disturbed by the probe. Each technique provides information specifically

on the type of motion which affects the spectroscopic property in the question, so that the different techniques reports on different aspects of the "fluid" condition. Thus ^{2}H and ^{13}C nuclear magnetic resonance spectroscopy provides rather specific information on the dynamics of hydrocarbon flexing motion and by the chemical synthesis of fatty acids with ^{2}H- and ^{13}C nuclei positioned at specific sites along the hydrocarbon chain a detailed understanding of bilayer structure can be obtained (Seelig and Seelig, 1980). By contrast, fluorescence polarisation spectroscopy of a rigid, rod shaped fluorescent molecule such as 1,6-diphenyl-1,3,5-hexatriene (DPH) indicates the extent of wobbling motion over the nanosecond time scale. This is influenced by bilayer structure over much of the length of the fatty acids at least equivalent to the length of the DPH molecule (ca 13 A°) rather than at a specific segment. Given these differences it is necessary to use several different techniques to characterize fully membrane fluidity or to use probes with different rotational properties. Each type of motion occurs over a rather different time domain and changes in another (Kleinfeld *et al*, 1981). Any of these methods are useful in a comparative experiment though attempts to convert derived motional parameters to absolute units of viscosity by comparisons of probe behaviour in hydrocarbon solvents of different bulk viscosities have been severely criticized largely because of the qualitative differences in the physical structure of the bilayer and bulk solvents.

Technical and theoretical advances have led to an improved understanding of these spectroscopic techniques and their interpretation. Nowhere is this more true than in fluorescence polarisation spectroscopy where early interpretations assumed that the rotational motion of rod like probes was unhindered so that polarization of fluorescence was viewed as an index of rotational rate.

More recent time-resolved studies have demonstrated more complex rotational

properties in that free rotations of the probes were constrained by the anisotropic structure of the bilayer (Von Blitterswijk *et al*, 1981). Thus DPH polarization is now thought to reflect primarily the degree of hindrance to free rotation rather than a rotational rate.

Finally, it is necessary to recognise that probe techniques generally provide average information on fluidity/order depending upon the distribution of probe positions within the membrane. The membrane is thus treated as though it were a single hydrophobic phase with discrete and definable properties, even though it is probably made up of a number of distinct microdomains each with different physical properties. The more common probe techniques, such as fluorescence polarization using DPH or electron spin resonance spectroscopy (ESR) using nitroxy labelled fatty acids, are not able to differentiate between different microdomains, a yield, a weighted average of the probe positions. This problem may be overcome by using probes with well defined positions in the so called "fluidity gradient". Thus, recently the emergence of different fluorescence probes like, 3(p(6phenyl)1,3,5-hexatrienyl)propionic acid (DPH-PA), which is an anionic derivative of DPH, and thus, reports exclusively the outer leaflet of the membrane (Kitagawa *et al*,1991) and a series of n-(9-anthroyloxy) stearic and palmitic acid (2-AS, 3-AS, 6-AS, 9-AS, 12-AS, and 16-AP). These are based upon a long chain fatty acid and localized within a membrane with the carboxyl end at the membrane water interface and terminal methyl group approaching the core of the bilayer (Cooper and Meddings, 1991) may help to probe separately microdomains with subtly different properties. In less disruptive techniques such as ^{1}H and ^{13}C NMR (Nuclear Magnetic Resonance), it is easy to study membranes and they may yield structural information indirectly, by investigating the changes in the motions of a small molecule partitioned into the membrane (Smith and Jarrell,1985) From a practical point of view this means that it is frequently not possible to link casually a change in the average membrane fluidity, either to changes in all

molecules in a population of probes, to changes in the distribution of probe between microdomains or to changes in the properties in some of the microdomains but not others. By Mantsch *et al,* (1981) it has been demonstrated that Fourier-transformation infra red spectroscopy (FTIR), is also applicable to study the structural and functional properties of membranes. At present, this is the only technique available, which introduces no foreign molecules into the membrane, to study its structural integrity.

Phospholipids are considered as polymorphic, assuming a variety of different phase organizations depending on temperature, pressure, degree of hydration and the chemical composition of the phospholipid itself (Chapman, 1975). Three phases have been identified in phospholipids and phospholipid mixtures of biological origin; these include *lamellar, hexagonal* and *cubic* phases. Transitions between these phases can be driven by changes in temperature (i.e. are thermotropic) and, as a result, temperature will determine the particular phase or combination of phases that coexist in lipid membrane. Although lamellar phases dominate membrane structure throughout the biological range of temperatures, hexagonal and cubic phases can form at the extremes of normal physiological conditions. Although it is not evident whether this phase transition occurs in living membranes or not, but certainly, they do occur in pure lipid membranes. The effects of temperature on the phase state of membrane lipids are best illustrated in homogeneous suspension of a single phospholipid molecular species. But, yet, it is not well understood about the phase behaviour of membrane lipids which are heterogenous and constitute several phospholipid molecular species.

1.5 Adaptation of biological membranes to temperature (with reference to aquatic poikilotherms)

Adaptation or acclimation of poikilotherms to temperature extremes are accomplished by compensatory adjustments in essentially all major aspects of an animal's physiology and biochemistry. The poikilotherms are often faced with the problem of maintaining a constancy of function amid a fluctuating ambient temperature. The functional constancy is immensely referable to compensations that alter the catalytic capabilities of the organism, and are aimed at maintaining energy production and performance relatively uninfluenced by temperature. Essential to the acclimation response, is a metabolic restructuring that involves the synthesis of correct molecule in the correct quantities to promote efficient catalysis and metabolic regulation within a particular thermal regime.

Since the majority of the biological processes depend on the functional integrity of the membrane systems, it is not surprising that membrane lipid composition also reflects the avenue of adaptation (evolutionary or genetically)or the acclimatization (phenotypic) of a single species in a thermal regime.

Poikilothermic organisms exploit diversity in lipid structure to fashion membranes with physical properties appropriate to the prevailing ambient temperature. The major structural lipids in biological membranes are phospholipids, and diversity in phospholipid structure arises from chemical heterogeneity in both the headgroup (giving rise to different phospholipid classes, e.g. phosphatidylcholine and phosphatidylethanolamine) and the acyl chain domains (giving rise to unique molecular species of phospholipid such as 16:0/18:1 vs 16:0/16:1) of the molecule. Thermal adaptation of membrane function thus involves a chemical restructuring of bilayer lipid composition to ensure that the components present are best suited to function within the constraints imposed by prevailing thermal environment. The

retailoring of membrane lipid composition is perhaps the most pervasive cellular response to temperature change. Prior to discussing the specific compositional adjustments believed to be responsible for thermal compensation of membrane function, the various forces involved in determining lipid phase behaviour will be discussed briefly to identify unifying concepts useful in understanding and interpreting the consequences of temperature induced alterations in membrane lipid composition. Inclusive of lipid molecules, cholesterol and proteins as well as their interactions also play a crucial role in the ordering of the membrane in response to the ambient temperature.

1.5.1 Factors determining the phase behaviour of membrane lipids:

A) Molecular geometry argument:

Israelachvili *et al,* (1980) propose that molecular geometry is a primary factor in determining the phase preference of lipids and that lipid geometries may be quantitatively described by a dimensionless packing parameter, P :

$$P = v/(a.l)$$

where, v = hydrophobic volume of acyl domain.

l = length of the acyl chain.

a = mean surface area (i.e. headgroup size) at the aqueous interface.

Lipids for which $P \leq 0.33$ possess a relatively large interfacial surface area and a relatively small hydrophobic volume and, therefore, assume an inverted conical geometry which packs most efficiently in the form of spherical micelles (Fig.); lysophospholipids, which possess only a single acyl chain, are typical of this class of amphiphile. Alternately, in lipids for

which $0.5 \leq P \leq 1.0$, the increased bulkiness of the hydrophobic domain relative to head group size results in a cylindrical geometry which packs most efficiently in the form of a bilayer. Diacyl lipids (e.g. phospholipids) are most likely to form bilayers since, for similar values of headgroup area (a) and chain length (l), these lipids possess a hydrophobic volume and packing parameter that are twice those of micelle forming lipids. Among phospholipids, anionic as opposed to zwitterionic species are more likely to accommodate the hydrophobic volume of two acyl chains in a lamellar phase due to the increase in effective head group surface area resulting from electrostatic repulsion. Finally, in lipids for which $P \geq 1.0$, the hydrophobic volume is larger than the head group area, resulting in a conical geometry that packs most efficiently in the form of inverted micelles (the $H_{\|}$ phase). Diacyl phospholipids possessing smaller head group (e.g. PE) exemplify this class of lipid that favour nonbilayer structures. It is important to appreciate that the molecular geometry is a dynamic attribute of lipid structure subject to perturbations by environmental factors, and that, in extreme cases,these perturbations may be sufficiently large to alter the most efficient packing arrangement. Viewed in this context, temperature induced restructuring of biological membranes may be necessary to restore an appropriate lipid geometry and to maintain an optimal packing arrangement between membrane constituents.

B) Radius of curvature arguments:

The concept of spontaneous radius of curvature (R_0) and the competition between this force and hydrocarbon packing constraints have consequently been developed as an alternative explanation for the phase behaviour of phospholipids (Epand *et al*,1989, Lindblom and Rilfors,1989, Seddon,1990, Shyamsunder *et al*,1988). The spontaneous radius of

curvature is determined by molecular geometry : conically shaped lipids possess a small R_0 value and will induce curvature into a phospholipid monolayer (since only cylindrical lipids pack efficiently to form a planer lamellar phase), thus explaining the propensity to form the H_{II} phase. However, although monolayers of conical lipids in the H_{II} phase are nearly relaxed with respect to curvature, the lipid chains are in an energetically unfavourable configuration due to the presence of hydrophobic interstices located between the H_{II} lipid cylinders (i.e. the acyl chain of lipid must in essence stretch to cover or fill this interstices). Phospholipid phase behaviour is thus presumed to reflect a balance between the tendency to assume the spontaneous radius of curvature on the one hand and the hydrocarbon packing constraints on the other : as R_0 gets larger (or the curvature smaller), formation of the H_{II} phase is prevented by hydrocarbon packing constraints (since the larger the H_{II} tube diameter, the larger the voids that must be filled to overcome this constraint). According to this view : (1) the presence of small quantities of hydrocarbon in a bilayer can reduce T_h (temperature of fluid/ H_{II} transition) by intercalating into the hydrophobic interstices, thereby, reducing the hydrocarbon packing constraints (Epand *et al*,1989); and (2) the principle factor driving transitions from lamellar to non-lamellar configuration and approach R_0.

An important insight to arise from the recognition that competing forces may be acting on a bilayer is expressed in the concept of *frustration*, where, each monolayer is prevented from adopting its equilibrium curvature by being coupled to the other. This differs fundamentally from the situation in which neither monolayer exhibits a tendency towards curvature. Although the H_{II} phase is not normally observed in biological membranes, the physical forces operating in pure lipid systems tending to drive the equilibrium towards a nonbilayer configurations certainly are. Viewed in this context, it may be the propensity

towards H_{II} phase formation rather than the realization of the phase which is an important feature of membrane structure and function (Epand,1990).

C) Bending energy computations :

Hui and Sen (1989) have recently combined the concepts of molecular geometry and spontaneous radius of curvature (R_0) to compute a bending energy expression (the average R_0 was computed from molecular geometries and the corresponding bending energy expressed as a function of R_0). With increasing bending energies, mixtures of lipids displayed increasing susceptibility to phospholipase A_2, facilitated lipid transfer by phospholipid exchange proteins, and enhanced activity of Ca^{2+}-ATPase. These authors concluded that biological membranes exist in a state of maximum allowable bending energy, just short of disruption.

1.5.2 Cholesterol modulation as a mechanism of thermal compensation :

Cholesterol exerts profound effects on the physical and functional properties of a membrane and is, therefore, a prime candidate to regulate membrane function in thermal adaptation and acclimation as well. However, a possible regulatory role for cholesterol in acclimation to thermal stress has not been definitively established and is a relatively little studied aspect of membrane adaptation to temperature extremes. In most cases, cholesterol content of membranes has been found to be inversely correlated with growth temperature including muscle mitochondria (Wodtke, 1978), hepatic microsome and erythrocyte membranes of carp (Wodtke,1983), outer mitochondrial membranes of goldfish brain (Chang *et al*,1989) and flight muscle mitochondria of *Schistocerca* (Downer *et al.*1981); however, in other cases, cholesterol do not vary with acclimation history, as it has been reported for

synaptic (Cossins,1977) and myelin membranes (Buckley *et al.*, 1985) of goldfish brain and membrane of flounder erythrocytes (Sørensen, 1990), indicating no consistent effect of growth temperature on the cholesterol/phospholipid ratio. Yet, in none of these studies have the functional consequences of variations in cholesterol content been evaluated.

