A. Watts (Ed.), *Protein–Lipid Interactions*

CHAPTER 4

Lipid–protein interaction in a biological membrane: Effect of cholesterol and acyl chain degree of unsaturation

Celina E. CASTUMA[1,*], M. Teresa LAMY-FREUND[2], Rudolfo R. BRENNER[1] and Shirley SCHREIER[3]

[1]*Instituto de Investigaciones Bioquímicas de La Plata (INIBIOLP), UNLP-CONICET, Facultad de Ciencias Medicas, 60 y 120, (1900), La Plata, Argentina,* [2]*Institute of Physics, Universidade de S. Paulo, C.P. 20516, CEP 01498, S. Paulo, Brazil,* [3]*Department of Biochemistry, Institute of Chemistry, Universidade de S. Paulo, C.P. 20780, CEP 01498, S. Paulo, Brazil*

Abbreviations

UDPGT	UDP-glucuronyl transferase
UDPGA	UDP-glucuronic acid
p-NP	*p*-nitrophenol
5-, 12-, 16-SASL	stearic acid containing a 2′,2′-dimethyl-*N*-oxyl-oxazolidine ring at carbons 5, 12, and 16, respectively
ASL	5α-androstan-17β-ol containing a 2′,2′-dimethyl-*N*-oxyl-oxazolidine ring at carbon 3
DHP	1,6-diphenyl-1,3,5-hexatriene
PC	phosphatidylcholine
PE	phosphatidylethanolamine
PI	phosphatidylinositol
PS	phosphatidylserine
DBI	double bond index
EPR	electron paramagnetic resonance
ESR	electron spin resonance

1. Introduction

The interaction between lipids and proteins in membranes is a two-way process, each component affecting the properties of the other. It has long been known that the organizational and motional properties of lipids are influenced by the presence of proteins. Likewise, the function of membrane proteins (enzymes, pumps, channels, receptors) is affected by the lipid environment.

* Present address: Department of Biochemistry, Stanford University School of Medicine, Stanford, CA 94305-530, U.S.A.

A large body of literature exists on the effects of lipids on the activity of membrane-bound enzymes. Varying phospholipid head groups, chain length and degree of unsaturation has been shown to influence the kinetic behaviour of enzymes. Cholesterol has also been found to have an effect on protein function, including enzyme kinetics.

Whether the observed effects are due to participation of the lipids under study in direct contact with the protein, involving annular or non-annular sites, or whether it is a long range effect, due to changes in the properties of the bulk lipid is still not clear in many cases. One example is the extensively studied transmembrane enzyme, the Ca^{++}, Mg^{++}-ATPase. Although evidence has been provided for the absence of cholesterol from the immediate neighbourhood of the protein [1], the sterol has been found to affect the enzyme kinetics [1,2]. A similar situation is verified with the nicotinic acetylcholine receptor, for which cholesterol is claimed not to be present in the boundary lipid [3], but whose function is affected by the sterol [4]. In both cases it has been proposed that cholesterol may occupy non-annular sites that allow direct contact with the protein [1,3].

In microsomal membranes, it has been found that several enzymes, such as fatty acid desaturases [5–8], glucose-6-phosphatase [5], and UDP-glucuronyl transferase [9–12] have their activities modulated by in vivo or in vitro manipulation of lipid composition.

The present chapter will focus on work done on UDPGT, an enzyme whose activity is strongly affected by the lipid environment. The effect of in vivo (dietary) cholesterol incorporation, as well as that of in vitro cholesterol incorporation and depletion, and the effect of changes in acyl chain degree of unsaturation in guinea pig liver microsomes have been analysed from the point of view of: (1) enzyme kinetics; (2) bulk lipid properties, by means of fluorescent probes; (3) lipid–protein interactions, by means of spin label probes.

2. *Effect of cholesterol and acyl chain degree of unsaturation on the kinetic properties of UDP-glucuronyl transferase*

UDP-glucuronyl transferases [13] are members of a family of isozymes, present in the endoplasmic reticulum of many tissues, that play a fundamental role in detoxication processes by conjugating metabolites of a variety of endogenous and xenobiotic compounds to glucuronic acid in order to increase their solubility and facilitate elimination. The main organ where glucuronidation takes place is the liver.

