MODIFICATION OF THYMOCYTES PLASMA MEMBRANE AFFECTS DESATURASES ACTIVITIES

Ph.D.Thesis.

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Szeged.

1987


B 2768

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ACKNOWLEDGEMENT

I am grateful to Dr.Tibor Farkas for the supervision throughout this work, for his helpful discussions and expert guidance, at the Institute of Biochemistry, Biological Research Center of the Hungarian Academy of Sciences.

I would like to thank the Institute of Biochemistry and Nutrition of the University of San Marcos, Lima, Peru, who made my stay in Szeged possible, to fulfill this work.

I wish to thank Dr. Árpád Párdutz, director of the International Training Course, who made a prolongation possible to complete this work.

My thanks to Endre Lehoczky and Anikó Pogonyiné of the Institute of Biophysic, József Attila University, Szeged for assistance with the polarization fluorescence measurements.

I also thank Anikó Vanicsek and Judith Baunoch for their skilled technical assistance.

The experiments were performed while the author was a fellow of the 13th International Training Course, supported by the Hungarian Academy of Sciences and UNESCO at the Biological Research Center, Szeged, Hungary.

ABBREVIATIONS

ANS	1 anilinonaphthalene-8sulfonic acid.
BSA	Bovine serum albumin.
CH	Cholesterol.
CH/PL	Cholesterol/phospholipid ratio.
Ci	Curie.
cpm	counts per minute.
DPH	1,6-diphenyl-1,3,5-hexatriene.
ESR	Electron spin resonance.
FA	Fatty acids.
GLC	Gas liquid chromatography.
g	units of gravity.
M	Molar.
ml	millilitre.
mCi	milliCurie.
NMR	Nuclear magnetic resonance.
P	degree of fluorescence polarization.
PC	Phosphatidylcholine.
PL	phospholipids.
17:0/17:0 PC	L- Diheptadecanoil phosphatidylcholine.
18:1/18:1 PC	L- Dioleyl phosphatidylcholine.
r.p.m.	revolutions per minute.
Sat/unsat	Saturated acids/unsaturated acids ratio.
sp.act.	specific activity.
TLC	thin layer chromatography.
uCi	microCurie.
UV	Ultraviolet light.
18:0	Stearic acid.

18:1	Oleic acid.
18:2	Linoleic acid.
18:3	Linolenic acid.

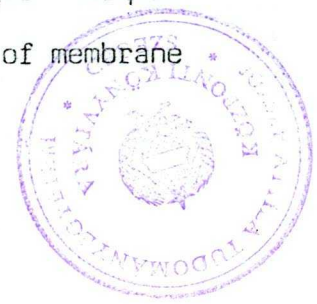
ABSTRACT

The concept of homeoviscous adaptation hypothesises that the membrane lipid composition varies in response to conditions that perturb phospholipid acyl chain fluidity, in such a way as to maintain a fluidity constant. An approach was made to modificate phospholipids plasma membrane with two different phosphatidylcholines.

The modifications were made by incubating whole thymocytes from rats, either with 17:0/17:0 PC or 18:1/18:1 PC. After incubation time, the fluidity and lipid composition of plasma and intracellular membranes were tested. The change in the physical state of plasma membrane by the saturated PC was detected by polarization fluorescence, with an increase in the "P" value, and a decrease in the mentioned parameter for 18:1/18:1 PC treated cells. The lipid composition of the plasma membrane in 17:0/17:0 PC treated cells, detected by Gas liquid chromatographic corresponded to high values of sat/unsat and the absence of the saturated PC was detected in intracellular membranes.

Δ^9 and Δ^6 desaturation of labeled fatty acids was tested and the treatment with saturated PC activated both desaturases, but not to the same extent.

The treatment with 18:1/18:1 PC led to a transition to a fluid state in the cell membranes, as reported by polarization fluorescence, but this PC was not incorporated into the membranes, according to the gas liquid chromatography results, and a possible extraction of membrane cholesterol is supposed.



The Δ^9 and Δ^6 desaturation of labeled fatty acids showed a slight inhibitory action.

A probable mechanism involving an information exchange between the endoplasmic reticulum and the plasma membrane in order to maintain the proper fluidity, is suggested to explain the results obtained.

1. INTRODUCTION

1.1. Influence of lipid membrane composition on membrane function.

The accepted picture of biological membranes is the fluid mosaic model of Singer and Nicholson (1972). This model hypothesises that the membrane lipids are in a fluid and relatively homogenous phase, and the globular proteins are embedded in the lipid fluid bilayer core. It is thought that a liquid crystalline phase is essential to protein function (Jost et al 1973).

Although the membrane lipids may exist in a non bilayer 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 planes of hydrated polar groups in an aqueous environment. Phospholipids are the major constituents of biological membranes and together with sterols and glycolipids form the matrix of the membrane.

The phospholipids of biological membranes of eukaryotes consist basically of phosphatidylcholine (PC) and sphyngo-myelin (SM) and the aminophospholipids phosphatidyl ethanolamine (PE) and phosphatidyl serine (PS). These lipids and the proteins constituents are distributed asymmetrically between the outer and inner leaflets of the lipid bilayer (Rothman and Lenard 1977) and the functional properties of biological membranes are accounted for by interaction between proteins and lipids. The connection between the lipid composition of the membrane and membrane function can be explained in terms of membrane fluidity.

There are many studies that prove the relationships

between the lipid composition of biological membranes and its physical properties with enzymatic activities (Carruthers and Melchor, 1986; Carriere and Le Grimellec, 1986; Wormann et al., 1986; Kreuter et al., 1984). Any change in the lipid composition of membranes (cholesterol or phospholipids) results in changes in the local environment of integral proteins (receptor molecules, enzymes, transport) (Brasitus and Dudeja, 1986; Rathiers and Chen, 1986; Zabrecky and Raftery, 1985; 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 requirement 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 group, the nature of the backbone (glycerol or sphingosine), the length of the acyl chains and the saturation of the acyl chains. Fatty acid chains as well the other variable factors in lipid composition appear to affect membrane-bound enzymes in a more general way by influencing membrane fluidity e.g.the activity of membrane transport proteins was found to increase with increasing unsaturation of the fatty acid (Overath, 1970).

1.2. The "fluidity" of the membrane.

Changes in the composition of lipids in the membrane bilayer can affect the ordering of the hydrocarbon chains and

the degree of molecular motion of different parts of the lipid molecules. The physical state of the lipids of the membrane is termed "fluidity". The term fluidity is widely applied to phospholipid bilayers and natural membranes, and combines in a single term the effect of lipid dynamics and acyl chain order. Lipid dynamics refers to lateral and rotational diffusion of the whole molecule as well as rotation about single carbon-carbon bonds. Acyl chain order of lipid packing refers to the average orientation of each carbon atom along the chain (Shinitzky, 1984). Some authors are of the opinion that this term may not be related to the degree of order (Seeling and Seeling, 1977).

In a more disordered membrane (liquid crystalline state) the acyl chains are not packed as tightly and therefore, holes or gaps can form between the chains. In the membrane acyl chain order decreases and mobility increases with increasing bilayer depth and no single value can be assigned to the fluidity of a membrane.

Nevertheless, fluidity has often been a useful concept for assessing the bulk lipid structure of the membrane, and it is technique dependent and most methods allow one to distinguish changes in lipid dynamics and lipid order. The order parameter S is a time and assemble average function to describe the overall organization at a particular portion, it varies from 0 (chaos) to 1 (perfect order). It supposes that molecular motion within the ordered environment is rapid. The definition of rapidity depends upon the method of measurement (^{2}H-NMR, ESR, etc) and its gives us the mobility of

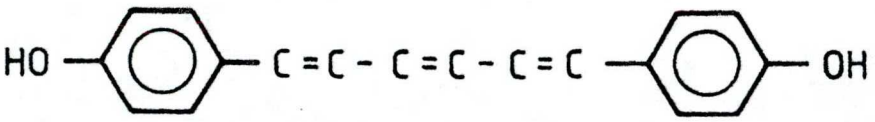
particular segments. Both the ordering and mobility of the chains are components of membrane fluidity (Smith and Jarrell, 1985).

In the study of molecular motion in membranes, the most commonly used techniques are fluorescence polarization and magnetic resonances (electron spin resonance and nuclear magnetic resonance), and each one measures rather different parameters.

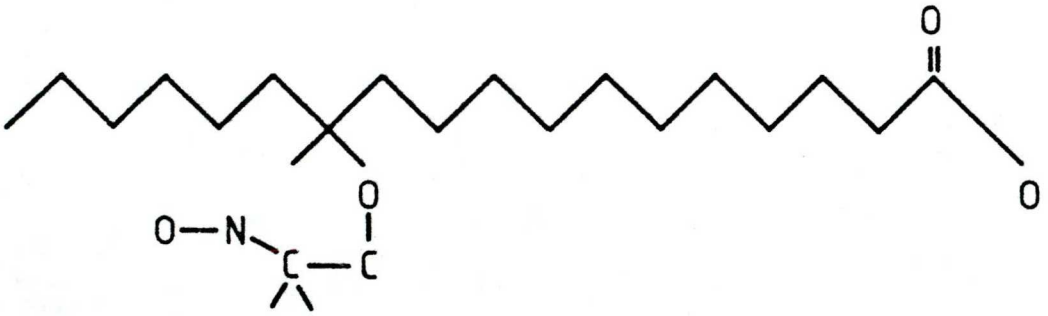
In fluorescence polarization a lipophylic fluorophore such as 1-6 diphenyl 1-3 hexatriene (DPH) (Fig. 1) with an elongated rectangular shape and stable trans configuration locates itself in the lipid environment of the membrane. The probe is excited with polarized light and the relaxation or depolarization of the fluorescence caused by the rotation of the probe in the lipid bilayer is measured. A highly ordered membrane with a high microviscosity will limit the rotation of probe molecules so that emitted light maintains a high degree of polarization, while a less ordered membrane with low microviscosity will allow rapid depolarization.

The phase transitions in lipids is characterized by a change in fluidity with temperature. The fluorescence probe DPH is partitioned into liquid and solid phase, and the temperature profiles of fluorescence polarization, reveal phase transitions (Shinitzky 1978).

Information obtained by magnetic resonances provides explanations about the dynamic processes, since nuclear magnetic resonance and electron spin resonance data reflects both the chemical environment of characteristic groups in a



(DPH) 1-6 diphenyl 1-3 hexatriene



(12-NS) 12 NITROXIDE STEARIC ACID

Fig.1.- DPH AND 12 NS

molecule and their motion. ESR studies of membrane have been conceived with lipid soluble spin labels which have been found to be localized in the hydrocarbon regions of phospholipids. A free radical as nitroxide, covalently linked to membrane components or to their analogues, is sensitive both to its detailed motions and the polarity of its environment. It is the unpaired electron of the free radical that allows an electron spin resonance spectrum to be measured. Changes in molecular motion are recorded as differences in ESR spectra. Lipid spin labels most commonly used are 12 nitroxide stearic acid (12-NS) (Fig.1) and 5 nitroxide stearic acid (5-NS) (Levine, 1972).

In the less disruptive techniques NMR, such as ^{1}H and ^{13}C are observed, so that 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).

1.3. Factors influencing membrane fluidity.

Membrane fluidity can be influenced by altering the proportion of Cholesterol/Phospholipids (more ordered the higher cholesterol concentration), sphyngomyelin/lecithin, saturated/unsaturated fatty acids (greater mobility, the higher degree of unsaturation) or by changes in the fatty acid composition.

The effects of PC/SM ratio and of CH/PL ratio on cellular functions are not identical. PC/SM mole ratio has large effects on cellular shape, protein and enzyme

activities, as has been studied in the age-dependent profile of myocites (Elishalom, 1985).

1.3.1. The role of Cholesterol.

Studies of the effect of compositional changes in bulk lipid structures have shown that acyl chain order increases with cholesterol content above the phase transition temperature and decreases below this temperature. For lipids in the liquid crystalline (fluid) phase, cholesterol increases the degree of acyl chain order significantly (Smith and Jarrell, 1985). For lipids in the tightly packed gel (rigid) phase, cholesterol addition leads to disordering and to the onset of molecular motion.

The distribution of cholesterol is highly disproportionate among the membranes of a cell. It is well known that cholesterol is found in relatively high concentrations in mammalian plasma cell membranes, less in endoplasmic reticulum, and lower yet in mitochondrial membranes (Cooper, 1978). Cholesterol distribution would seem to be maintained by a thermodynamic equilibrium according to membrane composition, which could determine the relative cholesterol content, depending upon their lipid and protein content (Yeagle and Young, 1986).

On the basis of calorimetry data it was suggested that cholesterol has a preference for sphingomyelin over phosphatidylcholine and for phosphatidylcholine over phosphatidylethanolamine which is observed when a phase transition to the gel state from the liquid crystal state

occurs (Van Dyck and De Krujff, 1976). There are also special roles for hydrocarbon chain length and the extent of unsaturation in the partitioning of cholesterol among membranes (Lange et al., 1979).