Cholesterol is a surface active steroid that is readily accommodated in most membranes up to molar ratios 1:1 (relative to phospholipid) and possibly higher (Finean, 1990). The steroid nucleus lies parallel to and buried within the acyl domain of the membrane, but the cholesterol molecule adopts a conical shape due to small area (19Å^2) occupied by the 3β-hydroxyl group located near the bilayer surface (in the vicinity of the ester carbonyl linkages of the phospholipids) and the larger hydrophobic end area (38Å^2) occupied by the flexible alkyl tail in the membrane interior (Rilfors *et al.*,1984). Consequently, cholesterol disturbs the packing of gel phase lipids and decrease the size of the cooperative unit involved in the gel/fluid transition, causing the phase transition to broaden and, in some cases, to disappear entirely (Yeagle, 1985) at high cholesterol levels, the relative proportions of *trans*- (acyl chains are fully extended in an all *trans* configuration to *gauche*-conformers (acyl chains are not fully extended with all *trans* configuration but having kinks or bends at different positions in the acyl chain) changes very little with temperature, consistent with a broadened phase transition (Quinn *et al.*,1989). Conversely, fluid phase lipids are ordered by cholesterol, particularly in that portion of the acyl chain closest to the head group (Meddings,1989; Yeagle,1985), and this ordering is evident in both the fluid lamellar and inverted hexagonal phases (Chen *et al.*,1990). For example at 48°C, 30 mol% cholesterol inhibits *gauche* rotamer formation in DPPC by factors of 9 and 6 at positions 6 and 4, respectively, of the acyl chain, thus demonstrating a strong ordering effect in the region of the bilayer where the sterol is presumed to insert; in contrast, the ability of

cholesterol to order the acyl chains was much reduced at position 12 (Davis *et al.*,1990). However, the influence of varying amount of cholesterol on the enthalpy of the gel/fluid transition indicates a broader sphere of cholesterol influence than that portion of bilayer nearest the aqueous interface (Singer, 1990). In addition to influencing membrane order and width of the gel/fluid transition, cholesterol also promotes formation of H_{II} phase (Seddon, 1990). As a result of these effects, the bilayer is thinned by cholesterol below the gel/fluid transition and thickened at higher temperatures (Ipsen *et al.*, 1990). Furthermore, addition of cholesterol to a fluid bilayer reduces the permeability to glucose, water and cations, this inhibits both Na+/K+ (Yeagle, 1989) and sarcoplasmic reticular Ca^{2+}-ATPase activities (Madden *et al.*, 1979). Recent studies, however, point towards markedly different interactions between cholesterol and saturated (from which most of the above generalities have been derived) as opposed to unsaturated phospholipids (Finean, 1989). For example, unsaturation moderates significantly the effects of cholesterol on the lateral diffusion of phospholipid analogs (reduced 4 fold by 30 mol% cholesterol in DMPC but uninfluenced in DOPC), alkyl chain motion and oxygen permeability (reduced in bilayers of DMPC but not DOPC) (Pasenkiewicz Gierula *et al.*, 1990). Thus in those cases where membrane cholesterol content is positively correlated with growth temperature, cholesterol may act to stabilize membranes at high temperatures and in this manner contribute to thermal compensation of function, but this hypothesis needs to be reevaluated in the light of the significantly reduced interactions between cholesterol and unsaturated species of phospholipids.

1.5.3 Phospholipid head group restructuring :

Polar lipids are one of the major constituents in the biological membranes. A particular feature of polar lipids of the membranes is that they are comprised, for most part,

of unbranched hydrocarbon residues, that differ in length and number of unsaturated bonds in the chain. They also differ in the distribution of a wide range of molecular species (described later in details) which differ in type and position of attachment of the hydrocarbon substituent to the backbone of the molecule. It has been recognised that biological membranes consist of a complex assortment of polar lipids, but why it is so, is still, to be deciphered. Considering the fact that different molecular species of different polar headgroups have a role in membrane adaptation, still we can't ignore that polar headgroup interaction, itself has a role in maintaining the structural and functional integrity of the membrane.

A) Patterns of headgroup restructuring:

Among all the assortment of polar head groups present in the phospholipid classes, phosphatidylcholine (PC) and phosphatidylethanolamine (PE) comprise the major amount (Hazel, 1979). These two phospholipids are most affable in response to temperature.

Membranes of cold adapted/acclimated poikilotherms routinely possess higher proportions of PE and, less commonly, reduced proportions of PC than membranes formed at higher growth temperatures ((Hazel, 1988). Consequently, the PC/PE ratio tends to be positively correlated with growth temperature (Chang *et al*,1988, Sørenson,1990, Pruitt, 1988, Hazel,1988, Anderson,1970, Miller *et al*,1976, Caldwell *et al*,1969, Wodtke,1976, Gulik *et al*,1990, Chang *et al*,1985, Van Den Thillart *et al*,1975, Christiansen,1984, Hazel,1984, Hazel and Carpenter,1985) and this correlation is equally strong in mitochondrial and microsomal membranes (correlation coefficient $\simeq$ 0.71), but less evident in total tissue phospholipid extracts (Hazel, 1989). Furthermore, in winter active and winter quiescent crustaceans, only the winter active species displayed increased proportions of PE (Pruitt,1988). In addition, for rainbow trout undergoing acclimation from 20 to 5°C, the

PC/PE ratio in renal plasma membranes declined from 1.71 to 0.78 within 8 hr of temperature change, indicating that adjustments in head group composition can occur quite rapidly (Hazel and Landrey, 1988). Similarly, proportions of PE in microsomal membranes of Sonoran desert fish, *Agosia*, declined from 12.1% in the cool of the morning to 2.7% in the heat of the afternoon (Carey and Hazel, 1989). The structure of brain gangliosides also changes in vertebrate poikilotherms as a consequence of thermal adaptation : the most complex of polar gangliosides (containing from 57 sialic acid residues) are found in Antarctic fish, while gangliosides of birds and mammals are significantly less polar (containing only 14 sialic acid residues) (Rahmann *et al*,1989). Thus in interspecific comparisons, the lower the environmental temperature, the more polar the composition of brain gangliosides (Rahmann and Hilbig, 1981,1983), however, similar adjustment in ganglioside polarity have been reported in axolotl brain following acclimation from 23 to 15°C (Irwin *et al*,1990). Temperature induced alterations in polar headgroup composition are also common in microorganisms. Over the entire temperature span of bacterial growth, there is a general increase in glycolipid content from psychrophiles to extreme thermophiles (Russell *et al*,1990). In the alga *Dunaliella*, the ratio of DGDG/MGDG doubled in cells transferred from 30 to 12°C (Thompson,1989). Increased ratios of DGDG/MGDG are a common feature of cold acclimation (Rilfors *et al*,1989, Thompson,1989).

B) Consequences of headgroup restructuring :

The common feature underlying most temperature-induced changes in headgroup composition is a reduction in both R_0 and the cross-sectional area of the head group relative to that of the hydrophobic domain (thus increasing the conical character of the lipid) as

temperature declines, resulting in increased proportions of nonbilayer forming lipids at cold temperatures. It is worth noting that these changes are frequently in a direction opposite to those predicted by homeoviscous theory. For example, the gel/fluid transition temperature for PE is generally about 20°C higher than that of PC of similar acyl chain composition (Yeagle *et al*,1986) due in part, to hydrogen bonding between headgroups of PE (but not PC) (Hauser *et al*,1981, Seddon,1990), the close packing permitted by the reduced steric bulk of the ethanolamine compared to the choline head group (Simon *et al*,1989), and the greater hydration of PC relative to PE (Brown *et al*,1986). Consequently, the orientational order all along the length of the acyl chain (as detected by ^{2}H-NMR) is higher in PE than PC and increasing proportions of PE lead to a progressive increase in acyl chain order in binary mixtures with PC (assuming equivalent acyl chain compositions) (Seddon,1990). However, because the headgroup of PE is less bulky than that of PC. PEs occupy smaller areas/molecule in a monomolecular film than PCs of similar fatty acid composition [35-42Å^2 for PE compared to 47-54Å^2 for PC (Hauser *et al*,1981) while the acyl domain is bulkier (due to greater proportion of Unsaturated Fatty Acids [UFA]), PC assumes a cylinder conformation which packs uniformly in the lamellar phase while PE adopts a conical form (of lower R_0) (Rand *et al*,1990) more rapidly accommodated in the H_{II} phase. Consistent with these observations, the bilayer/nonbilayer transition is sensitive to headgroup structure, T_h being reduced by increased proportions of PE or raised by elevated proportions of PC. Furthermore, N-methylation of PE extends the lamellar phase to temperatures at least 20°C above that of the parent phospholipid (Brown *et al*,1986). Thus, the replacement of PC by PE at cold temperatures, although not sufficient to induce formation of H_{II} or cubic phases is expected to displace the phase behaviour of the membrane to a point closer to that of the bilayer/nonbilayer transition. Accordingly, although an acute drop in growth temperature

increases the temperature interval between T_h and the growth temperature, compensatory adjustments in headgroup composition reduce T_h and thereby restore the proximity of the membrane to the bilayer/nonbilayer transition point. Similar arguments have been advanced to explain thermal modulation of the MGDG/DGDG ratio (Rilfors *et al*,1984).

There is a growing body of evidence to suggest that the balance between nonbilayer and bilayer forming lipids (or alternatively stated, the proximity to the nonbilayer phase transition) is an important factor in determining membrane function. For example, the presence of nonbilayer forming lipids increases the susceptibility of bilayer lipids to attack by phospholipases (Buckley,1985, Hui *et al*,1989) and modulates both the coupling efficiency between ATP hydrolysis and Ca^{2+} transport in reconstituted preparations of Ca^{2+}-ATPase (Navarro *et al*,1984) and the activity of the branched chain amino acid transporter (Driessen *et al*,1988). Furthermore, when intrinsic membrane proteins are reconstituted into exclusively bilayer forming lipids, there is an increased rate of ion leakage across the membrane that can be eliminated by the inclusion of non bilayer forming lipids in the reconstitution mixture (Quinn,1989). In addition, even minor variations in headgroup structure may also have profound effects on lipid miscibility, for while glucosyl glycolipids mixed freely with DMPC, those containing galactose displayed regions of solid/solid immiscibility in the low temperature region of the phase diagram (Koynova *et al*,1988).

1.5.4 Acyl Chain Restructuring :

A) Patterns of acyl chain restructuring :

Adjustments in various aspects of acyl chain composition are among the most thoroughly documented of membrane adaptation to temperature change. A nearly ubiquitous response to altered temperature is reduction in the proportion of saturated fatty acids (SFAs)

and a corresponding increase in the proportion of unsaturated fatty acids (UFAs) with cold exposure. This adaptation has been extensively documented in bacteria (Russell *et al.*,1990), multicellular poikilotherms (Hazel, 1989) and plants (Orr *et al.*,1990). A recent compilation of temperature-induced changes in the acyl chain composition of membrane lipids from a variety of metazoan poikilotherms (Hazel, 1988) established the following generalities : (1) SFA levels declines 19% on average, for 20°C drop in growth temperature, resulting in a 1.3- to 1.4-fold increase in the unsaturation ratio (UFA/SFA); (2) correlations between the unsaturation ratio of membrane phospholipids (PC and PE) and cell or acclimation temperature are negative and significant, but vary for different membrane fractions with the strength of the correlation ranking mitochondria > microsome > synaptosomes; and (3) the types of UFA which accumulate during cold adaptation may be either monoenes or poly unsaturated fatty acids (PUFA), with adjustment in PUFA levels predominating in winter-active (as opposed to winter-quiescent) species. Furthermore, interspecific differences between the fatty acid compositions of animals adapted to extreme thermal environments parallel with those in seasonally acclimatized animals, like brain fatty acids of Antarctic (Morris *et al.*, 1969) and total lipids of deep-water marine fish (Patton, 1975) possess higher proportions of PUFA and lower proportion of SFA than temperate water species.

B) Rationale for changes in acyl chain composition :

The introduction of a *cis*-double bond into an acyl chain can have a profound effect on both the molecular shape and physical properties of a phospholipid. Because rotation is restricted about a carbon-carbon double bond, a bend of ca.30° is introduced into the acyl chain (Smith *et al.*, 1984). Consequently, UFAs adopt a more expanded conformation occupying greater areas in a monomolecular film (Cullin *et al.*, 1971), pack less compactly,

and possess lower melting points than their saturated homologs. Substitution of UFA for SFA is thus expected to increase the bulkiness of the acyl domain, thereby increasing the magnitude of the packing parameter and enhancing the conicity of the phospholipid (especially PE). Since an acute drop in temperature increases the molecular order of the acyl chains and promotes closer packing of phospholipids by diminishing their hydrophobic bulkiness, elevated unsaturation in the cold will restore the expanded character of the hydrophobic domain and thereby offset the direct effects of cooling.