Some UDPGT isoforms have already been purified and characterized. They were seen to consist of a single polypeptide chain with molecular weight ranging from 50000 to 56000 [13]. Cloning and expression of a few isozymes, among

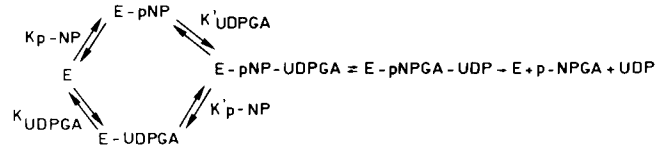

Fig. 1. Postulated reaction mechanism for UDP-glucuronyl transferase. K_{pNP} and K_{UDPGA} correspond to the dissociation constants for the binary complex. K'_{pNP} and K'_{UDPGA} correspond to the dissociation constants for the ternary complex. From Castuma and Brenner (1986) [11].

them, that which catalyses the glucuronidation of *p*-nitrophenol [14,15], has also been achieved. Analysis of the hydropathy profiles indicates that all UDPGTs contain hydrophobic signal sequences and a very hydrophobic stretch near the C-terminal region.

The combination of computer-based prediction and experimental work with proteases and antibodies leads to a model where only one transmembrane helical segment near the carboxy-terminal is present. The active site is located on the luminal side of the endoplasmic reticulum [13].

UDP-glucuronyl transferase catalyses the transfer of glucuronic acid from UDP-glucuronic acid to a second substrate (containing hydroxyl, carbonyl and amino groups) by a random ordered sequential reaction [16] (Fig. 1). In the figure, the second substrate is *p*-nitrophenol. UDPGT kinetic properties are modulated by lipid composition.

Castuma and Brenner have examined both the effect of cholesterol [10, 11] and of the degree of fatty acid unsaturation [9, 13] on the kinetics of UDP-glucuronyl transferase in microsomal membranes of guinea pig liver.

2.1. *Effect of in vivo modification of cholesterol content*

The cholesterol content in microsomes was altered in vivo by administering a cholesterol-rich synthetic diet over a 25-day period [10]. This procedure led to an approximate duplication of the sterol content (Table 1). Dietary cholesterol led to additional changes: a slight relative decrease of PC and a more pronounced decrease of PE caused a change in the PE/PC molar ratio (Table 1).

An examination of fatty acid composition indicated only slight variations in linoleic, palmitoleic and arachidonic acids. Moreover, the distribution of fatty acids in PC, PE, and PI was also essentially unaffected.

To study the effect of cholesterol on the different steps of the UDPGT reaction (Fig. 1), initial rates were measured as a function of variable UDPGA concentrations at several fixed *p*-NP concentrations, and vice-versa [10]. Double reciprocal plots of the results indicated that Km depends on the concentrations of the second substrate. Secondary plots of the intercept on the $1/V$ axis versus

TABLE 1
Effect of in vivo cholesterol incorporation on the lipid composition of guinea-pig liver microsomes (weight%)[a]

Lipids	Normal	Normal+cholesterol
Cholesterol	13.4±0.4	28.7±2.3
Cholesterol esters	2.8±0.1	4.6±0.8
Triacylglycerol	3.6±0.2	3.9±0.9
Phosphatidylcholine	47.8±1.8	41.5±1.9
Phosphatidylethanolamine	25.3±0.4	13.7±0.5
Phosphatidylinositol	7.1±0.1	7.6±0.6
Double bond index/saturated fatty acid[b]	2.97±0.03	3.04±0.04
Cholesterol/phosphatidylcholine (mol/mol)	0.31±0.10	0.46±0.08
Phosphatidylethanolamine/phosphatidylcholine (mol/mol)	0.57±0.04	0.36±0.06

[a] Results are the mean±SE of five animals analysed separately. Data from Castuma and Brenner [12].
[b] Double bond index/saturated fatty acid = Σ(number unsaturated mol × number double bond)/Σ(number saturated mol).