It is known that cholesterol can induce a liquid crystal state in lipids that could otherwise occupy a gel state. The tail of cholesterol is substantially more ordered than the corresponding region of the phospholipid hydrocarbon chains. Cholesterol does increase the bilayer thickness of membranes consisting of phospholipid with hydrocarbon chains up to 16 carbon atoms in length, but for the same carbon hydrocarbon chain, an interesting deviation from this behaviour is observed. (Beltman et al.,1984).

The distribution of cholesterol is therefore non uniform. In addition to this transverse asymmetry, is supposed an asymmetry in the lateral phase of the bilayer showing distinct domains of "solid" and fluid lipids. Regions of the membrane may be richer in some lipid species than others due to their mutual solubility properties. It appears that certain specialized lipids such as cholesterol are not only able to reorganize lipid packing in the bilayer, but by preferentially associating with certain classes of lipids serve to bring about regions or domains varying in "lipid composition" (Klausner et al.,1980; Kleinfeld et al.,1981).

To summarise cholesterol is a major component of the plasma membrane. It has a dual effect on membrane fluidity, increasing the mobility of hydrocarbon chains of the bilayer, below the phase transitions temperature and decreasing it

above this temperature (Hsia and Boogs, 1972). The lipids of functional cell membranes at physiological temperatures are in a fluid rather than a gel state. Cholesterol enrichment decreases lipid fluidity of the outer membrane leaflet. The location of cholesterol within the bilayer has been determined by examining its effects upon individual structural parameters (Huang, 1976; Phillips and Furner, 1974).

1.3.2. The role of phospholipids.

The plasma membrane possess an asymmetric distribution of lipids. The majority of them have the anionic phosphatidyl ethanolamine and phosphatidylserine in the cytoplasmic leaflet, and the outer leaflet predominatly contains the neutral phospholipids phosphatidylcholine and sphyngomyelin (Op den Kamp, 1974). It has been established that in the course of evolution, changes in phospholipid composition of the membrane were in the direction of increase in choline-containing phospholipid, while phosphatidyl ethanolamine remained constant (Drachev et al., 1985).

Phosphatidylethanolamine due to its primary amine headgroup, has the ability to form hydrogen bonds, and it is expected that the viscosity imparted by phosphatidyl ethanolamine is higher than that of phosphatidylcholine (Hirata and Axelrod, 1980).

The asymmetry with respect to lipid headgroup, creates a second type of asymmetry, since the fatty acyl chains of the predominantly exterior are more highly saturated than the

acyl chains of the predominatly interior phospholipids (Olashow et al., 1983). As saturated fatty acid chains are able to pack more efficiently than unsaturated ones, the lipids of the outer monolayer are more closely spaced than the lipids of the inner leaflet of the membrane. The mechanism by which lipid asymmetry is generated is as yet unknown. One probable mechanism is that lipid synthesis occurs on both leaflets of a given membrane bilayer. Different levels of synthesis perhaps depend on availability of substrate and would produce the asymmetric distribution. Studies with microsomes showed that phospholipid synthesis occurs only on the cytoplasmic face of the endoplasmic reticulum. This lipid asymmetry could have been generated as a result of different ratios of transbilayer movement of each lipid species following its synthesis (Bell et al 1981).

1.3.3. The role of sphingomyelin.

Sphingomyelin together with lecithin are the two major phosphorylcholine phospholipids. Despite the fact they have the same headgroup, their effects on membrane fluidity are markedly different. Natural phosphatidylcholine bears highly unsaturated acyl chains, and therefore produces high fluidity to lipid domains. Conversely, sphingomyelin is highly saturated and produces lipid domains of low fluidity (Shinitzky and Barenhoz, 1974). The rigidifying effect of sphingomyelin is also due to its amide linkage and the free hydroxyl group. Two important biological processes have been associated with changes in PC/SM. Both aging and

artheriosclerosis are characterized by substantial decreases in PC/SM (Shinitzky, 1984)

1.3.4. The degree of unsaturation.

Long chain fatty acids, which are components of phospholipids acyl chains are known to regulate membrane viscosity in the same way as cholesterol. The reports are consistent with the finding that cis unsaturated fatty acid increases the fluidity of phospholipids in biological membranes, partitioning into fluids regions increasing acyl motional freedom, whereas saturated fatty acids and trans unsaturated fatty acid do not, because they decrease the motional freedom of phospholipid acyl chains (Karnovsky et al., 1982).

It is already known that cis poly-unsaturated fatty acids have more effect than cis mono-unsaturated fatty acids. The partitions of membrane of acids with two or more double bonds seem to be less than those of monounsaturated fatty acids, because these acids are relatively more hydrophylic. However since these cause much more perturbation than the same amount of monounsaturated fatty acid, they seem to cause a large increase in the membrane fluidity. The fact that there is little difference in the effects of unsaturated fatty acids with two or more double bonds may be due to the balance of their membrane partition and membrane perturbing effects (Lee et al., 1986).

In addition not only the length and degree of unsaturation of the acyl chains are important. Switching of

fatty acids between the sn_1 and sn_2 positions or combining two saturated or unsaturated fatty acids within one molecule, also has important effects on fluidity (Lynch and Thompson, 1984).

The activity of acyl chain desaturases can modulate in vivo the composition and fluidity of the plasma membrane lipids (Martin et al.,1976; Kasai et al.,1976) and this mechanism is mainly associated with the endoplasmic reticulum but recent evidence has been founded linking Δ^6 desaturase to the mitochondrial fraction (Hughes and York, 1985). But other authors did not find an increase in fluidity when the desaturase activities were increased (Holloway and Holloway, 1977).

1.4. The homeoviscous adaptation concept.

The concept of homeoviscous adaptation hypothesises that the membrane lipid composition varies in response to conditions that perturb phospholipid acyl chain fluidity in such a fashion as to maintain the fluidity (Sinensky, 1974). These authors support this adaptive alteration of acyl chain composition in membrane cholesterol to phospholipid ratio. The main point of this hypothesis was the finding of a somatic cell mutant fibroblast defective in the regulation of cholesterol biosynthesis, because of a defect in the regulation of the enzyme 3 hydroxy 3 methyl glutaryl Co A (HMG-CoA) reductase by cholesterol in the culture medium it is also defective in the regulation of membrane fluidity (Sinensky, 1980). Phospholipids containing unsaturated fatty

acids have low melting points and greater molecular flexibility than their saturated counterpart. Consequently many organisms, including bacteria, plants and poikilothermic animals maintain optimal membrane fluidity and function according to environmental temperature by altering the proportion of unsaturated fatty acid in the phospholipids. This change is explained by homeoviscous adaptation.

It was earlier reported that the conversion from stearic acid into oleic acid, may result from the elevate level of the desaturase activity by de novo synthesis (induction theory) and an alternative theory was offered to explain the increased desaturase activity: membrane fluidity theory; activation of the desaturase is caused by reduced membrane fluidity (Kasai et al., 1976).

1.5. Means of controlling membrane fluidity.

The degree of unsaturation of the phospholipid acyl chains is a determinant factor in membrane fluidity. The presence of double bonds considerably increases its partial specific volume, increases the fluidity and decreases the degree of order in the system.

The effects of fatty acyl chain structure are evident on the temperature of transition between the gel and liquid crystalline phase. Transition temperatures decrease with decreasing chain length, unsaturation will also affect the exact fluidity of the bilayer in the liquid crystalline. The introduction of a first double bond into a saturated fatty acyl chain results in a very marked decrease in transition

temperatures, but the introduction of further double bonds results in a small decrease in transition temperature. Unsaturation ensures that lipids are in the liquid crystalline phase at physyological temperatures. In membranes, the bulk of the lipids are in a liquid crystalline phase, which is maintained against changes in environmental temperatures by changes in fatty acyl chain composition. The majority of phospholipid contains one saturated and one unsaturated fatty acyl chain, which ensures that the phase transition temperature of the bulk of the lipids in the membrane will be below physiological temperatures (Lee et al., 1986).

Evidence that desaturase activities participate in regulating the physical properties of membranes is available using the protozoarian Tetrahymenia by adjusting the growth temperature and varying the fatty acid composition by nutritional supplementation. It was concluded that microsomal desaturase activity responded to fluidity changes rather than the temperature changes (Martin et al., 1976; Kasai et al., 1976), and it has been confirmed with fluorescence polarization measurements (Dickens and Thompson, 1980). When the temperature decreases, the degree of unsaturation of membrane polyunsaturated fatty acid (PUFA) increases, and an increase in the thermal phase transition of cell phospholipid occurs, but without change in the total phospholipid composition. These changes have been observed in cultivated animal cells too. Nutritional experiments with rats have shown that Δ^9 desaturase activity is higher in rats fed on a

diet rich in saturated fatty acid compared with rats consuming a diet rich in polyunsaturated fatty acid (Jeffcoat and James, 1977).

Stearyl Co A desaturase activity has been extensively studied in liver, it has been shown to be a multicomponent membrane bound enzyme associated with the microsomal electron transport chain. The liver enzyme has been shown to have a lipid requirement for activity and is under dietary regulation (Holloway and Holloway, 1977).

Previous studies in aorta and liver microsomes demonstrated that the changes in stearyl Co A desaturase activity are accompanied by changes in the fatty acid composition of these microsomes (Holloway and Holloway, 1977). The authors estimated the fluidity of microsomal membranes from rat liver and aorta by direct measurement of microviscosity and fatty acid composition. They studied dietary induced changes in desaturase activity in liver microsomes and they found no association with fluctuations in membrane fluidity as the values for the microviscosity of these membranes were remarkably constant.

If the desaturase activity, is controlled by the fluidity of the microsomal membrane, it would be predicted to be stimulated under conditions of high microviscosity in order to produce more double bounds to return to the original microviscosity.

In contrast the dietary induction of acyl chain desaturases alters the lipid composition and fluidity of rat hepatocyte plasma membrane, as was reported by Storch and

Schachter (1984) and they related the increase in fluidity of the plasma membrane due to the starve-refeed regimen to both an increased unsaturation of acyl chain and a decreased CH/PL ratio.

Linoleic Acid 18:2 ^{9,12} is an essential fatty acid in most animals functioning in the maintenance of proper membrane fluidity and permeability and required as a precursor of PG, thromboxanes and leukotrienes. Δ^6 desaturation from linoleic acid to ϒlinolenic acid plays an important role in determining the fatty acid composition of animal tissues. The enzyme activity changes with substrate changes and product concentration, and by dietary factors, and hormones too (Brenner et al., 1977)

Studies relating fluidity to the CH/PL ratio show that when lipid fluidity of the cell membrane is reduced, an increase in mole ratio of CH/PL is observed. Under normal conditions the CH/PL ratio of any biological membrane remains constant presumably to maintain optimal function. At least half of the cholesterol molecules in the cell plasma membrane can exchange with the cholesterol pool of the serum (Shinitzky, 1979).

The regulation of cholesterol in cell membranes has been widely reviewed (Cooper and Strauss, 1984). For instance cells depleted of cholesterol may compensate by increasing the proportion of saturated fatty acids, and thereby maintain a functional optimum (Shinitzky, 1984). In another study cholesterol (low level) stimulated unsaturated phospholipid synthesis (Dahl and Dahl 1983). The unsaturated fatty acid

uptake is enhanced in sterol-starved cells supplemented with small amounts of cholesterol (Dahl et al 1981).

Cholesterol in biological membranes can regulate membrane fluidity by condensing the fatty acyl chains of phospholipids above and fluidizing them below the phase transition (Rothman and Engelman, 1972).

Although the principally reported cold-induced lipid alterations have been the unsaturation of polar lipid acyl chains, reports attributing a role to the active retailoring occuring with and alteration in acyl chain composition has been made (Lynch and Thompson, 1984). This is the case in Dunaliella, in which the correlation of species with one saturated chain and one unsaturated chain decreased while that of species with two unsaturated acyl chains increased. It is thought that these changes may be of short importance in reestablishing a functional degree of fluidity until more appropriate lipids are transported from the endoplasmic reticulum to the surface membrane.

1.6. Evidence of homeoviscous adaptation.

Bacteria (Sinensky, 1974), protozoa (Fukushima et al 1976) and animal cells (Cossins, 1977) have been shown to respond to changes in environmental temperature by altering the degree of unsaturation of the fatty acyl chains of their membrane phospholipids. Decreased temperatures tends to increase the degree of unsaturation and vice-versa, affecting the fluidity of the membrane.

The usefulness of the homeoviscous adaptation lies in

providing the optimal lipid environment for critical membrane functions including membrane bound enzymatic activity (Sandermann, 1978).

Important changes in the sterol content and sterol/phospholipid ratio occurs during adaptation to lower temperatures. But this ratio was found to reduce in carp mitochondrial membranes (Woodltke, 1978) and conversely increasing of sterol content was observed in mitochondria and microsomes at lower temperatures (Aaroson et al., 1982).

Attempts to incubate lymphocytes with unsaturated fatty acids resulted in increased mobility with DPH, whereas there was little change with saturated fatty acids (Stubbs et al., 1980) and these authors related this accumulation of unsaturated fatty acid with intracellular lipids droplets of triglycerides and higher incorporation of exogenous fatty acid had little effect on the DPH probe with respect to phospholipids.