The impact of acyl chain unsaturation can be highly specific and vary with both the number and position of the double bonds. Substitution of oleic acid (18:1) for stearate (18:0) at the *sn*-2 position of DSPC (to form DOPC) results in : (1) a shortened plateau region of constrained motion (extending only to C_7 position) in the *sn*-2 position and (2) a local decrease in membrane order in the immediate vicinity of the double bond (Mc Elhaney, 1984; Rance *et al.*, 1980). However, because double bonds introduce rigid elements into an otherwise flexible chain (Brenner, 1984), a double bond in the *sn*-2 chain can restrict the range of conformations available to the acyl chain in the *sn*-1 position (Macdonald *et al.*, 1985; Paddy *et al.*, 1985), this effect is, nevertheless, of secondary importance since in absolute terms, the *sn*-1 chain of 16:0/18:1 PC is less ordered than that of DSPC when compared at the same temperature. Additionally, not all double bonds have an equivalent impact on the physical properties of the phospholipid (Bell *et al.*, 1986). For example, substituting 18:1 for 16:0 at the *sn*-2 position of DPPC (to form 16:0/18:1-PC) reduces the gel/fluid transition temperature by 50°C, while a second double bond (in 16:0/18:2-PC) further depresses the melting point by 22°C; however, a third double bond (in 16:0/18:3-PC) actually increases the melting point by 3°C (Coolbear *et al.*, 1983). Similarly, T_m (temperature of gel/fluid transition) values for 16:0/16:1 and 16:0/22:6-PC do not differ

significantly (-12°C vs. -10°C, respectively), and the molecular area occupied by 16:0/18:3-PC in a monolayer film, is actually greater than that of 16:0/20:4-PC (Smith *et al.*, 1984). Therefore successive double bonds are expected to increase the cross-sectional area occupied by an acyl chain, they also constrain the freedom of rotation about other carbon-carbon single bonds in the chain and these two effects appear to counterbalance one another in PUFA (Cossins *et al.*, 1985). In contrast, the position of a double bond has a major influence on the physical properties of a lipid, with the impact being greatest when present in the centre ($C_{9\text{-}10}$ of an 18-carbon fatty acid) of the acyl chain (Ali *et al.*, 1989; Brenner, 1984). For example, although the melting point of 18:3 $\Delta^{6,9,12}$ (-10°C) is similar to that of 18:2 $\Delta^{9,12}$, it is 28°C lower than that of 18:3 $\Delta^{9,12,15}$.

Based on the above considerations, it seems that the most effective means of altering the physical properties of the membrane is to vary the proportion of SFA, whereas the type of UFA present appear to be of secondary importance. However, it is still obscure and it is difficult to explain the accumulation of long-chain PUFA in winter-active poikilotherms in these terms.

Cause and effect relationships between temperature-induced changes in acyl chain unsaturation and specific aspects of membrane structure and function have not been rigorously demonstrated.

1.5.5 Restructuring of Phospholipid Molecular Species :

A) Patterns of molecular species restructuring :

If the acyl chain composition of a membrane changes significantly, it follows that the distribution of phospholipid molecular species must also change. However the converse of this statement is not true, for significant changes in molecular species composition may

occur without an alteration in acyl chain composition. For example, a membrane composed of equal proportions of 16:0/16:0-, 18:0/18:0- and 18:1/18:1-PC has the same acyl chain composition as a membrane comprised of 16:0/18:0-, 16:0/18:1- and 18:0/18:1-PC even though the species compositions are obviously different. Thus, it is possible for fatty acids to be "reshuffled" to form new molecular species of phospholipids without altering the fatty acid composition of the membrane. The potential significance of acyl chain reshuffling to the thermal adaptation of membranes was first illustrated in *Tetrahymena*, for which adaptations in the fluidity of microsomal membranes (as detected by changes in DPH fluorescence polarization) preceded gross changes in acyl chain unsaturation during adaptation from 39 to 5°C (Ramesha *et al.*, 1983). Rapid changes in molecular species have been noted in the Sonoran desert teleost, *Agosia*, in response to diurnal warming : the percentage of diunsaturated species of PC declined from 7.23 to 2.46% in muscle microsome between the cool of the morning and the heat of the afternoon (a temperature difference of 13°C) (Carey and Hazel, 1989). Similarly, in rainbow trout undergoing acclimation from 20 to 5°C, proportions of monoenoic and disaturated molecular species declined significantly in renal plasma membrane (Hazel and Landrey, 1988). Collectively, these results suggest that the restructuring of phospholipid molecular species may be a common means of rapidly adjusting membrane composition to temperature challenge. Furthermore, since no input from the lipid biosynthetic apparatus is required (because the enzymes presumed to be responsible for this modification, those of the deacylation/reacylation are membrane resident), this mechanism may be metabolically inexpensive and particularly important when low temperature effectively stops the net phospholipid synthesis. The manipulation of preexisting lipids remains as the only alternative for the restoration of the function (Thompson, G.A., 1989)

Among metazoans, cold acclimation is generally associated with increased proportions

of long-chain PUFA-containing diunsaturated molecular species (Farkas *et al.*,1988; Hazel and Landrey, 1988; Hazel *et al.*, 1986; Malak *et al.*, 1989). Frequently, altered proportions of only a few molecular species account for the vast majority of the acclimatory response, suggesting that thermal adaptation of molecular species composition may involve a precise control of the balance between disaturated and diunsaturated phospholipid species (Farkas *et al.*, 1988).

B) Rationale for restructuring of phospholipid molecular species:

The significance of molecular species diversity lies in recognition that the *sn*-1 and *sn*-2 acyl chains of a phospholipid are not equivalent. Thus *sn*-1 remains perpendicular to the plane of the bilayer throughout its length, whereas the first methylene segment of the *sn*-2 chain is oriented parallel to the bilayer surface (Brenner, 1984). Consequently, for acyl chains of similar length, the *sn*-1 chain will penetrate further into the bilayer. As a result of this structural feature, the physical properties of a phospholipid are determined not only by the length and the unsaturation of the acyl chains, but also by their location on the glycerol backbone (Davis *et al.*, 1981, Lynch *et al.*, 1984). For example, the phase behaviour of 1-Stearoyl-2-Oleoyl-PC (SOPC- T_m = 3°C) differs markedly from that of a binary (1:1) mixture of DSPC and DOPC (two transitions at 22 and 30-53°C) despite having an identical acyl chain composition (Lynch *et al.*, 1984). Similarly, transition temperatures of OSPC and OPPC differ by 17.9°C, while those for SOPC and DOPC differ only by 9.9°C (Davis *et al.*, 1981). Furthermore, in addition to influencing physical properties, and phase behaviour, subtle differences in molecular species composition may also alter molecular interactions within the membrane and thereby influence function. For example, the condensing effect of cholesterol is more pronounced with mixed-chain (one saturated and one unsaturated acyl

chain) than diunsaturated species of PC, and only in the former instance is liposomal permeability reduced (Demel *et al.*, 1972). Additionally, enrichment of erythrocyte membranes with 16:0/22:6-PC stimulates Na^+/Li^+- exchange and furosemide sensitive Rb^+ uptake, while these activities are inhibited by 16:0/18:1-PC (Englemann *et al.*, 1990). It has been shown that double-unsaturated species diacylglycerols seems to be an important structural requirement for the high protein affinity of this compound in rat liver mitochondria (Schlame *et al.*, 1990).

From the above, it is apparent that subtle changes in molecular species composition can significantly influence both physical and functional properties of a membrane and, thus, may contribute to thermal adaptation of membrane function in pokilotherms. Molecular species restructuring may be especially significant during the early stages of adaptation to cold when lipogenic capacity is depressed (Dey *et al.*, 1992).

1.5.6 Role of Protein in membrane adaptation :

Structural and catalytic properties of proteins such as optimal temperatures, temperatures of thermal denaturation, substrate specificity, catalytic efficiency, and temperature dependence of kinetic constants (e.g. K_m) depend on the genetically determined primary structure of the protein in question. In terms of thermostability, organisms that have inhabited a cold environment for generations may have experienced mutations causing some of their proteins to become relatively heat labile; such mutations would not be lethal unless the organism were transferred to a warmer environment. It therefore is not surprising that variations in protein function associated with adaptation to a particular thermal environment are correlated in genetically determined differences in amino acid composition and sequence.

One of the well documented protein, which plays an important in cold adaptation

of pokilotherms, like fish is Thermal Hysteresis Protein (THP). This protein produces a thermal hysteresis (i.e. lower the freezing point of water below the melting point). They produce antifreeze proteins which lower the freezing point, but not the melting point, of water thus producing a difference between the freezing and melting points (DeVries, A.L., 1972; DeVries, A.L., 1986). These unique, usually repeating sequences of the antifreeze proteins with their preponderance of hydrophillic side chains (sugars in the glycoprotein antifreezes, hydrophillic amino acids in the other) apparently allow the proteins to hydrogen bond to the surface of a potential seed ice crystal, probably at step sites, and thereby inhibit crystal growth by forcing an increased radius of curvature of the advancing ice front. This effectively lowers the freezing point via the Kelvin effect (Raymond *et al.*, 1989; Knight *et al.*, 1991). Antifreeze proteins are most common in freeze avoiding species where they function to lower the freezing point and/or promote supercooling of body water. These proteins have been most thoroughly studied in cold water marine teleost (bony) fishes (Devries, A.L., 1972, 1986; Feeney *et al.*, 1986; Davies *et al.*, 1988; Davies *et al.*, 1990; Cheng *et al.*, 1991). Levels of thermal hysteresis activity generally range from 1-1.5°C in fish. In addition to depressing the freezing point without significantly lowering the vapour pressure, the antifreeze proteins can promote supercooling (Zachariassen and Husby, 1982) and inhibit ice nucleators (Duman *et al.*, 1991; Parody-Morreale *et al.*, 1988). Another effect of the antifreeze proteins, which is a potential advantage to freeze tolerant fish (those that can survive freezing of their extracellular water) is the recrystallization inhibition activity (Knight *et al.*, 1984; Knight *et al.*, 1988) of proteins even at concentrations well below those required to detect thermal hysteresis activity. Fish typically produce multiple sizes of THPs with similar primary structures (Cheng *et al.*, 1991). The largest known animal (fish) THP is ~34 kDa (Cheng *et al.*, 1991). The heat-shock phenomenon has also been reported in

cultured cells of Rainbow trout (RTG-2). A distinct set of heat-shock polypeptides have been found which is of 70 kDa (hsp 70). This induction of heat-shock polypeptide has been shown to be very fast, within 5 minutes after inducing with sodium arsenite (Kothary *et al.*, 1984). This heat shock protein has also been reported in *Salmo gairdnerii* (Kothari *et al.*, 1982). From a recent study on carp erythrocyte *(in vivo)*, it has been found that, during the up-shift of temperature, these erythrocytes rigidified their membrane (Dey *et al.*, 1992) and new proteins appear at 25°C which are at 68 kDa and 70 kDa. These bands were also present when the fish were shifted up from 5°C to 15°C, but it is pronounced at 25°C; probably they are also heat-shock proteins (unpublished data). Protein-lipid interaction is another factor which can influence membrane adaptation, some of them may rigidify or others to fluidize it. It has been shown, that, some of the proteins interacting with membrane phospholipids, effectively, lower the fluidity of artificial membranes (Papahadjopoulos *et al.*, 1975). It has been also shown by Monte Carlo simulation technique that, due to the strongly fluctuating nature of the main transition in lipid membranes, the perturbation of integral proteins may be of a significant range and extend up to a large number of lipid layers, depending on the temperature and the degree of mismatch (i.e. varying protein length in an artificial lipid membrane) between the lipid and protein hydrophobic thickness (Maria *et al.*, 1991). Lateral mobility of protein on the cell surface and a rapid rate of protein diffusion, comparable to that of lipid molecule in the same membrane can uncouple the cytoskeleton from the lipid bilayer rendering the same membrane more fluid or vice versa (David *et al.*, 1992). So it can be said that, in pokilotherms such as fish, heat-shock proteins and lipid-protein interaction are also playing role in membrane adaptation to temperature.

To conclude with the above, it can be said safely that proteins can play a vital role in thermal acclimation or adaptation in pokilotherms.

OBJECTIVES

1. To establish the necessity for adaptations in membrane lipid composition by assessing the effects of temperature on membrane structure and composition in cases where this environmental parameter plays a significant role in the life history of the organism.

2. To identify common mechanisms of adaptation to environmental temperatures by ascertaining those properties of lipid membrane structures and compositions which are subject generally to regulation.

3. To provide explanations for thermally-induced alterations in membrane lipid composition.

4. To assess the efficacy of such adaptations.

5. And, to specify those lipid molecular adjustments of general significance responsible for implementing changes in membrane lipid composition.

2. MATERIALS AND METHODS

2.1 Animals :

Table 1. lists the fish species involved in this study. 17 marine and 8 fresh water species which were investigated are shown and the environmental temperature range these fish experience is noted.

2.2 Chemicals :

1-Palmitoyl-2-Docosahexaenoyl-Phosphatidylcholine (PDPC) was obtained from Avanti Polar Lipids, Inc. (Alabaster,AL), 1-Palmitoyl-2-Oleoyl-Phosphatidylcholine(POPC), 1-Palmitoyl-2-Oleoyl-Phosphatidylethanolamine (POPE) and Dioleoyl Phosphatidylethanolamine (DOPE) are from Sigma Chemicals, (St. Louis, MO) and all other chemicals used were of analytical grade from Sigma Chemicals (St. Louis, MO) unless otherwise stated.