TABLE 2
Kinetic constants for the forward reaction of UDPGT in guinea pig liver microsomes[a]

Microsomes	K_{UDPGA} (mM)	K'_{UDPGA} (mM)	K_{pNP} (mM)	K'_{pNP} (mM)	V_{max}[b]	Hill coeff.
In vivo studies[c]						
Normal	12.3±0.4	10.6±0.2	0.12±0.03	0.10±0.01	10.5±1.1	0.40±0.02
Cholesterol-enriched	8.1±0.3	7.2±0.1	0.07±0.01	0.06±0.01	15.9±1.3	0.68±0.03
In vitro studies[d]						
Normal	12.0±0.20	10.3±0.30	0.17±0.01	0.12±0.01	10.1±1.3	0.46±0.03
Cholesterol-enriched	8.9±0.12	6.2±0.11	0.10±0.02	0.08±0.02	14.3±1.2	0.74±0.01
Cholesterol-depleted	13.1±0.20	10.9±0.1	0.20±0.01	0.1±0.01	8.7±0.8	0.38±0.01

[a] Results are the mean of five experiments±SE.
[b] In nmol min^{-1} mg $protein^{-1}$.
[c] Data from Castuma and Brenner [10].
[d] Data from Castuma and Brenner [11].

1/(concentration of the fixed substrate) yield $1/V_{max}$ and a second K_m' for each substrate. K_{mUDPGA} and K_{mpNP} are dissociation constants of binary enzyme-substrate complexes, while K'_{mUDPGA} and K'_{mpNP} are dissociation constants of the ternary complex [16].

Table 2 summarizes the kinetic measurements. It shows that the cholesterol diet led to an increase of the affinity for all the reaction steps, as well as an

TABLE 3
Cholesterol and phospholipid content of in vitro modified guinea pig microsomes[a]

Microsomes	Phospholipid/protein (μmol/mg protein)	Cholesterol/protein (μmol/mg protein)	Cholesterol/phospholipid molar ratio
Normal	0.38±0.02	0.14±0.01	0.368
Cholesterol-enriched	0.39±0.01	0.20±0.02	0.513
Cholesterol-depleted	0.37±0.02	0.10±0.02	0.270

[a] Results are the mean of five experiments±SE. Data from Castuma and Brenner [11].

increase of the enzyme specific activity. UDPGT from normal microsomes presented the typical non-Michaelis–Menten kinetics characteristic of this enzyme [17], displaying an apparent negative cooperativity of the enzyme for UDPGA. The cholesterol-rich diet led to an increase in the Hill coefficient, indicating a shift to michaelian kinetics. A similar effect was observed when animals were submitted to an essential fatty acid-deficient diet [9] (see below).

2.2. *Effect of in vitro modification of cholesterol content*

In order to examine the effect of cholesterol on the kinetics of UDPGT without modifying the phospholipid composition, an in vitro technique [5, 11] was employed that consisted of preparing liposomes of the extracted microsomal lipid containing either only the phospholipids or phospholipids plus cholesterol, and incubating these liposomes with normal microsomes in the presence of the 12 000 g supernatant of guinea pig liver homogenates, which contains lipid transfer proteins [18]. With this procedure, the cholesterol content of normal microsomes was either decreased or increased (Table 3), without modifying the remaining lipid composition.

The kinetic parameters for the UDPGT reaction of these systems are given in Table 2. The results for the cholesterol-enriched microsomes are similar to those found when cholesterol was incorporated in vivo. In contrast, cholesterol depletion led to a decrease of the Hill coefficient.

2.3. *Effect of in vivo modification of acyl chain degree of unsaturation*

A change in the degree of fatty acid unsaturation without altering the head group composition and the cholesterol/phospholipid mole ratio was accomplished by feeding guinea pigs a fat-deficient diet where glucose substituted for corn oil [9, 12]. Table 4 gives the lipid composition of normal and fat-deficient liver microsomes while Tables 5 and 6 give the fatty acid composition of total lipids and phospholipids (Table 5) and of sphingomyelin (Table 6). While essentially

TABLE 4
Lipid distribution of normal and fat-deficient guinea pig liver microsomes[a]

Lipid	Proportion of lipid (g/100 g total)	
	Normal	Fat-deficient
Cholesterol	9.5±0.4	9.2±0.3
Cholesterol esters	1.1±0.1	1.1±0.1
Triacylglycerols	2.6±0.2	2.5±0.2
Phosphatidylcholine	46.5±2.7	46.9±2.7
Phosphatidylethanolamine	24.9±1.6	25.3±1.4
Phosphatidylinositol	7.8±0.3	7.4±0.3
Phosphatidylserine	3.3±0.2	3.5±0.1
Sphingomyelin	4.3±0.2	4.1±0.2
Cholesterol/phospholipid (mol/mol)	0.31±0.02	0.30±0.01