The effect of dietary polyunsaturated fatty acid on the fluidity of human erythrocytes was tested and there were no changes in the membrane fluidity as investigated by ESR and DPH probes (Popp-Snijders et al., 1986). They showed no influence of the treatment although the increased unsaturation of PC and PE was observed, indicating a possible change in the phospholipids class separation. Similarly when subcellular membranes of specific tissues in rat were tested, they indicate little effect on the proportion of saturated to unsaturated fatty acid in the phospholipids of membranes studied under different diets (Gibson et al., 1984). In

lymphocytes and liver microsomes dietary experiments with polyunsaturated supplementation showed that fatty acyl group composition of lymphocytes and liver microsomes although of decreased order, the phospholipid composition compensated for the changes since no significant changes were detected by diphenylhexatriene (Conroy et al., 1986).

Also the experiments on head group are consistent with a phospholipid chain mediated homeoviscous adaptation as has been widely demonstrated in studies of transformed, cells line LM cells. Since PC is more fluid than ethanolamine with the same acyl chain composition (Schroeder et al., 1976), the ethanolamine analogues incorporation into the the LM cells led to compensation to the increased rigidity, that resulted from increased amino phospholipid content, because they decreased PC and PE. In this system the cells lowered PE. (Schroeder, 1980).

In isothermal experiments, LM cell membranes grown in the presence of ethanolamine had the same polarization values as the choline grown-cells , although the values were higher in microsomes and mitochondria from ethanolamine supplemented cells (Schroeder, 1978). This response of the cells when they are incubated with phospholipid expected to be more rigid, would indicate the probability that LM cells must be compensating for the rigidifying effect of the altered polar head groups, by altering the degree of unsaturation and/or chain length of the phospholipid acyl groups, because these parameters varied inversely with the degree of methylation. It has been found that the total phospholipid fraction of

membranes of LM cells grown in choline analogues did not contain higher percentages of unsaturated fatty acids or shorter chain length fatty acid, but when they tested the distribution of fatty acids among various phospholipids species from LM cell plasma membrane, microsomes, and mitochondria, this varied in degree of fatty acid unsaturation and in the ratio of long chain to short chain fatty acids in response to base analogues supplementation. It could be an important mechanism to compensate the changes in the membrane.

Phospholipids fatty acyl alterations appear to be one compensatory mechanism whereby LM cells with altered phospholipid polar head groups adapt and maintain constant fluidity properties. Manipulation of the membrane phospholipid acyl chain composition of LM suspension cells altered the fluidity properties of the membranes, high enrichment with saturated fatty acid increased polarization and supplementation with unsaturated fatty acid altered fluidity (Schroeder et al., 1979).

It is useful to compare the effects of two major compositional manipulations in LM cells, fatty acyl chain manipulation and polar head alteration. Manipulation of membrane phospholipid acyl chain altered fluorescence polarization, in contrast polar head group manipulation of LM cell membrane phospholipids neither altered polarization (except for ethanolamine supplemented cells) nor abolished or shifted characteristic temperatures.

To summarize LM cells seem to compensate or adapt well

to phospholipid polar head group manipulation, since compensating alteration in phospholipid composition acyl chain composition, and sterol/phospholipid ratio occur (Schroeder, 1978; Schroeder et al., 1976).

In the culture of neuroblastoma cells, the fatty acids profiles of the phospholipids were practically similar in the base supplement cells and control (Robert et al., 1977). The phospholipid distribution following base addition, and the elevated cholesterol levels provided the cells with an efficient means to regulate their membrane lipid composition.

However alterations in phospholipid composition or sterol/phospholipid ratio have not been found after fatty acid supplementation. Ferguson et al (1975) supplementing LM cell culture with linoleate as fatty acid bovine serum albumin complexes, noted an apparently compensatory mechanism, as a decrease of unsaturated fatty acids 16:1, 18:1, but when linoleate was supplied to cells grown at 28° C, there was no further increase in the unsaturated fatty acid composition of the phospholipids.

Glaser et al (1974) found that a probable regulatory mechanism that could compensate for the changes in the polar head could be driven by altering the sterol content since it was reduced in cells grown in a choline analogue and they observed no changes in the total fatty acid composition, but in cells supplemented with analogues as well as linoleate however linoleate incorporation was slightly increased in the phospholipid.

Since cholesterol at physiological temperature

increases order parameter, the studies performed on plasma membranes of a mutant defective in the regulation of cholesterol biosynthesis and the wild type, apparently showed no change because of an adaptative alteration in membrane phospholipid composition (Sinensky, 1980). It is supposed than an alteration in phospholipid composition has compensated for the change in cholesterol level, and an increase in unsaturated fatty acid content of the phospholipids was the observed response, specially the oleic/stearic acid ratio.

Evidence that alterations in fluidity affects the desaturases activities rather than acyltransferases was demonstrated in microsomal membranes of rat liver (Pugh and Kates 1984). By dietary manipulation the desaturases (Δ^9, Δ^6 Δ^5) activities were elevated, and it was correlated with decreased fluidity of membrane phospholipid.

When the experiments in isothermal circumstances were fulfilled in microsomal membranes, they indicate inhibition of desaturases in alcohol-induced fluidity changes in a substrate concentration dependence (Garda and Brenner, 1984) and the cholesterol incorporation in rat liver microsomes produced, higher microviscosity and increase in the fatty acids desaturation of rats (Garda and Brenner, 1985). These two experiments showing opposites responses to the desaturases evoked by different changes on membrane order, suggests that desaturase reactions may function as a self regulating mechanism of unsaturated acyl chain content and through changes of membrane fluidity.

1.7. Aim of this work.

The membrane bound enzymes and membrane transport processes are sensitive to membrane order. The observations mainly lead to the acceptation of homeoviscous adaptation to temperature by means of desaturase activities.

Previous works as we have seen above have been made mostly in the form of long term studies by dietary induction or supplementation in culture cells, in which the steady state membrane conditions had been re-established. However in these studies fluidity is the major factor regulating fatty acid desaturase apparently because alterations of phospholipid polar head group distribution was not of critical importance in the acclimation process. It was rather found to correlate with the degree of phospholipid fatty acid desaturation.

Here we tested conditions either to rigidity or fluidify of the plasma membrane, in the form of short-term experiments, and similar results were obtained. There is presence of adaptation homeoviscous, via desaturases activities, which is more relevant starting from the plasma membrane to endoplasmic reticulum, making conceivable, an exchange of information.

The possibility of exchange between plasma membrane and microsomes arises since the endoplasmic reticulum is the site of desaturase activity and the plasma membrane senses the changes observed. This supposition can only be tested by mean of a system that really ensure an external modification in whole cells.

Our approach was the use of two phosphatidylcholines with opposite effects. The 17:0/17:0 PC to rigidify the plasma membrane and 18:1/18:1 PC to fluidify it. The effects of these approaches are elucidated by the temperature dependent of polarization fluorescence and lipid composition. The effect on endoplasmic reticulum is given through desaturases activation.

2. MATERIALS AND METHODS

2.1. Materials.

1-^{14}C stearic acid (sp.act. 59 uCi/umol/1.7uCi/uml9, 1-^{14}C linoleic acid (60 uCi/umol-10 uCi/mol); all with more than 98% radiochemical purity were purchased from New England Nuclear (Boston, Mass. USA). L- α diheptadecanoyl phosphatidylcholine, L- αdioleyl phosphatidylcholine, and unlabeled fatty acid, were obtained from Sigma Chemical Company Co. (St.Louis,MO). DC Fertigplatten Kieselgel 60 plates were from Merck. Destillated water was used throughout the experiments. All other reagents were of analytical grade and obtained from Reanal Fine Chemicals (Hungary).

2.2. Methods.

2.2.1. Suspensions.

Four to five weeks old rats thymocytes were used in all experiments. Thymi were removed immediately after cervical dislocation and placed into cold Krebs-Ringer phosphate buffer. To obtain single cell suspensions the tissues were cut into small pieces, ground and agitated gently, in a glass tube for three minutes. The connective tissue was removed by filtering through gauze. The cells were then concentrated and counted. In all experiments thymocytes were used at a density of 10^9 cell /ml.

2.2.2. Membrane modification and isolation of plasma membranes.

Thymocytes were preincubated with either 17:0/ 17:0 phosphatidylcholine or 18:1/18:1 phosphatidylcholine, in a final concentration of 10 uM for 30 minutes. All incubations were performed in a shaking water bath at 37°C, using (1-3) x 10^9 cells.

The isolation of plasma and intracellular membranes was carried out according to the method of Koizumi (1981). Briefly,the cells were obtained by the same procedure, as described above. To obtain sufficient quantities of plasma membranes from young rats, five or seven rats were used per experiment. To ensure complete removal of contaminating erythrocytes, each cell suspension in phosphate buffer saline containig 5 mM $MgCl_2$ (pH 7.8) was centrifuged at 1,000 r.p.m for 10 minutes, two times; and nine volumes of 1 mM $NaHCO_3$, 2 mM $CaCl_2$ and 5 mM $MgCl_2$ (pH 7.8) were added to the packed cells. After 1-2 minutes hypotonic treatment, the cell suspension was adjusted to isotonic osmolarity by adding 10 times concentrated phosphate-buffered saline. After this treatment, the cells free from erythrocytes were prepared by centrifugation at 1,000 r.p.m. for ten minutes. The temperature was maintained below 4°C throughout the procedure.

The viability of the cells was determined by the trypan blue exclusion probe, and an aliquot was taken to count the cells. The viability was between 96-98 %.

The packed cells were suspended in 10 volumes of 1 mM

$NaHCO_3$, 2 mM $CaCl_2$ and 5 mM $MgCl_2$ solution to swell the cells. Ca^{+2} and Mg^{+2} were included to prevent disruption of nuclei. After 30 minutes thymocytes were homogenized by a tight fitting Dounce glass homogenizer, Kontes type B, (Kontes Glass Co, Vineland, NJ) with approximately 100 strokes. Phase contrast microscopy of the membrane fraction revealed vesicles of varying sizes.

Each homogenate was layered onto sucrose layers in a centrifuge tube, in which the sucrose solutions had been pipetted in the following order: 4.4 ml of 70%, 9.7ml of 42 % and 10.4 ml of 20 % of sucrose solutions. Each sucrose solution contained 2 mM $CaCl_2$ and 5 mM $MgCl_2$. The samples were centrifuged for 1 hour in the Beckman L 8M ultracentrifuge class H, using a SW 27 rotor at 52,000 x g. The hypotonic medium in the upper part was removed, and, at 20% sucrose layer containing the microsomal components, the fraction was aspirated carefully. The plasma membrane fraction, was obtained as a band in the interface between 20% and 42 % of sucrose layers. Mitochondria, cell debris and undisrupted cells were recovered at the interface of the 42 and 70 % sucrose layers, and nuclei were sedimented at the bottom of the tube. This plasma membrane fraction was diluted to isotonic sucrose concentrations with 1mM $NaHCO_3$ and centrifuged at 13,000 x g for 15 minutes, to remove contaminating microsomes. After centrifugation, the sediment was washed with 1mM $NaHCO_3$ solution to wash and centrifuged at 13,000 x g for 15 min. The pellet of plasma membranes was resuspended in a small volume of 1 mM $NaHCO_3$, and stored in a

liquid nitrogen tank, for posterior gas chromatographic and diphenylhexatriene analyses.

The microsomal fraction was resuspended at an isotonic sucrose concentration with 1 mM $NaHCO_3$ and centrifuged at 40,000 r.p.m. x 1 hour, in a Beckman L 8 M ultracentrifuge class H, using a Ti 60 rotor. The pellet, was then suspended in a small volume of 1mM $NaHCO_3$, and stored in liquid nitrogen for later analyses.

As the plasma membrane, the mitochondrial fraction was treated in the same manner.

The plasma membrane preparations were residually contaminated with other intracellular membranes as judged by marker enzymes made by Koizumi. Therefore the observed changes in lipid composition by gas chromatographic and DPH polarization are likely to reflect true differences in the physical properties of these membranes. We analized the purity of the fractions by CH/PL ratio determination.

2.2.3. Protein assay.

The protein concentrations of the resulting membrane vesicles were quantified using a protein dye binding assay (Bradforf, 1976) ; Bio-Rad Protein Assay Kit, Bio-Rad Laboratories, Richmond, Calif); bovine serum albumin (Sigma Chemical Co) was used as the standard.

2.2.4. Extraction of lipids.

Subcellular membranes and whole cells were extracted according to the Folch technique (1957). Chloroform :

methanol (2:1 v/v) was added to the sedimented thymocytes. Polar and non polar lipids were separated by single dimension thin layer chromatography, on precoated silica gel plates using petroleum ether:diethyl ether:acetic acid 85:15:1 as solvent. The spots, of phospholipids after visualization under UV light by spraying with 0.5% ANS in methanol, were removed and phospholipids were transesterified, in the presence of 5% HCl in absolute methanol in screwed tubes at 80^{0}C for 2.30 hrs. Separation of methyl esters was made by silver nitrate TLC or GLC.