2.3 Analytical techniques :

Lipids from livers, dissected fresh from animals, were extracted according to Folch *et al.* (1957) and air shipped to the laboratory in sealed vials flushed with BHT, 0.01% was added to the extract before flushing with N_2. In the laboratory the chloroform was evaporated and the samples were taken up in benzene and stored at -25°C until assayed. Phospholipids and neutral lipids were separated by silicic acid column chromatography by eluting the latter with chloroform and the former with methanol. Phospholipid subclasses were further separated by thin layer chromatography (TLC) on precoated fluorescence G-60 silica gel

plates (F_{254}, E. Merck, Darmstadt, Germany) according to Fine and Sprecher (1982) with an impregnation of boric acid, using the solvent system chloroform:methanol:water:ammonia in the ratio 120:75:6:2 (v/v). Spots were detected under UV at 254 nm. Authentic phospholipids (Sigma Chemicals, St. Louis, MO) were used to identify the polar headgroups. Phosphorous content was determined after scrapping the individual spots. At first they were digested using 0.5 ml HCL for 15 min at 180°C and then 0.6 ml $HCLO_4$ was added and kept at 180°C for 60 min. Reaction was stopped by adding 3 ml distilled water to it. After that 0.5 ml of 2.5% ammonium molybdate was added to it followed by addition of 0.5 ml of 10% ascorbic acid. The mixture was shaken thoroughly and incubated in a water bath at 100°C for 5 minutes. Absorbance was measured at 790 nm and phosphorous content was calculated in accordance with the standard (Rauser *et al.*, 1980). PC and PE were separated by TLC as mentioned above and eluted using the solvent chloroform:methanol:water in a ratio of 50:50:1 (v/v). Phospholipids were transmethylated in the presence of methanol containing 5% hydrochloric acid at 80°C for 2.5 hours. Segregation of the fatty acids was done on 10% FFAP on 80-100 mesh Supelcoport (Supelco,Bellefonte PA) in a 2m long column (2mm i.d.). A Hitachi Model 263-80 gas chromatograph connected to a Hitachi, Model, 263-80 data processor was used for analysis.

2.3.1 *Lipid hydrolysis by Phospholipase A_2* :

Distribution of different fatty acid molecules in PC and PE was determined by digestion with phospholipase A_2 from *Crotalus adamanteus* venom (Sigma Chemicals, St.Louis, MO). 2 mg of the purified PC and PE was dissolved in 1 ml of peroxide-free diethyl ether to which 5 IU (5 ng of protein) of enzyme was added, dissolved in 0.1 M Tris-

HCl buffer, containing 0.4 mM $CaCl_2$ at pH 8.9. Reaction was stopped after 30 minutes of stirring at 27°C by adding 0.5 ml of ultra-pure distilled water to the reaction mixture. After the ether phase was collected, chloroform:methanol (2:1 v/v) (with 0.5 ml of 20% 0.2 M KCl) was added to extract the residual lipid from the chloroform phase. The chloroform and ether phase was pooled, evaporated and dissolved in 200 μl of benzene. Free fatty acids and lysophospholipids were separated by TLC as described above, using the solvent chloroform:methanol:water (65:25:4 v/v). Authentic lysophospholipids and free fatty acids (Serdary Research Laboratories, London, Ontario, Canada) were used to identify the spots. The lipids were extracted by chloroform:methanol:water (50:50:1 v/v) and transmethylated and analyzed by gas chromatography as mentioned earlier.

2.3.2 *Molecular species determination* :

Determination of molecular species composition of the isolated PC and PE was done according to Takamura *et al.*(1986). The dinitro-benzoyl derivatives of diacylglycerols, obtained by Phospholipase C (from *Bacillus cereus* for PE and from *Clostridium perfringens* for PC) (Sigma Chemicals, St.Louis, MO).

Preparation of the Derivatives :

Dinitrobenzoyldiradyl-glycerol (DNB-DG) derivatives were prepared by Phopholipase C hydrolysis. Approximately 2 mg of PC and PE were dried under N_2 flow and dispersed by sonication in 1.25 ml of 10 mM Tris-HCL buffer (pH 7.5) containing 30 mM H_3BO_4. Then 200 units of phospholipase C and 2 ml of water saturated peroxide free diethyl ether were added. After shaking vigorously at 30°C for 3 hr, the diradylglycerol was extracted

twice with the water saturated ether. This incubation was enough for complete hydrolysis of PC and PE. The combined ether extract was dried under N_2 flow in vacuum. Then 25 mg of 3,5-dinitrobenzoyl chloride and 0.5 ml of dry pyridine were added. The mixture was heated in a sealed vial at 60°C for 10 min. The reaction was stopped by adding 2 ml of methanol-water (80:20 v/v) and 2 ml of water. For washing of the derivatives following steps were followed :

1) 2 ml of 0.1 M cold HCL was added.

2) Then it was washed three times with 2 ml of n-Hexane.

3) After each washing, upper hexane phase was collected and pooled which gave a total volume of 6 ml. After that, it was evaporated under vacuum and 3 ml of n-Hexane was added and washed with, 2 ml of 0.1 M HCL (three times), 2 ml of 0.1 M $NaHCO_3$ (once), 2 ml of 1 M NaCL (once) and 2 ml of distilled water (once) consecutively.

Separation of the Derivatives :

The DNB-DG derivatives thus prepared were separated into diacyl subclasses by single step TLC on fluorescence G-60 silica gel plates (F_{254}, G-60, E. Merck, Darmstadt, Germany) using the solvent system, Benzol/n-Hexane/Diethylether (50:45:5 v/v). The derivatised spot was visualized under UV light (254 nm) as dark spots against fluorescent background. Equal amounts (approximately 5-10 nmol) of the 12:0/12:0 (diacyl) DNB-DG derivative was applied as an internal standard. Then the band was taken out and extracted twice with chloroform/methanol (3:1 v/v) by stirring it for about 20 min.

Separation and Identification of the Molecular Species :

Separation was accomplished by High-Performance Liquid Chromatography [HPLC]

(Waters Associates, Model 440) at 254 nm with an UV detector on a Nucleosyl C-18 column (5 μm, 4 mm i.d. x 250 mm) using acetonitrile:2-propanol (80:20 v/v) of HPLC grade (Carlo Erba, Milano, Italy) as the mobile phase with a flow rate of 1.0 ml/min. The peaks were recorded and calculated using a data processor (Hitachi, Model 263-80) connected with the HPLC. Identification of peaks was based on the use of authentic standards [16:0/22:6, 18:0/22:6, 16:0/20:4, 18:0/20:5, 16:0/18:1, 18:1/18:1 and 18:0/18:1] (Sigma, St. Louis, MO) as well as on the basis of their relative elution times of Bell *et al.* (1991).

2.3.3 *Fluorescence anisotropy measurements* :

Preparation of Large Unilamellar Vesicle (LUV) :

To phospholipid (250 μg) dissolved in chloroform, 10μl of 10^{-6}M of 2-, 12-(9-anthroyloxy) stearic acid (2-AS and 12-AS) and 16-(9-anthroyloxy) palmitic acid (16-AP) (Molecular Probes, Inc. Eugene,Oregon) dissolved in tetrahydrofuran was added in a ratio of 200:1 (mol/mol) and mixed thoroughly. The mixture was dried under high vacuum for 30 minutes to remove the solvent completely. The dry lipids were then dispersed in 1.5 ml of 0.2 M Tris-HCl at pH 10. The lipid dispersion was vortexed vigorously and sonicated for 20 min in a bath type sonicator after adding another 2 ml of 0.2 M Tris-HCl and then extruded through 0.4-μm filter (Nuclepore, Pleasanton, CA) under high pressure (200 psi) applied from a cylinder of dry nitrogen. The extruded vesicles were centrifuged at 15000 rpm for 15 min. The pellet was collected and resuspended in 3.5 ml 0.2 M Tris-HCl at pH 7.4., according to the method described by Arindam Sen *et al.*(1991) using a pH jump from 10 to 7.4, with 0.2 M Tris-HCl which traps the vesicles in their unilamellar stage. These vesicles are labelled "supercritical" as under normal conditions identical lipid dispersions do

not form stable unilamellar bilayers but form a wide array of different phases like Multilamellar vesicles (MLV) with lipid particles, Q_{II} phases and H_{II} phases, depending on the ratio of PE to PC. But, it should be noted that there is currently no known method to determine whether or not these vesicles are symmetric with respect to lipid composition.

Anisotropy measurements :

Fluorescence anisotropy measurements were carried out using a computerised thermostated spectrophotofluorimeter, Hitachi MPF-2A. The sample was excited with vertically polarised light at 364 nm with a slit width of 6 nm and the vertically (I_{vv}) and horizontally (I_{vh}) polarised emitted light was read at 446 nm using a slit width of 12 nm. The computer programme was set so that, at each occasion the anisotropy was measured 10 times, thus each figure is the average of 10 measurements. The standard error in separate determination was always less than 0.005. Steady state fluorescence anisotropy (R_{ss}) was calculated according to the formula : $R_{ss} = I_{(vv)} - Z \,.\, I_{(vh)} / I_{(vv)} + 2\,Z \,.\, I_{(vh)}$, where Z : 0.92, calculated according to the formula $Z = I_{(vh)} / I_{(vh)}$. To correct the fluorescence intensity and anisotropy for light scattering, measurements were also made on unlabelled samples under the same conditions as the samples. These measurements were based on Kuhry *et al.* (1985), who showed that both fluorescence intensity and steady state anisotropy (R_{ss}) were influenced by the scatter in a way that :

$I_{(measured)} = I_{(fluorescence)} + I_{(scatter)}$, and

$R_{ss\,(measured)} = f\,R_{ss\,(fluo)} + (1 - f) \,.\, R_{ss\,(scatter)}$. Where,

f (a balanced fluorescence intensity factor) $= I_{fluo} / I_{fluo} + I_{scatter}$.

2.3.4 *Fourier-transformation infra red spectroscopy (FTIR)* :

Phospholipids (1mg) were dissolved in chloroform. The solvents were evaporated under dry nitrogen and the resulting thin film was hydrated carefully in 0.2M Tris-HCl buffer, pH 7.4 (Catersana *et al.*, 1991). Samples were placed on a Zns-Cleartran window (Spectra-Tech, UK) in a hollow thermostated cell, in a flow of liquid nitrogen, which gave a temperature stability better than 0.1°C. The temperature was monitored by a copper-constantan thermocouple located against the cell window, a continuous monitoring was done by a pyrometer (Mantsch *et al.*, 1981).

Spectra were recorded with a Philips PU 9800 Fourier transform spectrometer equipped with Deuterated Triglycine Sulphate (DTGS) detector. A pathlength of 4mm was used in all experiments. A total of 300 scans were combined, and resultant interferogram were triangularly apodized and Fourier transformed to obtain a resolution of $2cm^{-1}$ over the spectral range. Buffer spectra obtained under exactly the same condition, were subtracted from the vesicle spectra in order to eliminate the H_2O association band. In the study of band contours comprised of more than one component, a deconvolution technique was used along with band simulation. The observed band contour was calculated from a superposition of components that were either Gaussian, Lorentzian or a combination of these two line shapes, the method of Nilsson *et al.*, (1991) was followed.

2.3.5 *Electron Spin Resonance Spectroscopy (ESR)* :

Phospholipid (0.5×10^{-6} M) was mixed with 15 nmol 5-doxylstearic acid [5-(N-oxy-4',4'-dimethyloxazolidino)-stearic acid] in a ratio of 200:1 at room temperature for 5 min. The lipids were subsequently dried under a stream of nitrogen and placed under high vacuum

overnight to remove any traces of remaining solvent. The lipids were then dispersed in 0.2M Tris-HCl at 7.4 pH and sonicated for 20 minutes (forming multilamellar vesicles). Samples were contained in 1 mm o.d. micro-flat cell (Scanlon) accommodated within a standard quartz ESR tube which contained silicone oil for thermal stability and was temperature programmed from 5°C to 30°C. Spectra were recorded with a computerised ECS-106 (BRUKER) Electron Spin Resonance Spectrometer equipped with nitrogen gas flow temperature regulation. Each temperature dependent measurement was recorded during the heating cycle and repeated at least 20 times (20 scans at each temperature). Molecular order parameter (S) of the spin label was calculated from the splitting of the inner (2 $A_{\perp}$) and outer ($A_{\parallel}$) extrema according to the method and formula of Seelig (1966), as given below :

$$S_{zz} = \frac{A_{\parallel} - A_{\perp}}{A_{zz} - (A_{xx} + A_{yy})/2}$$

where A_{xx}, A_{yy} and A_{zz} denote the single-crystal principal values of the nuclear hyperfine tensor in the X, Y and Z direction respectively.

Principal values are :

A_{xx} = 0.615 mT, A_{yy} = 0.585 mT and A_{zz} = 3.365 mT.

Occasionally weak isotropic signals due to unincorporated labels were observed.