[a] Results are the means±SE of four independent experiments run in duplicate. The statistical significance was evaluated by the Student's *t* test. Data from Castuma and Brenner [12].

no change is observed in the distribution pattern of neutral and polar lipids from normal and fat-deficient microsomes (Table 4), a significant decrease in linoleic acid and a less marked decline of arachidonic and docosahexenoic acids, together with a great increase in the mono-unsaturated fatty acids, especially oleic, is observed for the fat-deficient microsomes (Tables 5 and 6). The double bond index is seen to significantly decrease for all classes of phospholipids (Table 5) and sphingomyelin (Table 6).

When the activity of UDPGT was examined, an increase in the Hill coefficient, towards michaelian kinetics, was observed for the fat-deficient microsomes [9]. The Hill coefficient increased from 0.39 for controls to 0.98 for animals fed a fat-deficient diet for twenty one days.

Arrhenius plots for UDPGT of normal and fat-deficient microsomes are given in Fig. 2 [12]. Both systems show two linear regions intersecting with a sharp transition (at 23.5°C for normal and at 18.3°C for fat-deficient microsomes). The activation energies E_a below and above the transition temperatures were 28 and 71 kJ/mole, respectively for normal microsomes, in agreement with the results of Pechey et al. [19]. For fat deficient microsomes the E_a values were 32 and 71 kJ/mole, respectively.

Both cholesterol addition and a decrease of fatty acyl chain unsaturation are known to cause an increase in bilayer lipid packing. In the studies of microsomal UDPGT activity both had similar effects on the enzyme kinetics, namely a switch from non-michaelian to michaelian.

TABLE 5
Fatty acid distribution of total lipids and individual phospholipids from normal and fat-deficient microsomes[a]

Lipid[b]	Source	Proportion of fatty acid (g/100 g total)										DBI
		$C_{14:0}$	$C_{16:0}$	$C_{16:1}$	$C_{18:0}$	$C_{18:1}$	$C_{18:2}$	$C_{20:3\omega6}$	$C_{20:3\omega9}$	$C_{20:4\omega6}$	$C_{22:5\omega3}$	$C_{22:6\omega3}$	
Total	Normal	0.9±0.2	14.1±0.9	1.2±0.2	26.1±1.3	9.8±0.6	34.5±1.2	1.0±0.1	0.6±0.1	9.4±0.4	0.8±0.1	1.6±0.2	1.30±0.05
	Fat-def	1.4±0.3	18.4±1.1	2.6±0.3[e]	25.3±1.3	21.7±0.9[e]	19.5±0.8[e]	1.2±0.2	0.7±0.1	7.5±0.3[e]	1.0±0.1	0.7±0.1[e]	1.07±0.02
PC	Normal	0.3±0.1	13.9±0.5	0.3±0.1	28.3±1.4	9.1±0.3	42.7±1.4	0.3±0.1	0.5±0.1	3.8±0.2	0.2±0.1	0.6±0.1	1.14±0.02
	Fat-def	0.5±0.1	15.1±0.7	1.1±0.2[e]	31.6±1.8	10.9±0.6	36.2±1.3[e]	0.5±0.1	0.4±0.1	2.9±0.1[d]	0.3±0.1	0.5±0.1	1.00±0.01
PE	Normal	0.6±0.1	9.4±0.8	1.2±0.1	28.9±1.8	8.4±0.4	32.6±1.7	0.5±0.1	0.8±0.2	13.2±0.5	1.6±0.7	2.8±0.2	1.51±0.06
	Fat-def	1.2±0.1[d]	12.3±0.6	2.4±0.3[e]	33.3±1.9	12.6±0.6[e]	23.9±1.1[d]	1.2±0.1[d]	0.5±0.1	9.6±0.3[e]	1.3±0.1	1.7±0.1[d]	1.26±0.03
PI	Normal	0.8±0.1	6.7±0.3	1.3±0.1	39.6±1.7	10.3±0.5	19.4±0.8	2.0±0.1	2.9±0.2	16.2±0.7	0.5±0.1	0.3±0.1	1.30±0.05
	Fat-def	1.3±0.3	7.5±0.5	2.2±0.2[e]	43.3±1.9	13.6±0.8[e]	10.9±0.5[e]	3.7±0.2[e]	3.0±0.1	14.0±0.6	0.3±0.1	0.2±0.1	1.03±0.02[e]
PS	Normal	1.5±0.1	7.2±0.3	1.3±0.1	37.8±1.8	9.2±0.6	27.7±1.3	1.1±0.1	1.5±0.2	10.2±0.6	1.3±0.1	1.2±0.1	1.22±0.02
	Fat-def	2.1±0.4	8.3±0.4	1.6±0.1	43.2±2.1	13.0±0.7[d]	19.3±0.9	1.5±0.1	2.1±0.2	6.7±0.3[e]	1.2±0.1	1.0±0.1	1.00±0.04