2.2.5. Fluidity measurements.

The fluorophore diphenylhexatriene (DPH), which monitors the dynamic behaviour of the hydrophobic regions of the membrane, was used for these determinations.
Diphenylhexatriene, is an almost ideal probe for measuring the fluidity of lipids, since it is hydrophobic, and located with its symmetry axis normal to the plane of the membrane, and this occupies the region near the center of this bilayer. The fluorescent behaviour of the dye 1,6 diphenyl 1,3,5 hexatriene as a function of temperature in phospholipid dispersions has been established by Andrich and Vanderkooi (1976).

A Perkin Elmer MP-44A spectrofluorimeter, equipped with a temperature controller was used. Plasma and intracellular membranes, suspended in buffer Tris pH=7.2, were incubated with DPH for 30 minutes, to allow stabilization of fluorescence intensity and reequilibrated to 10^{0}C and

polarization values were determined at approximately 2.5 min intervals

Diphenylhexatriene fluorescence polarization parameters, were measured using 375 ± 5nm and 420 ± 8nm for excitation and emission wavelengths respectively. The temperature changes were $1^{o}C$/min.

The degree of fluorescence polarization P, was calculated according to :

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

where $I_{//}$ and $I_{\perp}$ are observed intensities measured with polarizers parallel and perpendicular to the vertically polarized exciting beam respectively. G is a factor (1.42 in these experiments) used to correct for the inability of the instrument to transmit differently polarized light equally.

The temperature of the sample was regulated by circulating water and was read prior to each measurement by means of a digital thermometer.

2.2.6. Lipid composition analyses.

Membrane methyl fatty acid data were obtained to ascertain the extent of exogenous fatty acid enrichment. It was determined by gas liquid chromatography in a Hitachi apparatus, type 263-80 Gas chromatograph, using a column 15% Carbowax 20M on 80/100 Supelcoport from Supelco, Inc. (Bellefonte, Pennsylvania) with a range of 180-220^{o}C of the

programmed temperature, at a $1^{o}C/1$ min ratio.

The CH/PL ratio was determined by TLC in an Iatroscan TH-10 from Iatron-laboratories, using petroleum ether :ether :formic acid 85.15.1 as solvent. The percentages were calculated from peaks areas, against standards.

2.2.7. Δ^{9}-Δ^{6} desaturase assays.

1-^{14}C-18:0 and 1-^{14}C-18:2 were used to stimulate the conversion of stearic acid to oleic acid, or linoleic acid to linolenic acid respectively. Thymocytes (1-3 x 10^{9} cell/ml) in Krebs-Ringer buffer were labeled with 1-^{14}C-18:0 or 1-^{14}C-18:2, at $37^{o}C$ in a water bath with gentle shaking. The thymocytes were preincubated with the modifying membrane agents when tested, as described above.

The cells were then washed in a phosphate buffer, containig unlabeled stearate or linoleate, to remove unreacted label, and finally the lipids were extracted.

The lipids were extracted in the same way as aforementioned. Chloroform:methanol (2:1 v/v) was added to the sedimented thymocytes. Lipid class separation was made by TLC on silica G plates using petroleum ether:diethyl ether:acetic acid 85:15:1, as solvent. The spots, after visualization under UV light by spraying with 0.5% ANS in methanol, were removed into scintillation vials for counting. To determine the distribution of radioactivity among stearic acid and oleic acid (18:0/18:1) and linoleic acid/ ɣlinolenic acid (18:2/18:3), the lipids were transesterified in the presence of 5% HCl in absolute methanol in screw top tubes at

80°C for 2.30 hr. The separation of methyl esters according to the degree of unsaturation was performed by argentation thin layer chromatography (10 % $AgNO_3$ on Silica Gel G) using the same solvent system as for lipid class separation .The methyl stearate and methyl oleate, to assay Δ^9 desaturase, as the methyl linoleate and methyl linolenate, to assay Δ^6 desaturase, were cochromatographed, and were visualizated using aqueous Rhodamine B solution. The spots were removed into scintillation vials, and radioactivity was measured in a toluene based cocktail.

A Tri-Carb Packard automatic control liquid scintillation spectrometer model 574, was used for counting radioactivity in all experiments. Counts were corrected for quenching and counting efficiency.

3. RESULTS

3.1. Physical state of thymocyte membranes.

Fig 2.shows the temperature dependence of fluorescence polarization of DPH embedded in purified plasma membranes, microsomes, and mitochondria of untreated thymocytes. Higher P values indicate more ordered structures. There were two break points on the P versus 1/T plots indicating the onset and completion of phase separations that took place within the temperature range scanned.

The polarization of DPH fluorescence in membranes is a function of membrane fluidity and from the temperature dependent of polarization, form phase diagrams of the lipids. The sharp discontinuities in temperature dependence of DPH polarization, are observed at different temperatures according to lipid composition, because they correspond to the temperature at which these lipids undergo phase transitions as detected by differential scanning calorimetry (Chapmann, 1967).

From the Fig 2., it is evident that phase separation starts in both, the plasma membrane and microsomes around 27.5^{o}C, which is in good agreement with published data obtained for the former structures (Blazyk and Stein, 1972). However, plasma membranes obtained from the thymocytes completed phase separation at a higher temperatures than those obtained from rat liver (22.5 vs 19^{o}C). Phase behaviour of the mitochondria was similar to that of the aforementioned membranes, regarding the onset of phase separation. Phase

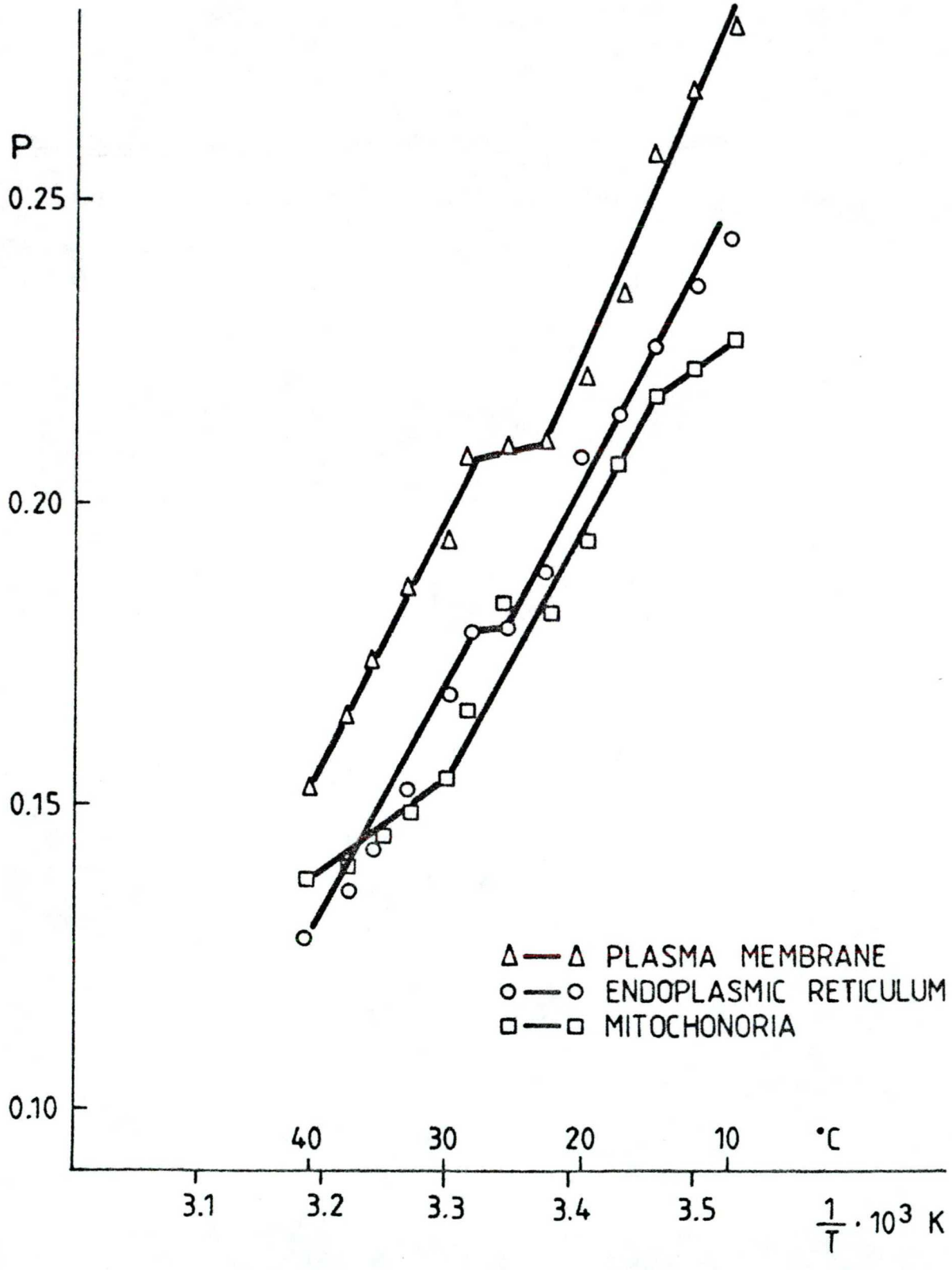


Fig.2.:- TEMPERATURE DEPENDENCE OF DPH FLUORESCENCE POLARIZATION (P) IN DIFFERENT MEMBRANES OF RAT THYMOCYTES.

separation in this system occured, however, at a more broad temperature range and was complete at considerably a lower temperature ($15^{o}C$), From the "P" values it may be concluded that plasma membranes were the most rigid structures followed by the endoplasmic reticulum and mitochondria. The fact that the plasma membrane is more rigid than the endomembranes, has long been demonstrated and is attributed largely to an elevated sterol to phospholipid ratio (Cooper,R.A.,1978). It is also demonstrated in these experiments (Table 1) where values of 0.47 were obtained for plasma membranes of control cells. In addition the high content of palmitic acid and the low content of polyunsaturated fatty acids in PC of lymphocytes membranes, makes these relatively more rigid than liver membranes (Ferber et al 1975).

In an attempt to modify the physical state of the plasma membrane of thymocytes they were incubated in the presence of vesicles, prepared either from 17:0/17:0 PC or 18:1/18:1 PC for one hour. It was hoped that during this time the vesicles would fuse with the plasma membrane and thus modify the "P" values accordingly (i.e.increase after 17:0/17:0 PC and decrease after 18:1/18:1 treatment).

Fig.3 demonstrates that this was the case, indeed. The most drastic effect was evoked by 17:0/17:0 PC. Not only the P values increased considerably, but also the onset of phase separation was shifted to higher temperatures ($40^{o}C$). The latter might be connected with the high phase separation temperature of the phospholipid used, being over $50^{o}C$. The shape of the plot did not assume the expected one (compared

Table 1.Effects of the membrane-modificating agents on the cholesterol/phospholipid ratio in rat thymocyte membranes

Fraction	Protein (mg/ml)	Control cells	17:0/17:0 PC treated cells	18:1/18:1 PC treated cells
Plasma membrane	0.400	0.47	0.06	0.18
Microsomes	0.920	0.30	0.06	0.015
Mitochondria	4.6	0.33	0.27	0.22

CH/PL ratio was determined as described in materials and methods. Results are the average of three experiments.