TABLE-1

Lists of different marine and fresh water fish species investigated

Species	Origin	Temperature°C
Marine fish		
Carnax calla	South-West India	23-26
Epinephelus bleekeri	South Chinese Sea	25-27
Lutaganus sebeae	South Chinese Sea	25-27
Mugil passia	South-West India	20-25
Nemipterus hexodon	South Chinese Sea	25-27
Nemipterus javonicus	South-West India	20-25
Pomadysys hasta	South Chinese Sea	25-27
Sardinella longipes	South-West India	20-25
Tachisurus yella	South-West India	20-25
Clupea herengus	North Sea	5-7
Clupea herengus	Baltic Sea	5-7
Scorpaenichthys marmoratus	Seattle (USA)	10
Hexaganus stelleri	Seattle (USA)	10
Hipoglossus hypoglossus	Seattle (USA)	10
Oncorhynchus kisutch	Seattle (USA)	10
Oncorhynchus tshawskaya	Seattle (USA)	10
Ophioidon elongatus	Seattle (USA)	10
Sebastosomus melanos	Seattle (USA)	10
Fresh water fish		
Abramis brama	North Finland	7-10
Acerina cernus	North Finland	7-10
Coregonus loverostris	North Finland	7-10
Esox lucius	North Finland	7-10
Catla catla	W.Bengal (India)	20-25
Cirrhina mrigala	W.Bengal (India)	20-25
Clarius lasera	W.Bengal (India)	20-25
Labeo rohita	W.Bengal (India)	20-25

3. RESULTS

1. Fatty acid composition of phospholipids in livers of fresh-water and marine fish adapted to contrasting thermal environment in their history of evolution:

Fig. 1. shows the average values of the most common fatty acids in total phospholipids of liver from fish investigated. Although, the number of species investigated was limited, we propose, that the data are representative for fish adapted to cold and warm environments. This inference is obtained from the low SE values for each fatty acids for fish from analogous habitat. It is apparent from Fig. 1. that the average fatty acid composition in liver phospholipids of CAMF (cold-adapted marine fish) and CAFF (Cold-adapted fresh water fish) are almost identical, and the same is true also for WAMF (warm-adapted marine fish) and WAFF (warm-adapted fresh water fish). The level of saturated fatty acids, and as a consequence, their ratio to unsaturated fatty acids are identical in fish from similar temperature conditions but consistently higher in fish living in warmer water (Table 2) The difference is highly significant in marine fish ($P>0.05$), while with fresh water fish it did not reach of $P<0.05$. In contrast, 18:1 was more abundant in both, CAMF and CAFF than in their counterpartners but the sum of 18:1 and 18:0 was almost identical. It is notable, that 20:4 exhibited significantly higher values in WAMF and WAFF than in fish from cold environment, but the opposite is true for 20:5 and 22:6 did not show any variation with the thermal adaptation. There is no notable change in any of the polar headgroup composition irrespective of their origin. Major polar headgroups are PC (70-75%) and PE (20-25%) in total liver phospholipids.

TABLE-2

Average composition of total saturated-, saturated/unsaturated-, total n-6- and total n-3 fatty acids of total phospholipids from fresh-water and marine fish adapted to contrasting temperatures.

	CAMF n=8	WAMF n=9	CAFF n=4	WAFF n=4
Total sat	21.4±2.2**	30.6±3.6**	23.2±2.5[1]	29.0±4.2[1]
sat/unsat	0.27±0.03**	0.44±0.07**	0.3±0.03[1]	0.41±0.08[1]
Total n-6	5.0 ±2.2[§]	9.5±3.3[§]	9.7±4.0[§]	21.2±6.1[§]
Total n-3	43.5±2.9[§]	32.1±11.3[§]	43.0±2.6[1]	39.5±6.1[1]
Double bond index	283.2	239.4	278.1	245.9

CAMF:cold-adapted marine fish, **WAMF**:warm-adapted marine fish, **CAFF**:cold-adapted fresh-water fish, **WAFF**:warm-adapted fresh-water fish.
** $p<0.001$, [§] $p<0.05$, [1] NS.

Isolated PC and PE showed the same trend as the total phospholipids. However, there were idiosyncratic differences between the two phospholipids irrespective of their origin. PE from cold adapted fish retained significantly less 16:0 and more 18:1 than that from warm adapted fish and 18:0 was more abundant than that in warm adapted fish. The level of 20:5

was significantly higher in liver PE from cold adapted fish, whereas, the level of 22:6 was invariable with temperatures, which was also true for PC (Fig. 2A & 2B).

2. Molecular architecture and composition of phosphatidylcholines (PC) and phosphatidylethanolamines (PE) :

To reveal whether, the demonstrated differences in gross fatty acid composition of PC and PE are also reflected at the molecular level these phospholipids were subjected to phospholipase A_2 digestion. This approach was prompted by our earlier observations on several fresh-water fish showing that PE responded to temperature change with more 18:1 was directed towards the *sn*-1 position and was accumulated when the fish were exposed or acclimated to reduced temperature (Farkas *et al.*, 1989).

Fig 3 (A and B)., shows the positional distribution of fatty acids of PC and PE of representative CAFF (*Coregonous loverortris*) and WAFF (*Cirrhina mrigala*) as well as of representative CAMF (*Oncorhynchus kisutch*) and WAMF (*Nemipterus hexodon*). In PC of both, WAFF and WAMF palmitic acid predominates in the position *sn*-1, accompanied by stearic acid. Some palmitic acid was detected also in the position *sn*-2. Polyunsaturated fatty acids accumulated in the *sn*-2 position, as a rule, but in CAFF and CAMF considerable amounts of 20:4, 20:5 and 22:6 also appeared in the *sn*-1 position. It was striking that both, CAFF and CAMF enriched oleic acid in the position *sn*-1 of PE at the expense of stearic acid. The ratio of stearic to oleic acid was above unity in WAMF and WAFF (*Pomadysys hasta*, *Labeo rohita* respectively). HPLC separation of PE into individual molecular species (Table 3) confirmed the accumulation of 18:1/22:6 and 18:1/20:5 species in CAFF and CAMF. The level of these (18:1/20:5 and 18:1/22:6) species in phosphatidylethanolamine

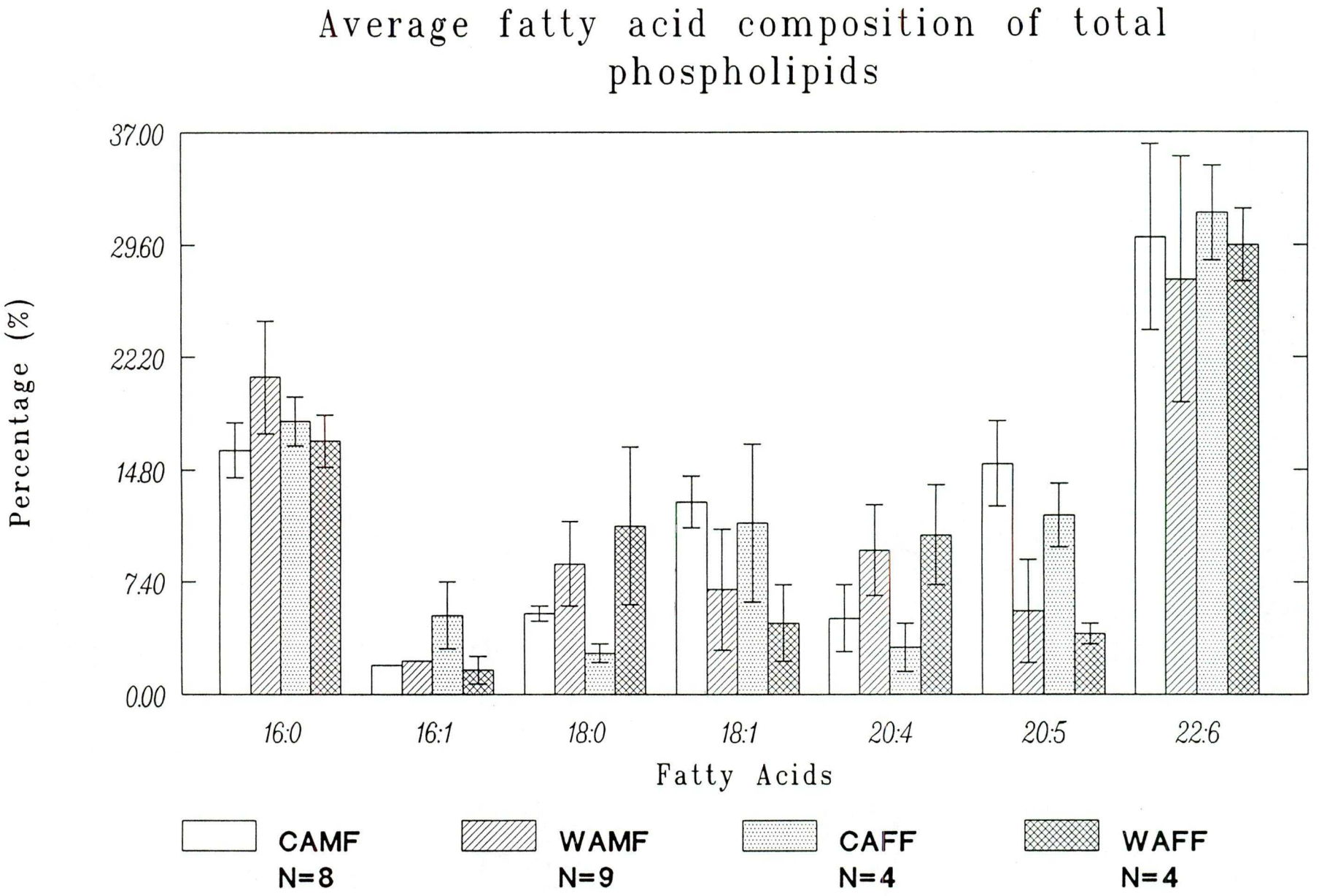

Average fatty acid composition of total phospholipids
Percentage (%)
37.00
29.60
22.20
14.80
7.40
0.00
16:0
16:1
18:0
18:1
20:4
20:5
22:6
Fatty Acids
CAMF
N=8
WAMF
N=9
CAFF
N=4
WAFF
N=4

Figure 1

Figure 2

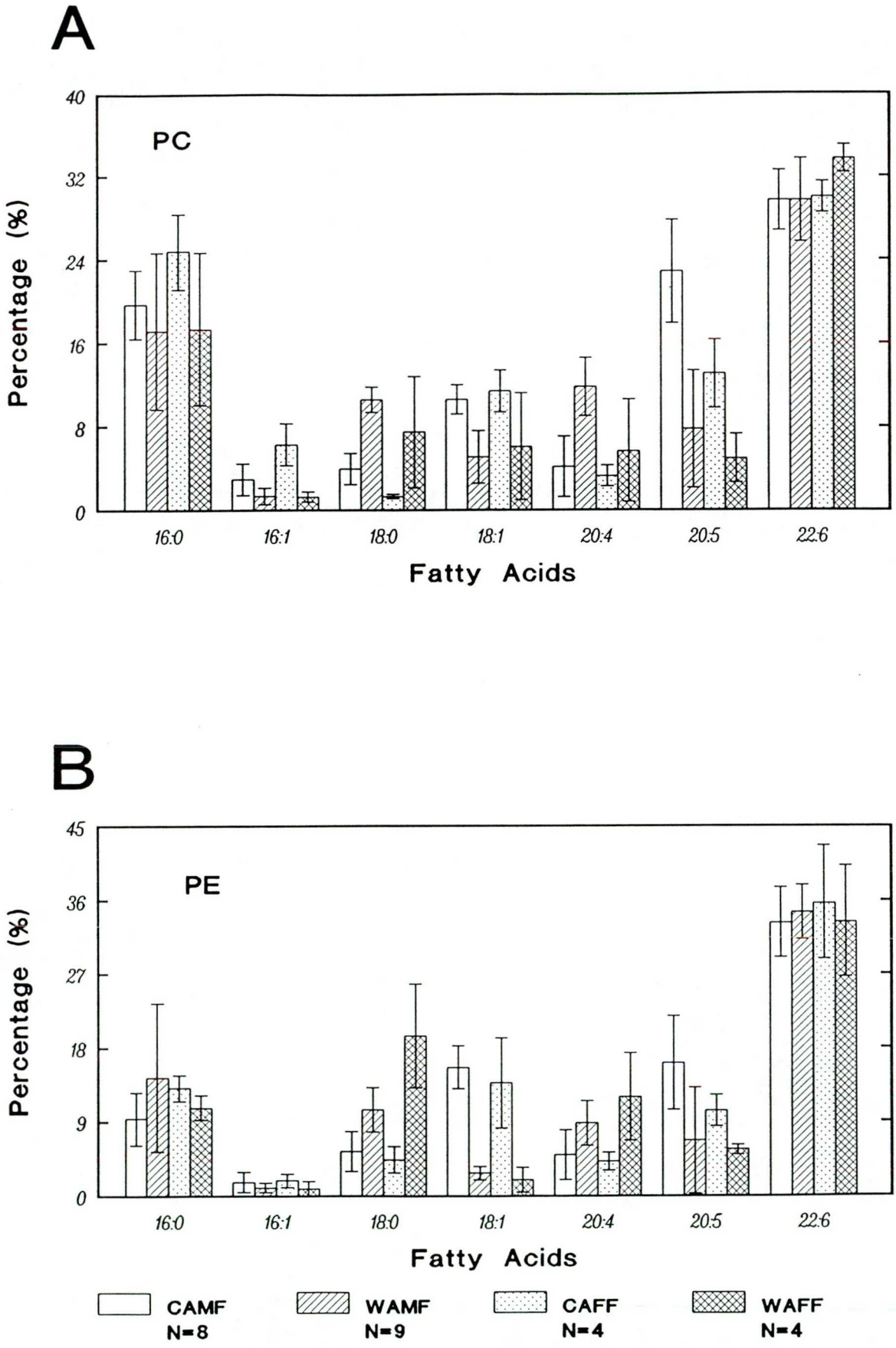


Fig.2. Average fatty acid composition of PC(A) and PE(B) from CAMF, WAMF, CAFF and WAFF (only the major fatty acids are shown).

of CAMF (*Scorpaenichthys marmoratus*) and CAFF (*Coregonus loverostris*) is 33.9 and 24.0% respectively, which can be contrasted to 7.0 and 7.1% in the WAMF (*Pomadysys hasta*) and WAFF (*Catla catla*) respectively.