[a] Results are the means±SEM for five independent experiments run in duplicate. Data from Castuma and Brenner [12].
[b] Fat-def, fat-deficient.
[c,e] Statistical significance evaluated by Student's *t* test: (c) $P < 0.001$; (d) $P < 0.005$; (e) $P < 0.01$.

TABLE 6
Fatty acid distribution of sphingomyelin from normal and fat-deficient microsomes[a]

Source	Proportion of fatty acid (g/100 g total)									DBI
	$C_{14:0}$	$C_{16:0}$	$C_{16:1}$	$C_{18:0}$	$C_{18:1}$	$C_{18:2}$	$C_{20:0}$	$C_{20:1}$	$C_{20:2}$	$C_{22:0}$	$C_{22:2}$	$C_{24:0}$	$C_{24:1}$	
Normal	1.4±0.1	10.8±0.5	1.4±0.1	30.7±1.4	9.1±0.4	17.3±0.8	1.2±0.1	0.7±0.1	2.5±0.2	19.3±0.9	1.8±0.1	2.2±0.2	1.6±0.1	0.67±0.002
Fat-def	1.6±0.1	11.3±0.6	2.0±0.2	33.8±1.5	9.3±0.5	8.9±0.4[b]	1.6±0.2	0.9±0.1	3.0±0.1	21.2±1.2	2.0±0.1	2.6±0.1	1.8±0.1	0.51±0.001

[a] Results are the means±SEM for five independent experiments run in duplicate. Data from Castuma and Brenner [12].
[b] Statistical significance evaluated by Student's *t* test: $P < 0.001$.

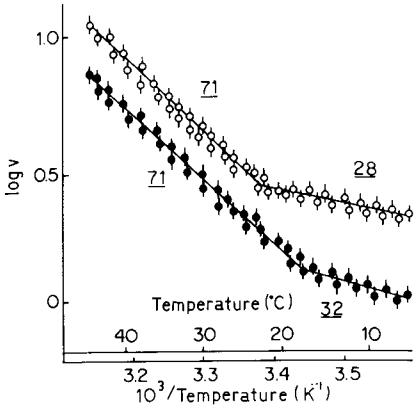

Fig. 2. Arrhenius plot of UDP-glucuronyl transferase activity in normal (open circles) and fat-deficient (solid circles) microsomes. The values underlined are the activation energies, E_0 (kJ/mole). Adapted from Castuma and Brenner (1989) [12].

2.4. *Significance of the kinetic data*

Vessey and Zakim [20] demonstrated that UDPGT displays negative homotropic cooperativity toward its natural substrate, UDPGA. The kinetic properties of the enzyme have been found to be modulated by the lipid composition [21–23] and packing [22,24–26]. A specific requirement for choline phospholipids has been demonstrated [21–23]. Moreover, isolated and delipidated UDPGT from pig liver presents non-michaelian kinetics when reconstituted with gel phase PC unilamellar vesicles, and a switch to michaelian kinetics was observed upon going from the gel to the liquid crystalline phase [26]. In addition, Hochman and Zakim [25] have reported that UDPGT is in a gel phase lipid environment at 37°C in pig liver microsomes.

It has been proposed that changes in the Hill coefficient of membrane-bound enzymes reflect changes in membrane organization and in protein–lipid interaction [27].