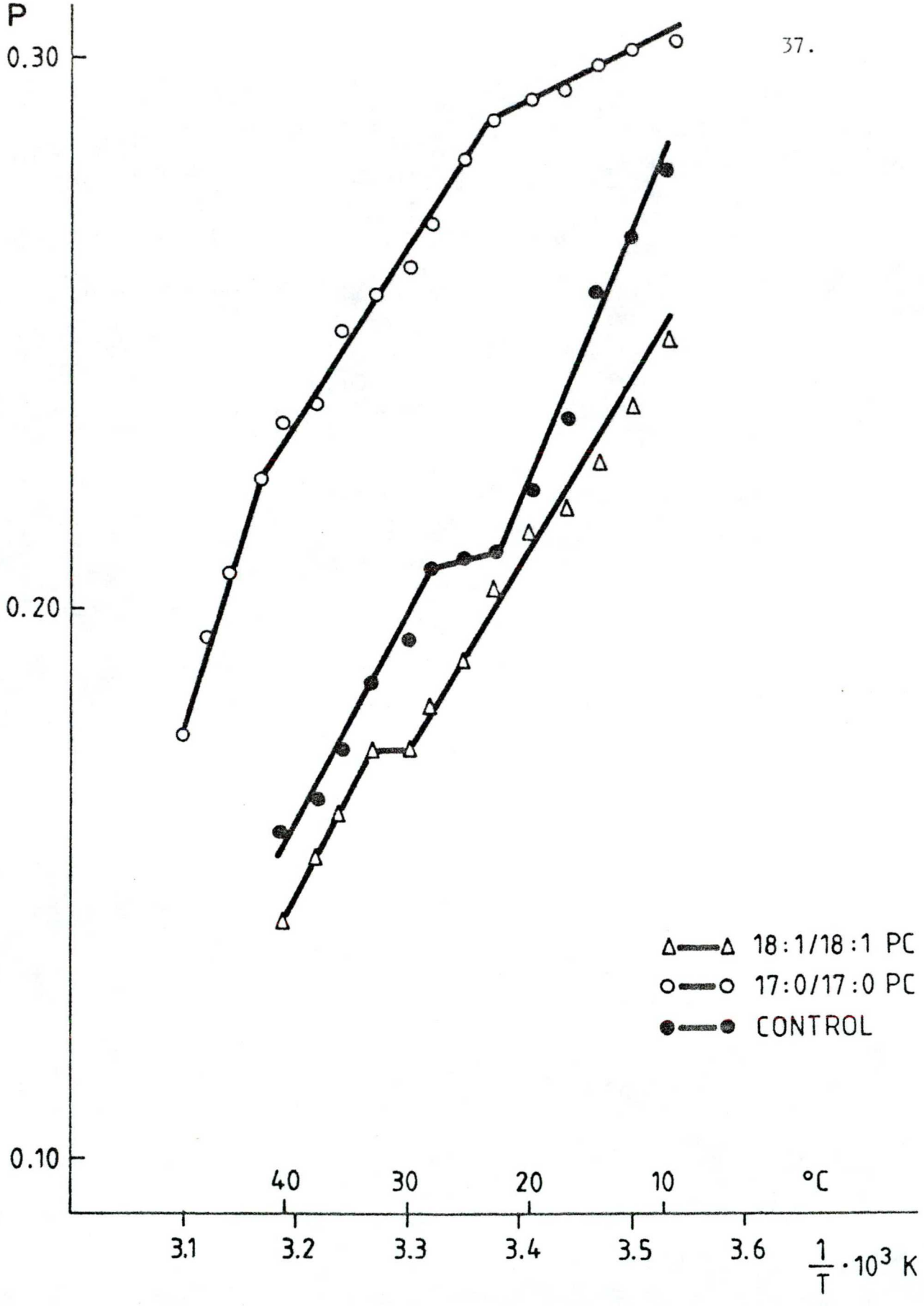


Fig. 3.: -PLOTS OF THE EFFECT OF MEMBRANE MODIFICATION ON DPH-FLUORESCENCE POLARIZATION VS TEMPERATURE OF PLASMA MEMBRANES OF RAT THYMOCYTES.

with the control) below the completion of phase separation. No explanation can be offered for this fact, but similar shapes have been obtained also for other systems. As also expected, incubation of the thymocytes with 18.1/18:1 PC resulted in a decrease of the "P" values, indicating successful fluidization.

However, the effects was not so drastic. Although the plasma membranes became significantly more fluid, they exhibited phase separations at higher temperatures, than the controls. As this phospholipid shows phase separation at $-20^{o}C$, a shift to lower temperatures of phase separation in the treated plasma membranes was anticipated. Again no explanation can be offered maybe these structures attempted to compensate the fluidifying effect of 18:1/18:1 PC by reorganizing their molecular architecture in a manner resulting in higher phase separation temperatures.

Fig.4 shows that incubation of the thymocytes affected microsomal ordering in the same way as observed for the plasma membranes, that is incubation in the presence of 17:0/17:0 PC resulted in a rigidification, and in the presence of 18:1/18:1 PC in a fluidization. On the other hand the shift of the onset of phase separation in the case of 17:0/17:0 PC was not so pronounced, but still significant and a downshift in the onset of phase separation in the case of 18:1/18:1 PC incubation was observable.

Mitochondria reacted similarly to the other membranes. From Fig 5, we concluded than an appreciable rigidification account for cells incubated with 17:0 /17:0 PC

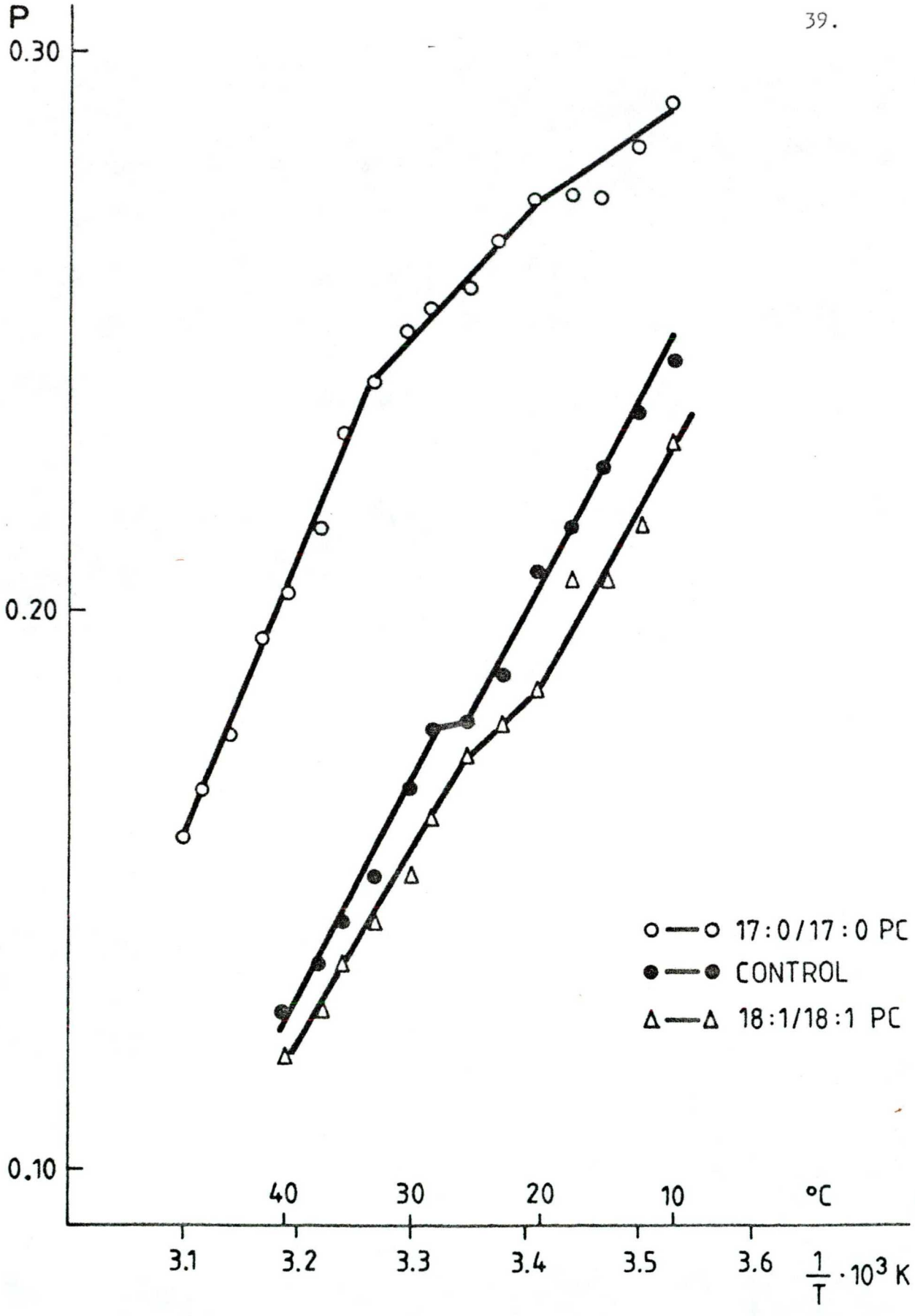


Fig.4.: - PLOTS OF THE EFFECT OF MEMBRANE MODIFICATION ON DPH-FLUORESCENCE POLARIZATION VS TEMPERATURE OF ENDOPLASMIC RETICULUM IN RAT THYMOCYTES

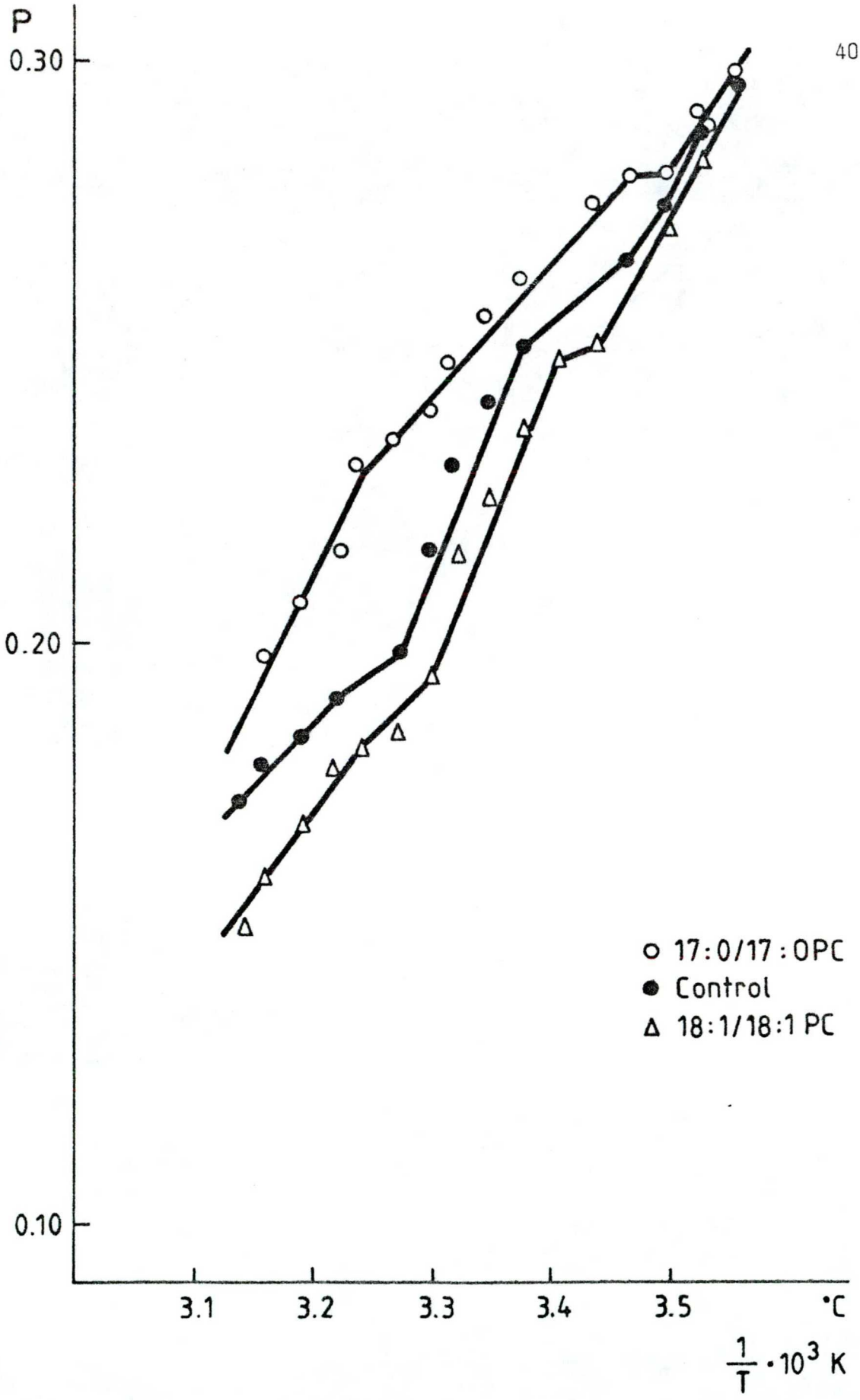


Fig. 5.: PLOTS OF THE EFFECT OF MEMBRANE MODIFICATION ON DPH-FLUROSCENCE POLARIZATION VS TEMPERATURE OF MITOCHONDRIA MEMBRANES OF RAT THYMOCYTES

and a fluidization for cells incubated with 18:1/18:1 PC, when they are compared with the controls.

3.2. Fatty acid composition of thymocytes membranes.

Fatty acid methyl esters obtained from the different membranes were subjected to gas-liquid chromatography. A gas chromatographic tracing of the control plasma membrane fatty acids is represented in the Fig 6a. As is seen palmitic (16:0),stearic (18:0), oleic (18:1 n-9), linoleic (18:2 n-6),and arachidonic acid (20:4 n-6) are the major fatty acids.The fatty acid composition of the purified membranes is given in table 2.

Incubating the thymocytes in the presence of 17:0/17:0 vesicles, resulted in an appreciable amount of 17:0 in the plasma membrane fraction (15%), leading to an increase in the saturated/unsaturated fatty acid ratio, which is regarded to reflect the fluidity relationship in such structures. With regard to the extremely low value as compared to the control value for CH/PL (table 1), we only suppose, that it may be due to the high proportion of phospholipid by the new attachment to the membrane, that decreases this ratio so drastically.