TABLE-3

Major molecular species in liver phosphatidylethanolamine of representative WAFF and CAFF as well as WAMF and CAMF

Molecular species	WAFF	CAFF	WAMF	CAMF
22:6/22:6	0.2	0.8	1.8	1.4
18:1/20:5	0.3	8.7	0.5	3.6
18:1/22:6	6.8	25.2	6.5	20.4
16:0/22:6	16.0	19.7	23.3	21.3
16:0/20:4	1.7	1.6	1.0	4.0
18:0/20:5	trace	4.3	5.8	4.9
18:0/16:1	3.1	1.6	trace	trace
18:0/22:6	11.0	10.3	7.5	2.5
18:0/20:4	20.4	12.6	26.6	3.4
18:1/18:1	3.4	0.6	2.6	trace
16:0/18:1	17.6	5.8	7.8	3.2
16:0/16:0	10.1	trace	3.2	trace
16:0/18:0	4.6	0.4	1.0	----
18:0/18:0	2.9	0.5	1.0	----
18:0/18:1	1.0	----	0.6	0.4

WAFF: *Labeo rohita*, CAFF: *Coregonus loverostris*

WAMF: *Pomadasys hasta*, CAMF: *Scorpaenichthys marmoratus*

The origins of all of these fish are from distant geographical regions and they most probably also vary in their feeding habits. On the basis of the presented data, it can be proposed that the specific differences in their phospholipid molecular composition and architecture of PE is a real manifestation of thermal adaptation of membranes at a structural level.

Figure 3

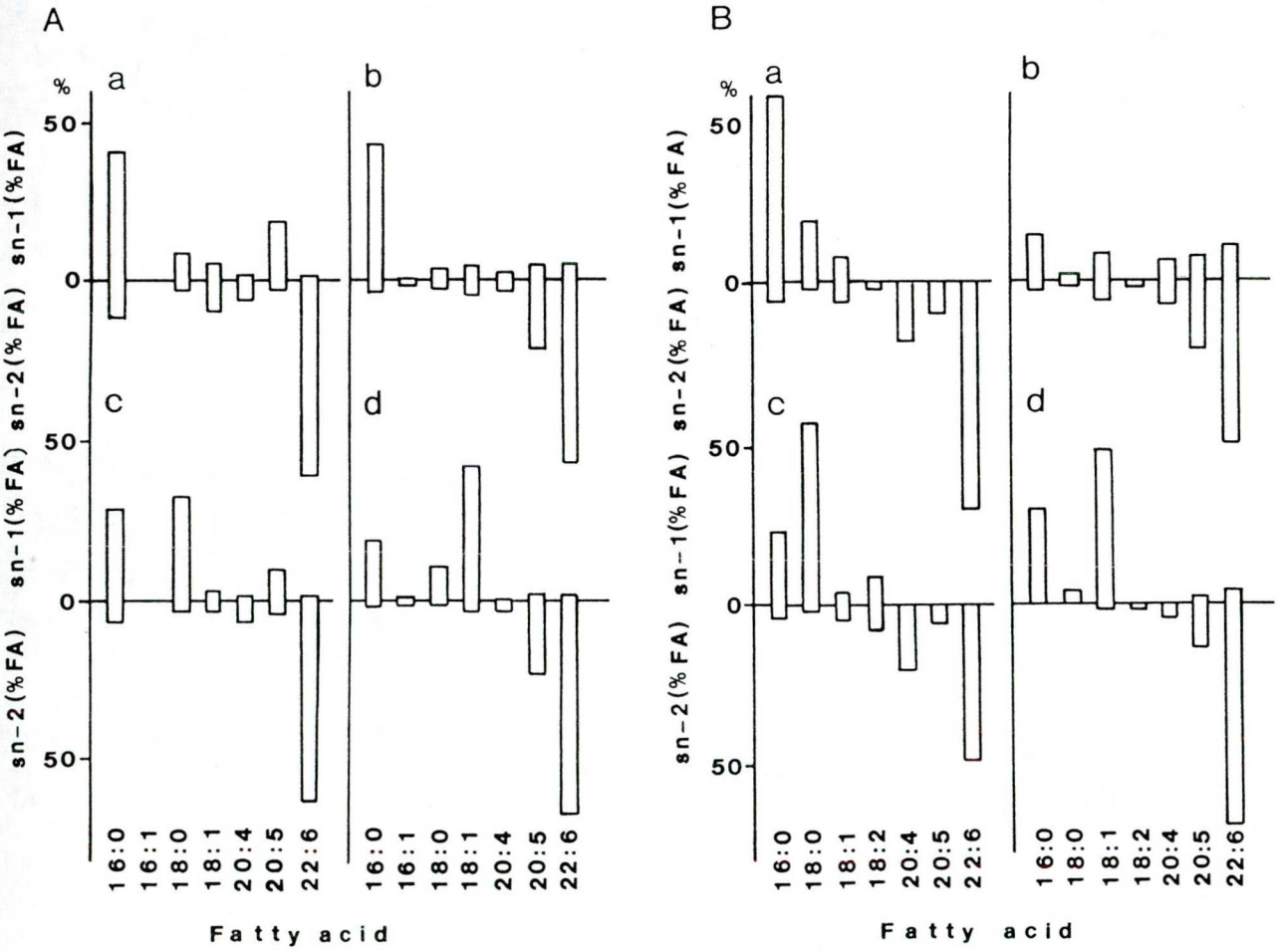


Fig.3.A. Positional distribution of major fatty acids in PC (a & b) and PE (c & d) of a representative WAMF (*Nemipterus hexodon*, a & c) and a representative CAMF (*Onchorchincus kisutch*, b & d).

Fig.3.B. Positional distribution of major fatty acids in PC (a & b) and PE (c & d) of a representative WAFF (*Cirrhina mrigala*, a & c) and a representative CAFF (*Coregonus loverostris*, b & d).

3. Physical properties of phospholipid vesicles obtained from fish adapted to cold and warm environments :

As the thermal environment of the investigated fish species ranged from 5 to 27°C, it was of interest to see whether specificity shown in the molecular architecture of individual phospholipids was manifested in the physical properties of membrane phospholipids in relation to the adapted temperature. Two different approaches were used: a probe technique (ESR and fluorescence polarisation) to study the hydrophobic core and a non-probe technique (FTIR) to study primarily the polar headgroup interaction.

Fig.4, shows the temperature dependency of the order parameter "S" of 5-doxyl stearic acid incorporated into phospholipid vesicles of representative CAFF (*Abramis brama*) and CAMF (*Lutganus sebeae*) as well as WAFF (*Labeo rohita*) and WAMF (*Pomadysys hasta*). Higher values represent more ordered and rigid structures. Two indicative features can be observed. Despite, the overall similarity in fatty acid composition of liver phospholipids in marine and fresh water fish from the same thermal environment, vesicles of the latter proved to be more rigid than the former. In addition, the compensation of the ordering state is far from completion. It can be calculated from the values of Fig.4, that for perfect compensation of membrane ordering state, the order parameter should have been around 0.635 in the phospholipids of CAFF and 0.610 in those of CAMF at 25°C, instead of the measured values, 0.740 and 0.690 respectively. The calculated homeoviscous efficacy is around 7-10%, which is far below the observed values on isolated native membranes (Cossins, 1983; Cossins and Prosser, 1982; Cossins *et al.*, 1980; Tsugawa *et al.*, 1990; Lagerspetz *et al.*, 1984; Harper *et al.*, 1990; Lagerspetz, 1985). One can note that, the signals coming from 5-doxyl stearic acid are an echo of the ordering state at the 5th carbonic atom segment of the acyl

chain, the upper half of the bilayer. Utilizing this finding, that the fluidity in artificial vesicles increases towards the methyl end of the acyl chains, the interior of the bilayers of these vesicles would expected to be less ordered.

Figure 4

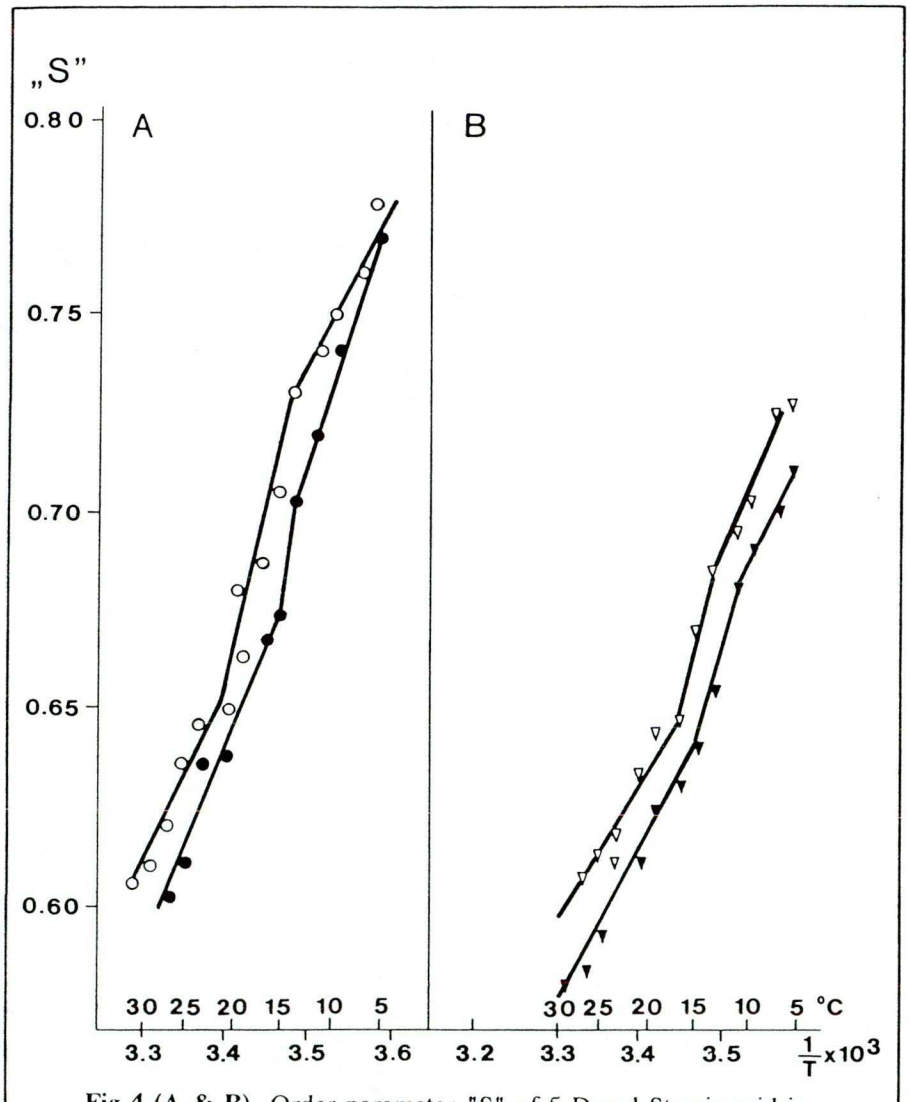


Fig.4 (A & B). Order parameter "S" of 5-Doxyl-Stearic acid in phospholipids from livers of representative WAMF (○), CAMF (●) as well as in WAFF (▽) and CAFF (▾).

To reveal the effect of 18:1/22:6 PE accumulating at lower temperature, model experiments were carried out. Vesicles were prepared from PE of WAMF (*Pomadysys hasta*) and CAMF (*Ophioidon elongatus*) incorporated with 1-Palmitoyl,2-Docosahexaenoyl

phosphatidylcholine (PDPC) in a ratio resembling the concentration of native phospholipids (i.e. 75% PDPC + 25% native PE from WAMF and CAMF respectively). The effect was studied by fluorescence anisotropy using different N-(9-anthroyloxy) fatty acid labels (2-AS, 12-AS and 16-AP) reporting defined segment of the bilayer (Cooper *et al.*, 1991; Squier *et al.*, 1991; Mateo *et al.*, 1991). Incorporation of PE from WAMF to PDPC resulted in a more rigid structure than PDPC alone in each membrane segment (C-2, C-12 and C-16) and at each temperature measured between 5-30°C (Fig.5A & 5B). This rigidifying effect of PE on phospholipid bilayers has been described (Michaelson *et al.*, 1974). Increasing the proportion of 18:1/22:6 PE by substituting PE from CAMF abrogated this rigidifying effect of PE and rendered the PDPC vesicles more fluid in the C-2 segment, than with PDPC alone, but did not affect the deeper regions (C-12 and C-16). Comparison of vesicles constituted from PDPC + PE from CAMF demonstrated close adaptation of fluidity over temperature in the upper segment of the membrane. This trend is similar in the case of fresh-water fish (Fig. not shown). Model studies on pure PDPC and POPC vesicles with different anthroyloxy fatty acids (2-AS, 12-AS and 16-AP) labels showed also that latter vesicles are less fluid mainly in the upper zone of the bilayer (Fig.6).