The results in Table 2 indicate that lipid composition manipulation affects the kinetic behaviour of guinea pig liver microsomal UDPGT. Procedures that lead to an increased packing of bulk lipids such as cholesterol increase – in vivo and in vitro – as well as a decrease in acyl chain degree of unsaturation promote a shift to non-michaelian kinetics. In contrast, cholesterol depletion causes a decrease in the Hill coefficient. This would mean that the lipids in the immediate vicinity of the enzyme change from a more gel-like to a more liquid crystalline state in the former case, whereas the opposite occurs in the latter case. Therefore, the effect of lipid composition manipulation upon the enzyme environment seems to be opposite to that in the bilayer region.

Changes in the cooperative behaviour of membrane-bound enzymes due to essential fatty acid deficiency [28–30], cholesterol administration [31], and hormonal treatment [32], have been reported. The free energy change for an allosteric change has been proposed to be of the order of 1–3 kcal/mole [33]. Siñeriz et al. [34] estimated that a variation in the interaction energy as low as 700 cal/mole would be enough to cause a significant change in the Hill coefficient.

In variable temperature studies, breaks in Arrhenius plots have been correlated both with phase transition [35] and with phase separation [36] of the bulk lipid. However, the non-linearity of enzyme activity as a function of temperature has also been ascribed to phase separations or phase transitions in the surrounding lipid [37]. The shift to lower temperature (Fig. 2) of the break in the enzyme activity profile of fat-deficient microsomes is consistent with a decrease in packing of the neighbouring lipids, concomitantly with a tighter arrangement of the bulk lipids (section 3).

The picture that emerges from the kinetic studies in conjunction with the physical studies of the bulk bilayer properties by fluorescence spectroscopy (section 3) is corroborated by spin label studies of microsomal membranes that reveal that the ESR spectra due to motionally restricted lipid in cholesterol-enriched microsomes are indicative of a greater degree of motion than that found in normal microsomes (section 4).

3. *Fluorescence studies of bilayer properties in normal and modified microsomes, and in extracted lipids*

3.1. *Spectral behaviour of fluorescent probes*

DPH, pyrene, *trans*-parinaric acid and merocyanine 540 were used to monitor the effects of cholesterol addition and depletion, and the decrease of acyl chain unsaturation upon the rotational (DPH) [38] and translational (pyrene) [39] mobility of lipids in microsomal membranes and in total lipid and phospholipid extracts. *Trans*-parinaric acid and merocyanine 540 are sensitive to the presence of gel phase lipid in a liquid crystalline system (lateral phase separation) [40] and to the degree of lipid packing [41], respectively.

Table 7 shows the effect of in vivo cholesterol enrichment on the following properties of DPH incorporated in microsomes: fluorescence anisotropy r_s [42], microviscosity η [38], and order parameters S_{DPH} [42]. The decrease in rotational mobility upon addition of cholesterol was accompanied by a decrease in translational mobility, as indicated by a decrease in the I_E/I_M ratio in pyrene fluorescence spectra (where I stands for intensity and the subscripts E and M for excimer and monomer, respectively).

TABLE 7
Effect of in vivo cholesterol incorporation on acyl chain degree of unsaturation and DPH properties derived from fluorescence spectra[a]

Microsomes	Double bond index/Saturated fatty acid	Fluorescence anisotropy r_s	Microviscosity η	Order parameter S_{DPH}
Normal	2.97±0.03	0.112±0.004	1.14	0.392
Cholesterol-enriched	3.04±0.04	0.131±0.003	1.36	0.452

[a] Results are the mean±SE of five animals analysed separately; fluorescence experiments were carried out at 37°C. Data from Castuma and Brenner [10].

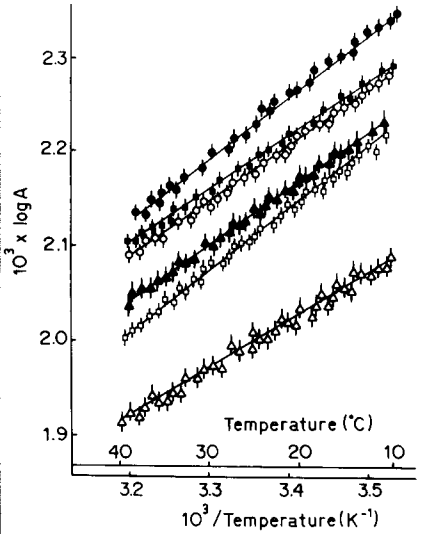

Fig. 3. Fluorescence anisotropy A of DPPH as a function of temperature in microsomes (circles) extracted total lipids (squares), and extracted total phospholipids (triangles). Open (closed) symbols correspond to normal (fat-deficient) microsomes. Adapted from Castuma and Brenner (1989) [12].