Fig 6b is a chromatographic tracing of that part of the chromatogram where the fatty acid in question appeared. Evidently, this is reflected in elevated P values, and in general in rigidifying of plasma membrane as has been shown in Fig 3. Despite the massive accumulation of 17:0, in the plasma membrane, the microsomal fraction did not contain any

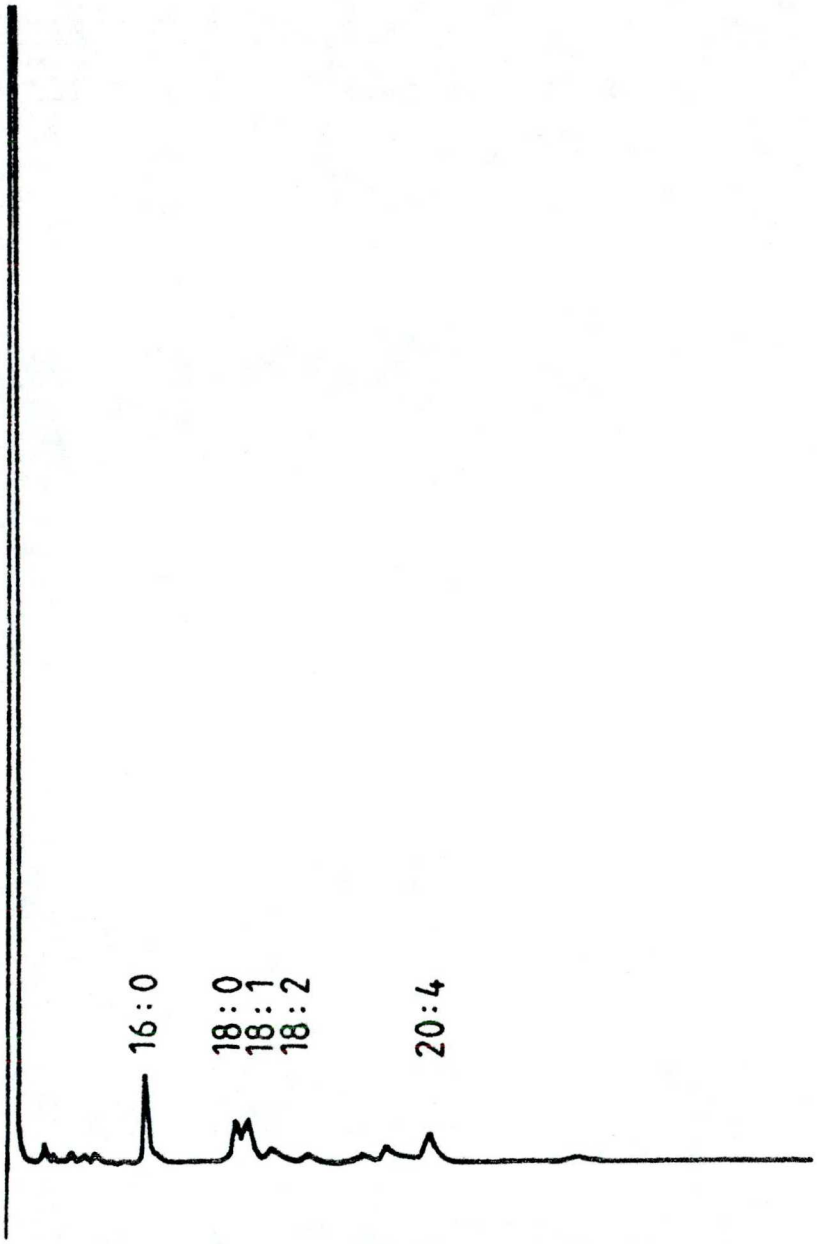


Fig. 6a.: -GAS CHROMATOGRAM OF FATTY ACID METHYL ESTERS FROM THYMOCYTE PLASMA MEMBRANE CONTROL CELLS

Table 2. Rat thymocytes plasma membrane phospholipid fatty acid composition[a]

Fatty acids[b]	Control cells[c]	17:0/17:0 PC treated cells	18:1/18:1 PC treated cells
12:0		12.88	
14:0		1.65	2.04
16:0	41.08	34.83	34.57
17:0		15.52	
18:0	10.32	5.16	5.89
18:1	12.54	4.97	12.45
18:2	8.69	4.22	10.08
18:3	1.07	3.44	3.14
20:3	7.56	2.80	5.40
20:4	18.70	8.41	26.47
Sat/Unsat[d]	1.05	3.37	0.74

a:Fatty acid methyl esters were prepared from total membrane lipid as described (see mat.meth.) and analized by gas-liquid chromatography.
Only percentages of major fatty acids are given. The difference between the sum of any column and 100 represents the percentage of other fatty acids not reported here.
b:Fatty acids are identified by chain length (number before colon) and degree of unsaturation (number after colon).
c:Values given for the fractions represent a single determination from a pooled sample from three animals.
d:Sat/unsat=sat/unsat ratio

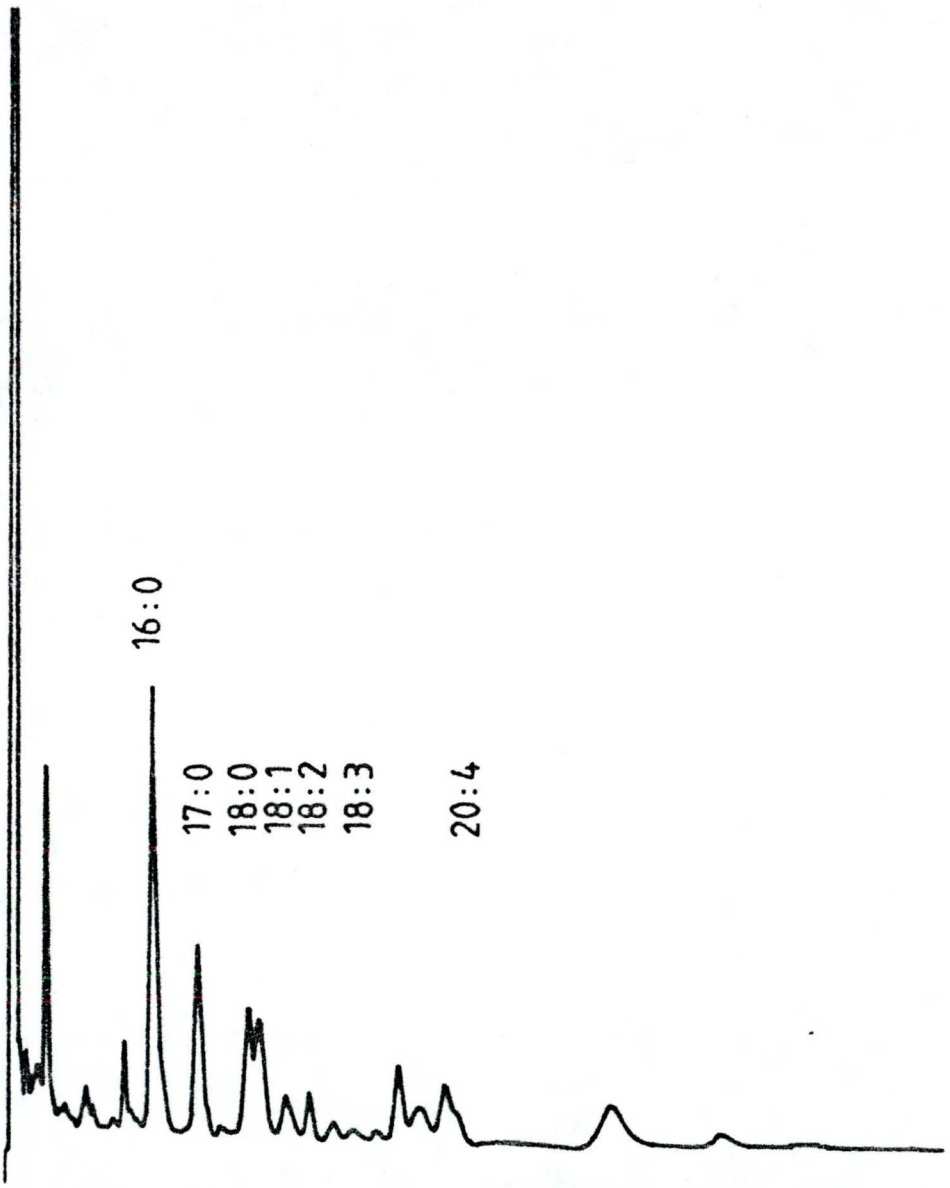


Fig. 6b.: -GAS CHROMATOGRAM OF FATTY ACID METHYL FROM ESTER THYMOCYTES PLASMA MEMBRANE OF 17:0/17:0 PC TREATED CELLS

of it. Fig 6c shows such a chromatogram, the place where the 17:0 should have appeared, is indicated by an arrow. It was easy to detect 17:0 in the fatty acid mixture, because it is not a normal membrane component and in general, it does not overlap with any other fatty acid. Table 3 gives the phospholipids fatty acid composition for microsomal membranes. Curiously, although there was no 17:0 incorporation, the sat/unsat ratio was increased in relation to the control, likely by an increase of short chain saturated fatty acids.

It was hoped that incubation of thymocytes with 18:1/18:1 PC will be reflected by the elevated amount of this fatty acid. Table 2 shows, that the percentage of oleic is similar to the control cells. An unexpected but significant increase in the percentages of 18:3 and 20:4 is also reported. Similarly, no oleic acid accumulation was observed in the microsomal fraction, as is demonstrated in the table 3. The same is true also for the mitochondria. Table 4 gives the exact fatty acid composition data for mitochondria, in cells with 17:0/17:0 PC, and 18:0/18:0 PC in relation to control.

3.3. Δ^9-Δ^6 desaturases assay.

Our earlier results were in order to obtain the optimal conditions for the proper radioactivity label incorporation. The thymocytes were prelabeled at a relatively high cell density. The uptake of radioactivity by the cells showed the peak of incorporation at 90 min.of incubation. The conversion

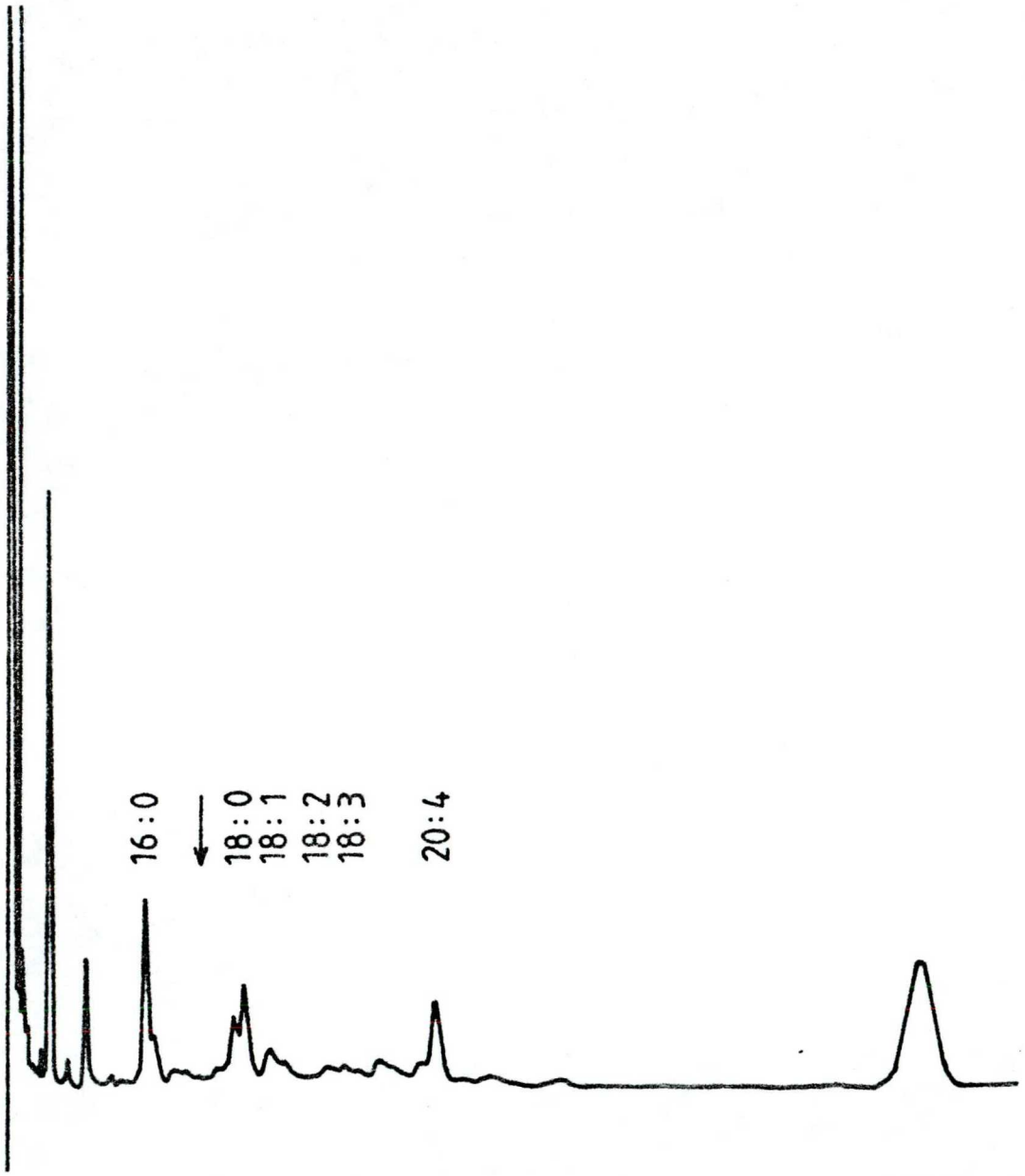


Fig. 6c.: -GAS CHROMATOGRAM OF FATTY ACID METHYL ESTERS FROM THYMOCYTES MICROSOMAL FRACTION OF 17:0/17:0 PC TREATED CELLS

Table 3. Rat thymocytes microsomal phospholipid fatty acid composition.