This finding is further supported by experiments in which PCs prepared from both CAMF and WAMF were cross combined with PEs of both origin (Fig. 5C). Since fluorescence anisotropies of PCs were almost identical in each respective membrane segment the values were averaged and jointly presented in Fig.5C. As expected on the basis of Fig.5A, PEs from WAMF rendered the combined vesicles more ordered than PE from CAMF. The difference was most drastic at the C-2 segment of the membrane, but persisted also in the deeper regions, albeit less pronounced. In the uppermost segment of the bilayer this combination of PC and PE mimicked an over 100% compensation to temperature.

Figure 5

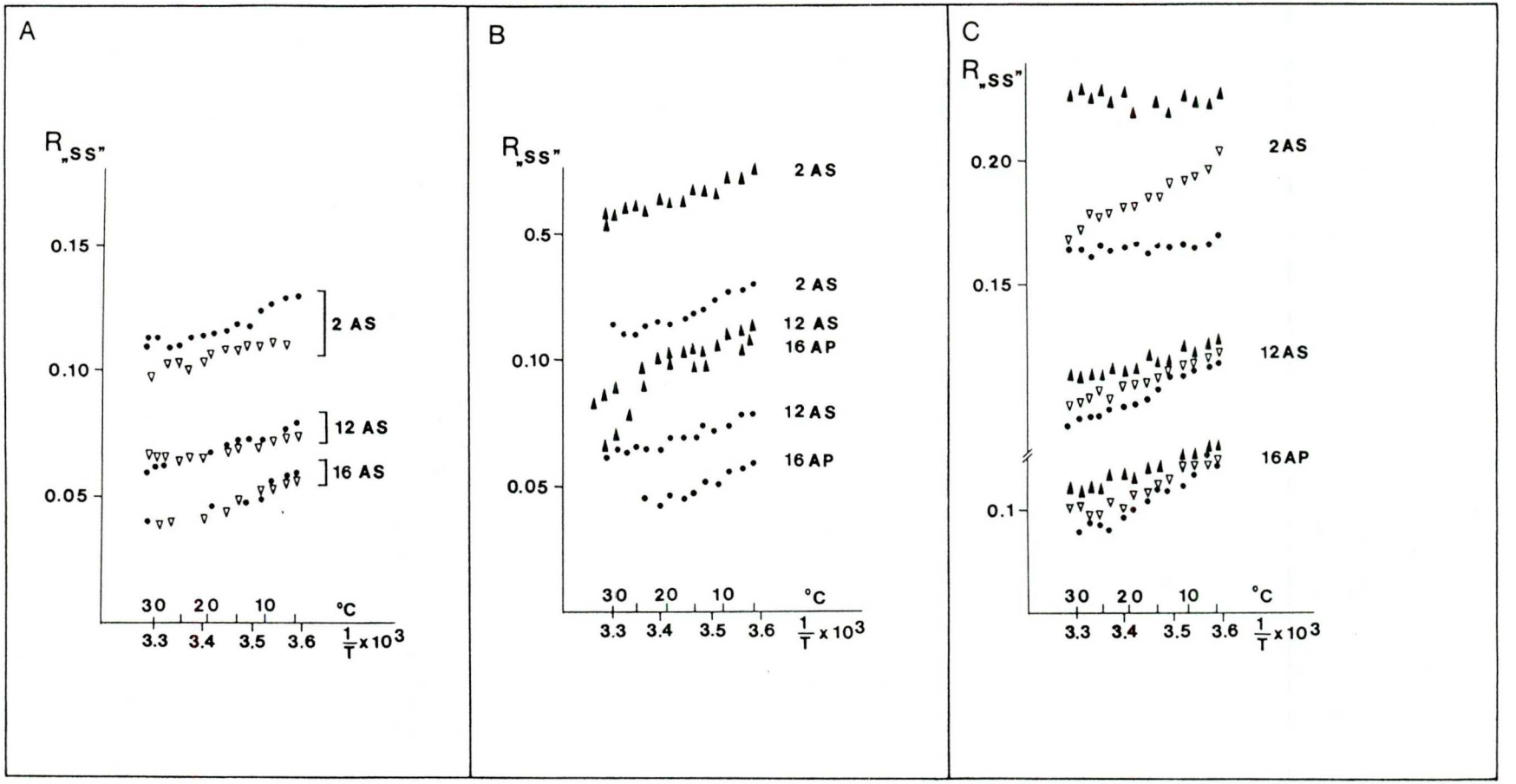


Fig.5A. Effect of PE from CAMF on fluorescence anisotropy of different anthroyloxy fatty acids in PDPC vesicles.
● : PDPC alone; ▽ : PDPC 75% + CAMF PE 25%.

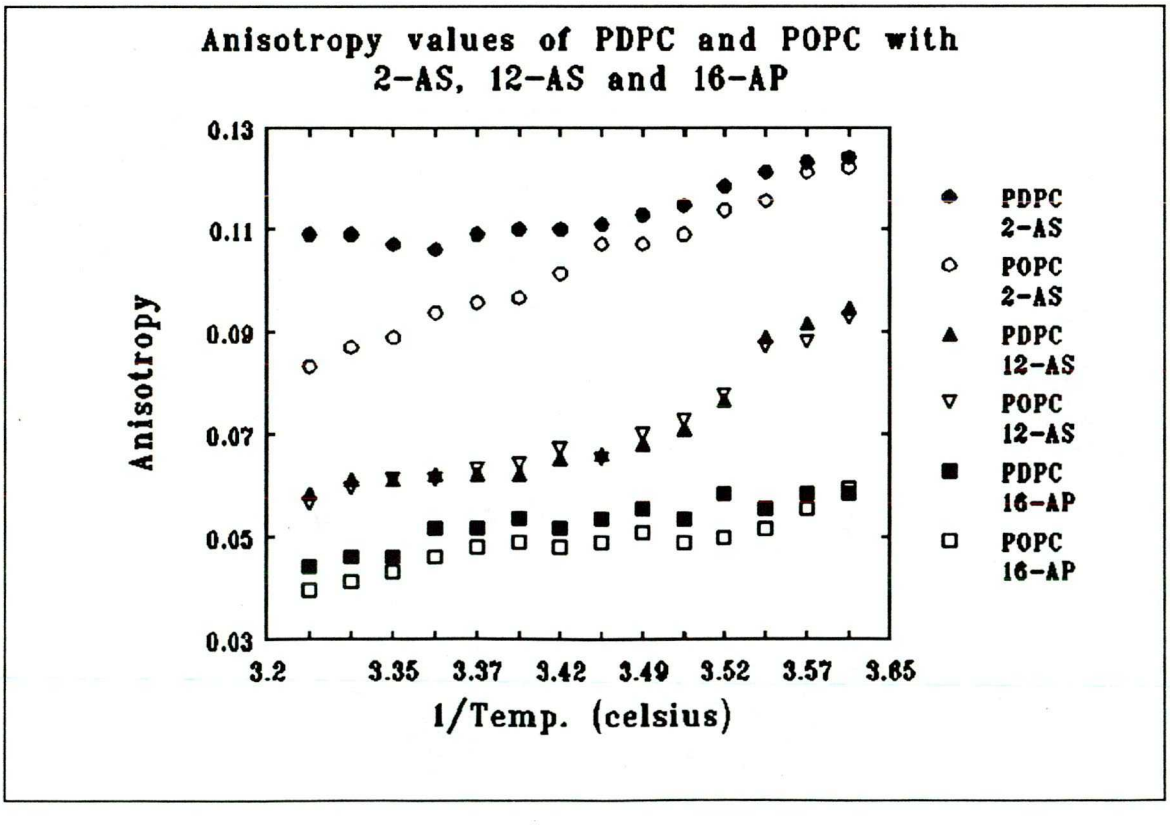
Fig.5B. Effect of PE from WAMF on fluorescence anisotropy of different anthroyloxy fatty acids in PDPC vesicles.
● : PDPC alone; ▲ : 75% PDPC + 25% WAMF PE.

Fig.5C. Effect of PE from CAMF and WAMF on fluorescence anisotropy of different anthroyloxy fatty acids in isolated fish PC (from both warm and cold adapted) vesicles. As there is no difference in the PCs from warm- and cold-adapted species, average values are represented here.
● : PC only; ▽ : 75% PC + 25% CAMF PE;
▲ : 75% PC + 25% WAMF PE.

The observation that PE from CAMF rendered the vesicles from native PCs as compared to vesicles made from PDPC more ordered, points to a complex interactions between different PCs and PEs present in the system.

From these model experiments, it can be concluded that 18:1/22:6 PE accumulating at lower temperature in fish livers is a good candidate to regulate membrane fluidity. It also follows from the data presented, that this effect is most pronounced in the upper half of the bilayer, while in the deeper region the physical properties of 22:6 (present also in PC) is dominating.

Figure 6



To verify the above observation, we employed a non-probe technique (FTIR), where a particular interaction can be detected by a frequency shift for the specific vibrational mode and changes in the line shape (Pimentel *et al.*, 1960). Without going into the detailed result, we present here the most striking observations.

The methylene stretching modes (ν_s-CH_2 at 2853 cm^{-1} and ν_a-CH_2 at 2923 cm^{-1}) are almost unaltered for CAMF (*Scorpaenichthys marmoratus*) and WAMF (*Pomadysys hasta*) measured at 5°C and 25°C (Fig.7A). The picture is same in the case of CAFF (*Coregonus loverostris*) and WAFF (*Catla catla*) (Fig.7A). This suggests that in the hydrophobic core of the membrane there is no change in terms of ordering of the membrane due to higher unsaturation. This was also shown by Nilsson *et al,*(1991). As the signal is coming from all the methylene stretches irrespective of their segmental ordering, we can say that the recognised methylene peak is an average signal coming from the -CH_2 stretches.

The carbonyl stretching mode (ν- C=O) at 1738 cm^{-1} (Mantsch *et al.*, 1981) exhibits a significant change between CAFF and WAFF (same species, as mentioned above) (Fig.7B). Direct confirmation about the reduction in the frequency due to the influence of the double bond incorporation in the α-β position has been shown by various authors (Bellamy, 1975; Thompson and Torkington, 1945; Hartwell *et al.*, 1948; Hampton and Newell, 1949; Rasmussen and Brattain, 1949). From Fig.7B it is obvious that there is a shift towards lower frequency in favour of CAFF and CAMF (shift is 0.623cm^{-1} for CAFF and 4.155cm^{-1} for CAMF), suggesting the incorporation of higher unsaturation at the *sn*-1 position as demonstrated by Phosplipase A_2 digestion of PEs. The observed broadening of the C=O stretching band in cold water fish is due to the vibrational dephasing mechanism (Rothschild, 1984).

The phosphate stretching mode in the frequency domain of 1000-1300 cm^{-1} (ν_s-Po^{-2} at

1088 cm^{-1} and ν_a-Po^{-2} at 1240 cm^{-1}) (Nilsson *et al.*, 1991) also shows a shift of 3 cm^{-1} at ν_s-Po^{-2} and a shift of 13 cm^{-1} at ν_a-Po^{-2} towards higher frequency in favour of CAMF and 1.706 cm^{-1} at ν_s-Po^{-2} and 9.809 cm^{-1} at ν_a-Po^{-2} towards CAFF (Fig.7C).

The above results nullify the postulated role of 22:6 in the position *sn*-2 in membrane adaptation to temperature. They illustrate the existence of a polar head group interaction and a general role of unsaturation at the *sn*-1 position (supporting the fluorescence and ESR study) in controlling membrane ordering state in thermal adaptation.

Figure 7

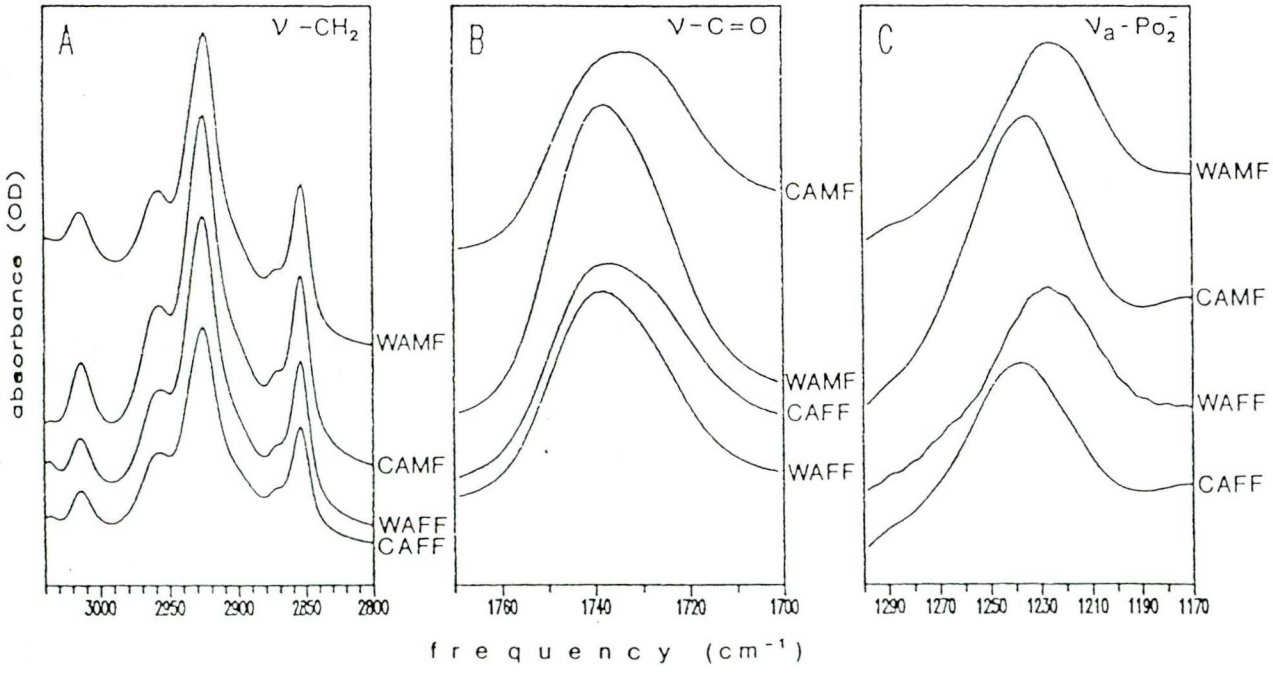


Fig.7. FTIR spectra of phospholipids from representative CAMF (*Scorpaenichthys marmoratus*), WAMF (*Pomadasys hasta*), CAFF (*Coregonus loverostris*) and WAFF (*Catla catla*).