A decrease (0.102±0.003) and increase (0.154±0.001) in fluorescence anisotropy of DHP were observed for in vitro cholesterol-depleted and enriched microsomes, respectively when compared to normal (0.112±0.002). Likewise, the slope of the I_E/I_M ratio versus pyrene concentration increased (0.028) and decreased (0.017) for cholesterol-depleted and enriched microsomes, respectively, when compared to normal (0.025).

Fig. 3 displays the thermotropic properties of normal and fat-deficient microsomes, total lipids and phospholipids labelled with DPH. The straight lines do not suggest the occurrence of a phase transition. The higher anisotropy for

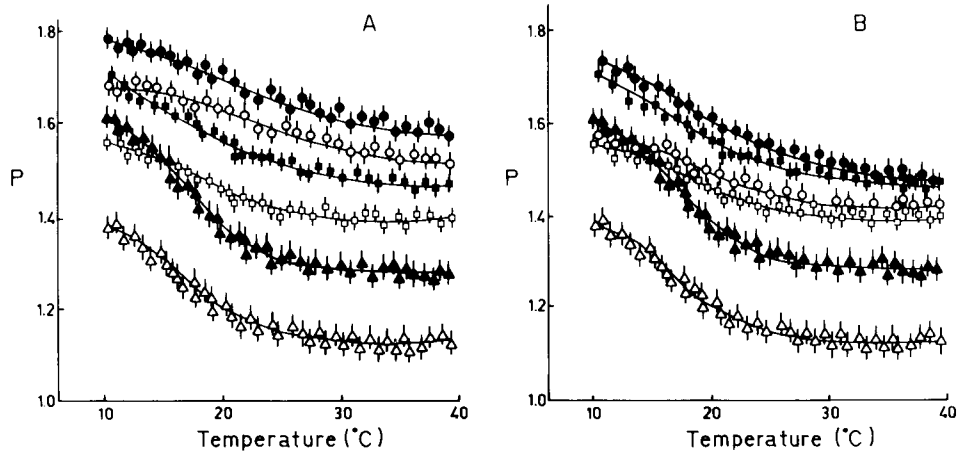

Fig. 4. (A) Polarization ratio P for *trans*-parinaric acid as a function of temperature in microsomes (circles), extracted total lipids (squares) and extracted phospholipids (triangles). Open (closed) symbols correspond to normal (fat-deficient) microsomes. (B) Polarization ratio P for *trans*-parinaric acid as a function of temperature upon cholesterol addition (in the same amount as found in the original membrane) to total phospholipids from normal (open circles) and fat-deficient (solid circles) microsomes. The other symbols are the same as in (A). Adapted from Castuma and Brenner (1989) [12].

the microsomal preparations is indicative of membrane proteins playing a role in controlling the physical state of lipids [43]. Similarly, the higher anisotropy for total lipids is an indication of the rigidizing effect of cholesterol. These results are in accordance with those of Garda and Brenner [5] for rat liver microsomes. Finally, the fluorescence anisotropy of DHP in fat-deficient systems is always higher than that of normal ones, indicating a decrease of rotational mobility in the less unsaturated environment.

In agreement with these results, the lower I_E/I_M ratio in the pyrene fluorescence of fat-deficient systems indicates a decreased translational mobility caused by the decrease in the degree of acyl chain unsaturation.

Trans-parinaric acid shows a preference for gel phase lipids. The polarization ratio P, obtained from fluorescence spectra of this probe increases rapidly with the appearance of a small percentage of gel phase lipid in a liquid crystalline system.

Fig. 4A displays the P values as a function of temperature for *trans*-parinaric acid in microsomes, total lipid and total phospholipid extracts. An abrupt change in P is observed for the phospholipid dispersions, suggesting the occurrence of lateral phase separation. The midpoints of these transitions were 19°C and 23°C for dispersions originating from normal and fat-deficient animals, respectively. In contrast, the change in P is relatively smooth for total lipids and

microsomes. That cholesterol is at least partly responsible for the latter result is demonstrated by adding the sterol to the extracted phospholipids. Fig. 4B shows that, upon addition of cholesterol, the systems display a behaviour very similar to that of the total lipids.