Fatty acids	Control cells	17:0/17:0 PC treated cells	18:1/18:1 PC treated cells
12:0	1.49	24.62	
14:0	2.53	11.36	3.04
16:0	35.91	20.68	40.21
18:0	1.81	4.05	8.33
18:1	14.30	11.30	12.25
18:2	18.83	4.78	8.57
18:3	10.66	0.87	2.14
20:3		6.01	5.77
20:4	14.43	16.29	19.66
Sat/unsat	0.71	1.54	1.06

Conditions were the same as described in Table 2.

Table 4. Rat thymocytes mitochondria phospholipid fatty acid composition.

Fatty acids	Control cells	17:0/17:0 PC treated cells	18:1/18:1 PC treated cells
12:0	6.84	4.68	
14:0	2.78	3.02	1.59
16:0	25.81	32.67	25.37
18:0	6.48	4.72	8.01
18:1	14.06	12.53	11.60
18:2	10.79	11.72	11.98
18:3	1.27	3.45	7.20
20:3	9.50	7.17	13.26
20:4	22.39	20.00	20.95
Sat/unsat	0.72	0.82	0.53

Conditions were the same as described in Table 2.

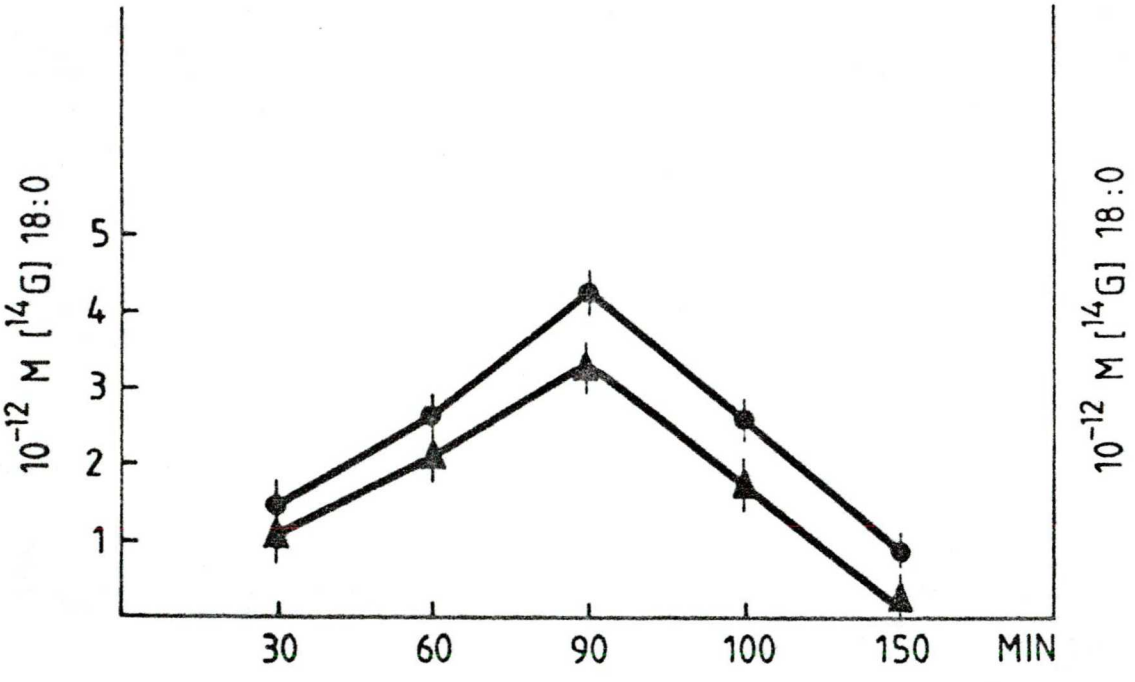


Fig.7.: TIME CURVE FOR UPTAKE OF 14C STEARIC ACID AND FOR FORMATION OF 14C OLEIC ACID. THYMOCYTES (2×10^9 cell/ml) WERE INCUBATED IN THE PRESENCE OF 14C 18:0 (26 µCi/umol. AT DIFFERENTS POINTS OF INCUBATION, THE DISTRIBUTION OF INCORPORATED RADIOACTIVITY AMONG SATURATED ● AND MONOUNSATURATED ▲ FATTY ACID WAS DETERMINED AS DESCRIBED IN MATERIALS AND METHODS. EACH POINT REPRESENTS MEANS OF THREE SAMPLES ±SD.

of stearate to oleate by Δ^9 desaturase activity followed the same pattern (Fig. 7).

The distribution of radioactivity among the phospholipids and free fatty acids fraction is shown in the Fig 8. 90% of the radioactivity was found in the PL fraction within 60 minutes, the rest of the counts were distributed in the diacylglycerol, cholesterol and triglycerides fractions (data not shown).

Based on the above results,preincubation times of 90 minutes were chosen for the experiments. We used the probe with cells incubated without any membrane modificating membrane (control), and cells with saturated PC,17:0/17:0 PC and unsaturated PC,18:1/18:1 PC. The cells, at the same amount 1-2 x 10^9/ml were preincubated with either 17:0/17:0 PC or 18:1/18:1 PC, in a range of 25 to 100 ul, before the addition of the respective labeled fatty acid. The results for the desaturation of 1-^{14}C-18:0 are showed in tables 5 and 6.

After the treatment with saturated phosphatidylcholine, there was no significant change of Δ^9 desaturase activity showing a decreased sensitivity since it was greatly activated when 100 ul of 17:0/17:0 PC were used in the incubation. The results with dioleoylphosphatidylcholine on Δ^9 desaturase showed a slight inhibition as we expected, since we considered this would act as a fluidifying agent, and thus there was no necessity of more desaturation.

The desaturation of 1-^{14}C-18:2 by the Δ^6 desaturase, when the cells were preincubated with both types of PC,

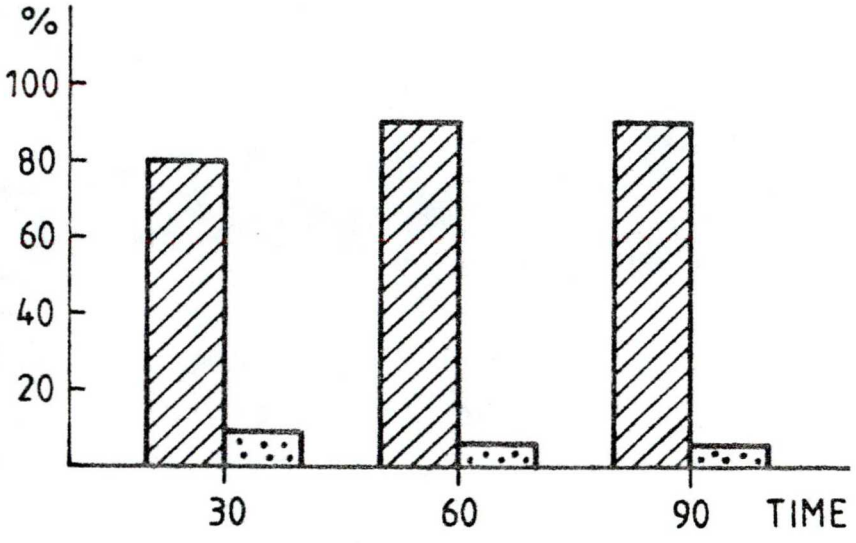


Fig. 8 .: - DISTRIBUTION OF INCORPORATED RADIOACTIVITY AMONG PHOSPHOLIPIDS AND FATTY ACIDS FRACTIONS. THYMOCYTES ($1-2 \times 10^9$ cell/ml) WERE INCUBATED IN THE PRESENCE OF 14C-1-18:0 (26 µCi/umol) FOR DIFFERENT PERIODS. LIPIDS CLASS SEPARATION WAS PERFORMED AS DESCRIBED IN MATERIAL AND METHODS. VALUES ARE GIVEN AS A PERCENTAGE OF TOTAL INCORPORATED RADIOACTIVITY

PL FA

Table 5.

	Control	Cells treated with 17:0/17:0 PC.		
		25 ul	50 ul	100ul
18:0	95.21	93.52	94.89	92.18
18:1	4.76	6.47	5.11	7.81

Δ^9 desaturase assay:distribution of incorporated radioactivity among fatty acid fractions. Thymocytes (x 10^9 cell/ml were preincubated with or without (control) 17:0/17:0 PC for thirty minutes, then incubated in the presence of ^{14}C 18:0 (1.7 uCi/ml) stearic acid, as described in materials and methods.

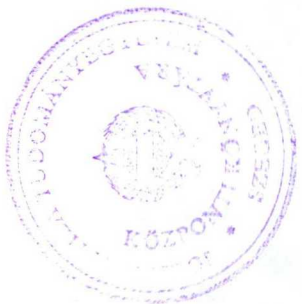


Table 6.

	Control	Cells treated with 18:1/18:1 PC.		
		25 ul	50 ul	100ul
18:0	94.70	96.05	96.57	97.46
18:1	5.29	3.94	3.43	2.54

Δ^9 desaturase assay:distribution of incorporated radioactivity among fatty acid fractions. Thymocytes (x 10^9 cell/ml were preincubated with or without (control) 18:1/18:1 PC for thirty minutes, then incubated in the presence of ^{14}C 18:0 (1.7 uCi/ml) stearic acid, as described in materials and methods.

Table 7.

	Control	Cells treated with 17:0/17:0 PC.		
		25 ul	50 ul	100ul
18:2	67.73	48.20	59.81	52.63
18:3	32.27	51.80	40.19	47.37

Δ^6 desaturase assay:distribution of incorporated radioactivity among fatty acid fractions. Thymocytes (x 10^9 cell/ml were preincubated with or without (control) 17:0/17:0 PC for thirty minutes, then incubated in the presence of ^{14}C 18:2 (10 uCi/ml) linoleic acid, as described in materials and methods.

Table 8.

	Control	Cells treated with 18:1/18:1 PC.		
		25 ul	50 ul	100ul
18:2	55.67	64.88	58.18	71.36
18:3	44.33	35.12	41.82	28.64

Δ^6 desaturase assay:distribution of incorporated radioactivity among fatty acid fractions. Thymocytes (x 10^9 cell/ml were preincubated with or without (control) 18:1/18:1 PC for thirty minutes, then incubated in the presence of ^{14}C 18:2 (10 uCi/ml) linoleic acid, as described in materials and methods.

is shown in tables 7 and 8.

Suprisingly, this desaturase was more clearly stimulated by addition of 17:0/17:0 PC, than the Δ^9 desaturase. With regard to the effect of 18:1/18:1 PC on this desaturase, we observed also a slight inhibition (table 8) as in stearyl Co A desaturase.

4. DISCUSSION.

It is very important for the cells to maintain a constant membrane fluidity under changing physiological or environmental conditions. Since Sinensky's pioneering work (Sinensky, 1974) a great number of publications have appeared which show that different organisms possess the capacity to maintain optimum membrane physical state (Cossins, 1977; Dickens and Thompson, 1980). Control of desaturase activity of the endoplasmic reticulum plays a crucial role in these processes. In previous experiments about regulation of fluidity membranes Skriver and Thompson (1976) suggested than the desaturase activity of microsomes was regulated by membrane fluidity, and with later findings made by Martin et al (1976) and Kasai et al.(1976), provided evidence that microsomal fluidity is regulated by induction of desaturase biosynthesis. These made it possible to suggest a model for the mechanism of fatty acid desaturase, which suggest that the enzyme immerse more deeply into the fluid lipid matrix thus rendering the active sites inaccesible for the substrate and vice-versa when the membrane becomes more rigid (Thompson, 1980). This model is shown in the Figs 9 and 10.

Borochov and Shinitzky (1976) proposed a fluidity controlled lateral displacement model. As is shown in the Fig.11 this hypothesis implies a viscosity treshold for certain membrane proteins. Upon increase in microviscosity, the proteins will associate by "lateral displacement".