A : ν-CH_2 (methylene) stretch. **B :** ν-C=O (carbonyl) stretch. **C :** ν_a-PO_2^- (phosphate anti-symmetric) stretch.

4. DISCUSSION

Differences in the fatty acid composition of liver phospholipids of marine and fresh water fish (evolutionary) adapted to contrasting temperatures resemble those achieved during seasonal acclimation of several fresh water fish. In both cases the adaptation/acclimation process is characterised by a change in saturated to unsaturated fatty acid ratio and in number of double bonds in the acyl chains. The observation that the average fatty acid composition of phospholipids in CAFF and CAMF as well as in WAFF and WAMF were not statistically different, can be interpreted as an overall effect of temperature on lipid metabolism and membrane lipids in poikilotherms like fish. However, the characteristic differences concerning the level of arachidonic-, and eicosapentaenoic acids in warm-, and cold adapted fish might be traced back to nutritional factors. In the diatoms abundant in cold waters of both type, 20:5 is predominating while in warm waters green algae, containing also 18:2, which can be converted easily to longer chain and higher unsaturated homologs by fish (Tocher *et al.*, 1989; Muci *et al.*, 1992; Kissil *et al.*, 1987). Nevertheless, these responses do not seem to be sufficient to provide a perfect compensation of the hydrophobic core of the membranes for the thermal differences. According to the ESR measurements (Fig.4) this compensation is around 10% and unrelated to FTIR measurements (Fig.6A). Indeed, we are aware of the fact that the total phospholipids we are working with, represent only the average membranes in the cell and characteristic differences might exist between the different membrane types as well in their physical states. Moreover, the phospholipids we used in this study lacked the membrane proteins and sterol known to affect the physical properties (Conroy *et al.*, 1986).

The fatty acid composition data and the HPLC analyses of the molecular species

composition of the purified PC, constituting 60-70% of the total phospholipids thus fabricating the membrane backbone, proved to be highly unsaturated in fish from all habitats. For an effective control of membrane fluidity a proper balance of highly unsaturated-, and saturated species is anticipated. The thermotropic phase transition of PDPC is close to -12°C and POPC is close to -20°C (Collebar *et al.*, 1983). On the other hand the disaturated species like DPPC having high transition temperature (+41°C) are low in all fish phospholipids investigated so far (Bell *et al.*, 1989; Hazel *et al.*, 1986; Hazel *et al.*, 1986). The reported decrement and increment of the molecular species of POPC and PDPC respectively in individual membranes of some fresh water fish (Hazel *et al.*, 1986; Hazel *et al.*, 1986) probably do not contribute effectively to the control of membrane physical state. Accumulation of PDPC might not have a great impact on the packing properties of the bilayer because its molecular area is about 15% greater than that of POPC (Evans *et al.*, 1987). A recent study (Applegate *et al.*, 1991) showed that the polyunsaturated fatty acids in the *sn*-2 position of phospholipids may favour formation of more condensed structures in contrast to those containing 18:1 in the same position. Model studies on pure POPC and PDPC vesicles with different anthroyloxy fatty acid (2-AS, 12-AS and 16-AP) labels showed also that latter vesicles are less fluid mainly in the upper zone of the bilayer (Fig.6). Studies on total phospholipids from CAMF, WAMF, CAFF and WAFF with anthroyloxy fatty acid labels showed that, liver phospholipids from cold-adapted fish are more ordered in the upper-half of the bilayer (at 2-AS position) than their counterpartners from warm temperature.

The most apparent difference between PC and PE was a significantly higher proportion of oleic acid in the latter, which was directed mostly into *sn*-1 position in cold adapted fish from both habitats. Molecular species composition showed that the level of 18:1/22:6 and

18:1/20:5 species have increased three fold and ten fold respectively in cold adapted fish. Accumulation of this species during cold adaptation might be a general phenomenon since it has been demonstrated also for some fresh water fish (Farkas *et al.*, 1989; Hazel *et al.*, 1986, 1988). Although, it cannot be excluded that it restricts only certain membrane types, Cossins *et al.* (1980) found that PE from microsome of a cold acclimated fish (*Lepomis cyanellus*) were more rich in oleic acids than from warm acclimated fish, while no accumulation of this fatty acid was found in mitochondria. Thus the possibility cannot be ruled out that elevated levels of oleic acid in PE of CAFF and CAMF reflects adaptational changes taking place in microsomal membranes. Since phospholipids originating from these structures make out only a portion of the total phospholipids, accumulation of oleic acid in them might be quite considerable.

A fundamental difference between PE and PC is in their molecular shapes : the former are conic while latter are cylindric (Cullis *et al.*, 1979). Substitution of 16:0 by 18:1 in *sn*-1 position will further accentuate the conicity of PE due to the introduced *cis*-double bond. Although, no information is available about the surface area and phase behaviour of PE, like 18:1/22:6, we can anticipate that replacement of 16:0 by 18:1 in *sn*-1 position favourably affects both the phase state and the packing properties of membranes during low temperature adaptation. It has been proposed that elevated level of conic shaped molecules are required to maintain functional integrity of biomembranes in cold (Wieslander *et al.*, 1980) and in accordance with this, accumulation of PE during cold adaptation has been demonstrated for a variety of species, tissues and membrane types (Farkas *et al.*, 1984; Thillard *et al.*, 1981; Wodtke, 1981; Durairay *et al.*, 1984; Hazel and Carpenter, 1985). The present results indicate that fish can increase not only the number of conic shaped molecules but also the conicity of the existing conic shaped molecules in the course of cold adaptation.

The presence of PE in the membranes may render them more ordered due to molecular interactions in the polar head group region (Michaelson *et al.*, 1974). FTIR results also support this interaction, where there is a considerable shift of asymmetric PO^{-2} stretch (Fig.6C) towards higher frequency in favour of cold adapted fish, indicating bonding of phosphate group with other molecule. There is ample evidence in the literature demonstrating that such shifts occur when phosphate-water hydrogen bonds are weakened and the number of water molecule hydrogen-bonded to the phosphate group decreased. This has been shown for PC (Arrondo *et al.*, 1984; Meiller and Diaf, 1988), PE (Send *et al.*, 1988) and for PS (Dluhy *et al.*, 1983; Casal *et al.*, 1984, 1989). Since such shift is indicative of some dehydration of the phosphate group (Castresana *et al.*, 1992). It can be concluded that in all probability other bonding can occur only with the available free proton with the amine of PE and PS which can bind with the extra electron available in the oxygen of the polar headgroup nearest to it, thus giving higher degree of conicity to PE molecule. They might interact as either ion pairing or proton transfer complexes. However, this conicity of PE is increased by the interaction of proton transfer complexes, as because this configuration is more stable than the other for its smaller dissociation constants due to hydrogen bonding between the cationic and anionic moieties (Vinogradov and Linnel, 1971). Although, it is not true that there is no such bonding like this in warm-adapted fish, in comparison to cold-adapted fish they show lower amounts of such bondings, which in turn is giving the phosphate groups of warm water fish more hydrogen bonding to the water, which is showing lower wave number (Seddon *et al.*, 1983), thus giving lower degree of conicity to PE molecule. This interaction, however, might be weakened in function of the type of acyl chains present in PE. Studies on model membranes, not presented here, showed that mixing POPE to POPC in a ratio of 25:75 brought about an increase in the anisotropy of different anthroyloxy (2-AS, 12-AS, 16-

AP) fatty acids, which was not observed when 16:0 in position *sn*-1 of PE was replaced by 18:1 (DOPE). Similarly, as demonstrated by the Fig.5A, PE purified from a WAMF (*Pomadysis hasta*) being poor in 18:1/22:6 species, exerted a rigidifying effect on PDPC vesicles, whereas PE from CAMF (*Ophioidon elongatus*) fluidised the uppermost segment of the same bilayer and did not influence the deeper regions, which also comply with the FTIR results (Fig.6A). It should be noted that the above mentioned effect is solely due to the unsaturation in *sn*-1 position of PE. FTIR results showed almost no change in the hydrophobic core (Fig.6A) where 22:6 is supposed to predominate. But in *sn*-1 CO-O-C stretching (1178 cm^{-1}) there is an increase of 3.0 cm^{-1} and 2.56 cm^{-1} towards higher frequency in favour of CAMF and CAFF respectively (Figure not shown). Even the C=O stretch shift towards the lower frequency supports this fact, because amount of 22:6 in *sn*-2 position of all the fish investigated remained almost constant, which is evident from phospholipase A_2 digestion. So, this shift can only occur due to a change in unsaturation in *sn*-1 position. The content of 18:1/22:6 species in the model system was similar to that in the native phospholipid extracts and according to the Fig.5C, this amount contributed a 100% homeoviscous efficacy in these vesicles. Despite this fact, the fluidity of vesicles of native phospholipids from CAMF relative to WAMF and CAFF relative to WAFF remained almost unchanged, can be explained by the presence of several different PE exerting a contracting effect in the polar head group region (described earlier), and seen with the model system consisted of WAMF PE and PDPC as well as with CAMF PC and WAMF PE (Fig.5A,B&-C). Thus based on these observations, we propose that molecular species like 18:1/22:6 and 18:1/20:5 of PE play an important role in keeping the membrane physical state unchanged to changing thermal conditions. The incorporation of this molecular species of PE (18:1/22:6) into the membrane cannot be traced back to dietary factors. Studies on marine and fresh-

water copepods adapted to cold environments (unpublished data) show that they do not accumulate this molecular species of PE, thus it is an adaptive response by fish for survival under the altered thermal environments. From these observations it also follows that the extent of compensation of membrane fluidity to thermal changes varies vertically in the hydrocarbonic core of the membrane bilayer, being the most efficient near the polar head groups. This explains why other labels, distributing evenly in the membrane, such as 1,6-diphenyl-1,3,5-hexatriene (DPH), show only partial compensation. Moreover, we propose that fatty acids in *sn*-1 position but not in the *sn*-2 position play this role which is in agreement with the results of G. Cevec, (1991), showing that *sn*-1 chains are more influential than the hydrocarbon attached at *sn*-2 positions, and 22:6 in *sn*-2 position has only a mechanical function in maintaining the membrane integrity.

5. CONCLUSION

From the above investigations, experiments and results it can be concluded that overall turnover in phospholipids and in their fatty acids compositions is not a real manifestation in thermal adaptation. Even saturated to unsaturated ratio probably has nothing to do with membrane adaptation to temperature. The theory of thermotropic phase transition in lipid membrane is not applicable in this respect, as it doesn't exist in any natural membrane under any condition considering their survival. It is feasible only to study this kind of phase transition in pure lipid system, but it should not be extrapolated in any case to natural membrane. If lipid molecules have any role in membrane lipid adaptation, then probably, molecular species like 18:1/22:6 and 18:1/20:5 phosphatidylethanolamine are playing the key role. More precisely, oleic acid (18:1) is the master molecule playing this role in adaptation only when it is attached in the *sn*-1 position of phosphatidylethanolamine and not in any other phospholipid. Docosahexaenoic acid (22:6) in contrast has only a mechanical property. It's higher unsaturation has no role in membrane adaptation as it has been suggested previously by several authors. All the previous interpretations and experiments on lipid membrane adaptation based on simple model experiments with one or two molecules of lipids are oversimplified explanations for membrane adaptation to temperature, because natural membrane, physically and chemically is highly heterogenous. Still it should not be divulged to say that oleic acid is the bandmaster of membrane orchestra in controlling natural membrane structure.

Results obtained from the investigation points to a complex genetic regulation even in lipids in the membrane of those organisms, those who are adapted to a constant temperature

in their course of evolution. Membrane and lipid biochemists often consider the 'retaiolring' of membrane lipid as the only major factor playing role in membrane adaptation and they escape from the point that lipids form only the matrix of any natural membrane and they try to overlook the interaction with other biomolecules which are present in the membrane or can be synthesised or can be conformably changed within a scale of nanosecond level (e.g. protein). At this point, it is speculative to say that lipid molecules fashion themselves only to furnish membrane an appropriate environment for other biomolecules controlling membrane function and structure.

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