Whatever is the real mechanism operating in the

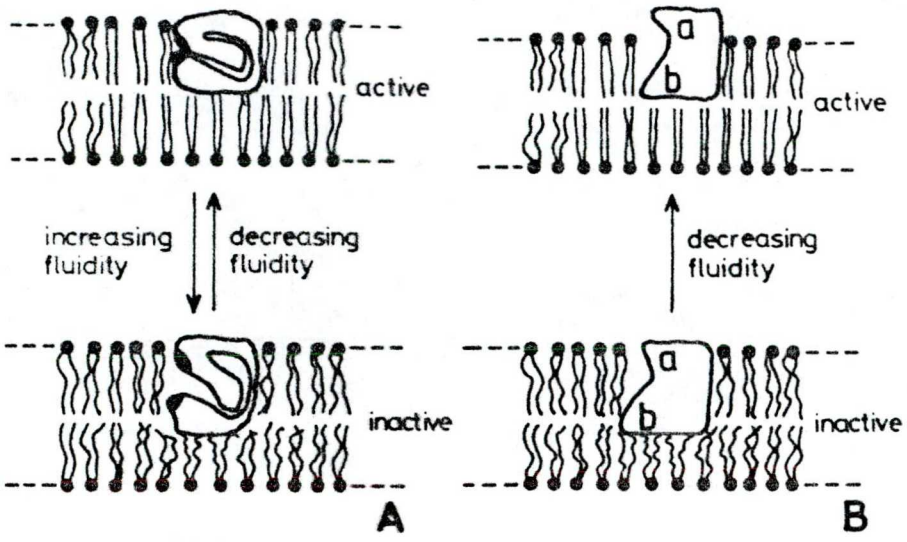


Fig 9 A. A POSSIBLE MECHANISM FOR THE ENHANCEMENT OF FATTY ACID DESATURASE ACTIVITY DUE TO A PROTEIN CONFORMATIONAL CHANGE NEAR THE ACTIVE SITE (HATCHED REGIONS)

Fig.10 B A POSSIBLE MECHANISM FOR THE ENHANCEMENT OF FATTY ACID DESATURASE ACTIVITY DUE TO A MOVEMENT OF THE ENZIME PERPENDICULAR TO THE PLANE OF THE MEMBRANE, PERHAPS ORIENTING THE ACTIVE SITE (b) IN A MORE FAVORABLE POSITION RELATIVE TO THE PHOSPHOLIPID-BOUND FATTY ACID SUBSTRATE

(FROM THOMPSON, G.A. Ir., MEMBRANE FLUIDITY, EDITE BY MORRIS KATES AND ARNIS KUKSIS, THE HUMANA PRESS, INC. NJ. 1980)

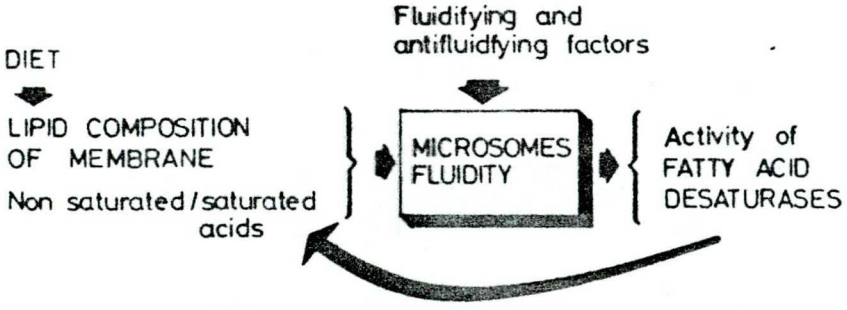


Fig. 10. HYPOTHETICAL SELF REGULATORY MECHANISM OF MEMBRANE FLUIDITY IN WHICH FATTY ACID DESATURASES ARE INVOLVED. FROM BRENNER RODOLFO R., PROG. LIPID. RES, 23, 69, 1984

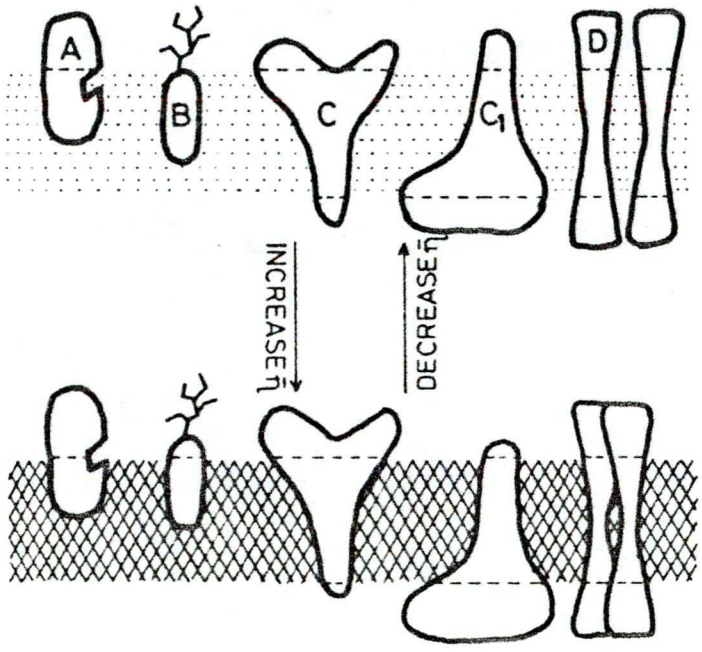


Fig. **11.** MODES OF VERTICAL AND LATERAL DISPLACEMENTS OF MEMBRANE PROTEINS MEDIATED BY CHANGES IN LIPID FLUIDITY. - A - C_1 DESCRIBES DIFFUSIBLE MEMBRANE PROTEINS IN WICH SMALL CHANGES IN LIPID MICROVISCOSITY WILL INCREASE THE EXPOSURE OF THE PROTEIN EITHER ON THE OUTER SIDE (A, B and C) OR ON THE INNER SIDE OF THE MEMBRANE, MODULATING THEIR ACTIVITY.
D - LATERAL DISPLACEMENT OF A MEMBRANE PROTEIN, WHERE INCREASE IN LIPID MICROVISCOSITY WILL INCREASE PROTEIN - PROTEIN INTERACTION.
(FROM SHINITZKY MEIR.., PHYSIOLOGY OF MEMBRANE FLUIDITY, CRC PRESS, INC - BOCA RATON, FLA, 1984)

endoplasmic reticulum, the question still remains open as to how these structures sense the changes taking place in the plasma membrane. The plasma membrane, being a permeability barrier, is not expected to allow exogenous stimuli in form of different chemical agents, hormones; etc to reach the endoplasmic reticulum. On the other hand it being the most rigid membrane structure of the cell, it must sense early these compounds or stimuli. As the desaturation takes place exclusively within the endoplasmic reticulum, an exchange of information is anticipated between the two membrane systems in order to maintain the optimum fluidity relationships for all the membranes.

In our approach to this question, phospholipids were selected to perturb integrity of the plasma membrane. Phospholipids were preferred over other compounds (like benzyl alcohol, short chain aliphatic alcohols, phenylethanol, alkyl ammonium salts or free fatty acids) for the following reasons: 1)Phospholipids (phosphatidylcholines in this case) are normal membrane compounds. If they are composed of specific fatty acids (like 17:0), they will be easy to detect in the membranes. 2)Their flip-flop rate is rather low (several hours depending on their fatty acid composition) so they are not expected to accumulate in the internal membranes during the experiment. 3)They would fuse with the plasma membrane, most probably in the first few hours, staying within the outer leaflet; and thus affect membrane physical state according to their physical properties.

Data of Fig 3 and table 2 indicate that these premises were at least partially met. Incubating thymocytes with 17:0/17:0 PC, an appreciable amount of 17:0 fatty acid was detected in plasma membrane structure. Moreover, its accumulation resulted in a very pronounced ordering (rigidification) as indicated by higher P values and shift of the phase separation temperature to a higher temperature region, although no 17:0 was detected in the microsomal or mitochondrial fractions. Unexpectedly, however, we could not detect (within the sensitivity of GLC determinations) any accumulation of 18:1 in the plasma membrane following incubation of thymocytes with 18:1/18:1 PC. We do not believe that this is a result of some experimental error, because the incubations conditions were the same as in the case of 17:0/17:0 PC. It is most probable that these rather fluid phospholipid molecules will not incorporate selectively into the plasma membrane, but interacting with it, they remove some cholesterol, which is supported by the very low CH/PL ratio when it is compared with the control, thus rendering these structures more fluid. An indirect support to this assumption can be taken from Sinensky's experiments; this author used a mixture of PC, PE and some neutral glycerides to remove membrane bound cholesterol and did not report that their phospholipid were incorporated, and this they explain as the effect of the membrane fluidizer (Lyte and Shinitzky, 1985).

The most significant result of these investigations, is that the physical state of the endomembranes (endoplasmic

reticulum, mitochondria) was comparable to that taking place previously in the plasma membrane, upon incubating the cell with PC vesicles, and even more important, that this took place without incorporation of any phospholipid molecules disturbing the surface membrane. The third important observation is that the endoplasmic reticulum gave an intelligent response in terms of homeoviscous adaptation to the changes in its own and the plasma membranes physical state. It is also obvious that the different desaturases responded differently to this stimulus: the Δ^9 desaturase was less sensitive than the Δ^6 one. We cannot give, but a rather teleological explanation for the fact that the phase separation temperature of palmitoyl/oleyl PC is higher than that of palmitoyl/linolenyl PC; thus the formation of the latter may contribute more efficiently to adjusting membrane physical state to the new situation. An alternative supposition can be gained that either this desaturase could have been inhibited by preexisting γlinolenate in the membrane, as has already been shown by Alaniz et al.(1986) or that Δ^9 desaturase incorporating the first double bond on saturated acids, needs major stimulation than Δ^6 desaturase via physical state modifications. This last possibility is supported by the highly rigidification-dependence responsiveness of the enzyme showing an effective activation only when 100 ul of 17:0/17:0 PC was used.

It is also possible to assume that the change in the fatty acid desaturase of the microsomes with the membrane physical characteristics was not necessarily reflected in the fatty acid composition, as we can deduce from the results of

tables 2 and 3, concerning the 18:1 and 18:3 percentages.

It is highly probable that there is an exchange of information between the endoplasmic reticulum and the plasma membrane concerning the physical state of the latter in order to maintain proper fluidity relationships within the cellular membranes. It seems to be evident that this is a two directional process i.e. the newly formed fatty acids/phospholipids will be exported from the endoplasmic reticulum to, among others, the plasma membrane which would stop sending message as soon as its own physical state is in equilibrium with the new circumstances. Perhaps this is the very early step in all kind of adaptation processes. It has been shown that activation of desaturases takes place in many poikilotherm species (Sinensky, 1974; Nozawa et al.,1974; Schroeder, 1978; Dickens and Thompson, 1984), and also that formation of unsaturated fatty acids, at least in fish is rather sensitively regulated according to the environmental temperature (Torrengo and Brenner, 1976). It may be postulated that plasma membrane or certain domains (regions) of it serve as a thermoreceptor in this regard. Another observation supporting the role of physical state of plasma membrane in controlling desaturase activity in endoplasmic reticulum comes from recent observations made on mitogen-stimulated lymphocytes (Shinitzky, and Inbar, 1975). Concanavalin-A in early phase of its binding to its receptor makes fluid the plasma membrane (Muller et al.,1979), and this is followed by a decrease of Δ^9

desaturase activity of the endoplasmic reticulum (Pajor et al.,1986).

Further efforts are needed to learn something about the nature of this supposed messanger. The possibility that the endoplasmic reticulum takes samples from the plasma membrane (the machinery is provided by the presence of the phospholipid exchange proteins) can be ruled out by the fact that no 17:0 could be detected in this structure. However, it remains open the question whether incubation of the cells with 18:1/18:1 PC removed some cholesterol from the plasma membrane and this was replaced by the endoplasmic reticulum. However it is difficult to imagine that the cell possess two different mechanism for the same purpose. We are inclined to the opinion that this messenger is not lipidic in nature. An indirect support to this theory is that the cold induced activation of desaturases in fish liver slices could be blocked by cycloheximide (Farkas, 1984), if we assume that cycloheximide blocked the formation of this messenger and not the desaturases itself. Because the induction of desaturase enzymes takes several hours (8-10) and the response to cycloheximide was immediate in those experiments, this latter possibility seems unlikely. However, in such a case the information has to go first to the protein synthesizing machinery of the cells and this may also be time consuming and does not serve the purpose: i.e. a rapid answer.

It might be interesting in this connection that Brenner has observed a protein factor in the cytoplasm which is required to the optimum desaturase activity in the

endoplasmic reticulum (Leikin and Brenner, 1986). Without adding 100 000 x g supernatant to the microsomal preparation their activity was rather low (Marra et al.,1986). Little is known about the nature or origin of this protein fraction but as a working hypothesis we can put forward that this protein is a component of the plasma membrane, and its release from it, is governed by the physical state of the plasma membrane in such a way that when it becomes more rigid, it will be forced out and thus appear in the cytosol. The endoplasmic reticulum being more fluid than the plasma membrane, would incorporate it changing by this way its own physical state, becoming more rigid. This would create the situations favouring desaturation of various fatty acids. However, this theory operates with a lot of uncertainities and does not explain what happens with this protein when the new equilibrium in the physical state is reached. Perhaps it will be removed from the endoplasmic reticulum and turned over or reincorporated in the plasma membrane.

The results presented in this work only shows a conceivable mechanism, to explain the fate that, an external physical state modification of the plasma membrane provoked a response in the endoplasmic reticulum, by means of the desaturase activation in order to maintain the fluidity in a optimum state.

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