The fluorescence intensity of merocyanine 540 increases with the decreased degree of lipid packing in membranes. When the probe was incorporated in microsomes and phospholipid extracts of normal and fat-deficient animals, the results were in agreement with those obtained with the other fluorescent labels, revealing a tighter packing of the acyl chains in membranes from fat-deficient animals. In addition, a phase transition was also detected at about 20–22°C for the phospholipid dispersions. A phase transition was also sensed by *trans*-parinaric acid and merocyanine 540 in bilayers prepared with the phosphatidylcholines from both normal and fat-deficient liver microsomes. The midpoints of these transitions were 19°C and 26°C, respectively.

3.2. Significance of the fluorescence data

The above results indicate that all probes used to examine the motional and organizational properties of the bulk bilayer region of normal and modified microsomes and of dispersions prepared from total lipids or from phospholipids gave results (Table 7) consistent with the known effects of cholesterol upon bilayer packing [44].

Several reviews have pointed at the lack of a simple correlation between acyl chain degree of unsaturation and membrane lipid packing [45, 46]. Nevertheless, the present data (Figs. 3, 4) clearly indicate an increase in fluorescence anisotropy in systems derived from fat-deficient animals.

The higher degree of organization caused by increasing cholesterol and decreasing degree of acyl chain unsaturation was verified in all preparations investigated. Nevertheless, the kinetic data suggested that the lipid surrounding the UDPGT displayed a behaviour opposite to that of the bulk lipid (section 2). In order to analyse the properties of the lipid interacting directly with protein, spin label experiments were performed (section 4).

4. Spin label study of the effect of cholesterol on lipid–protein interactions in microsomal membranes

4.1. Spectral behaviour of spin label probes

The cholesterol modulation of lipid–protein interactions in microsomal membranes was examined making use of spin labeling [47], a long time established technique for this purpose [48,49].

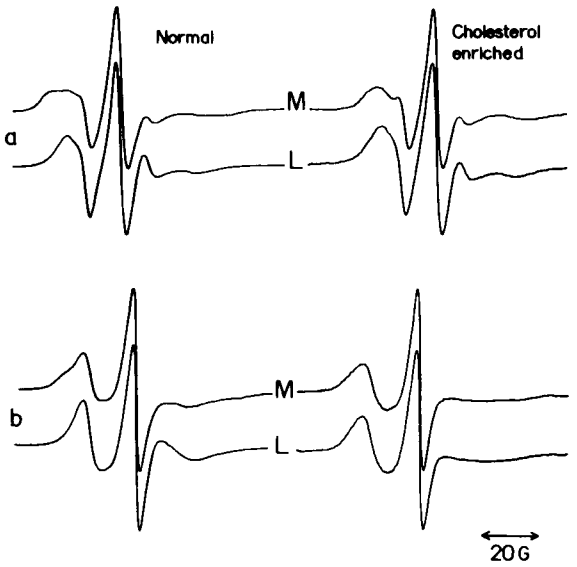

Fig. 5. EPR spectra of (a) 12-SASL and (b) ASL in normal and cholesterol enriched microsomes (M) and their extracted lipids (L). From Castuma et al. (1991) [47].

Guinea pig liver microsomal membranes containing 18 mole% cholesterol were enriched by the in vitro technique [5, 11], yielding a sterol content of 33 mole%. The procedure did not cause loss of membrane integrity as indicated by measurements of mannose-6-phosphatase latency [50].

The normal and cholesterol-enriched microsomes and their extracted lipids were spin labeled with 5-,12- and 16-SASL and ASL, and their spectra examined as a function of temperature. Spectral subtractions were only performed with 12-SASL and ASL.

The ESR spectra of 12-SASL and ASL in normal microsomes (Fig. 5) revealed the usual two-component spectra, one corresponding to the fluid bilayer – which is similar to the spectrum obtained for liposomes prepared from the extracted lipids – and another indicating the existence of a motionally restricted lipid population, which is resolved in the outer wings of the spectrum.

Fig. 5 displays the spectra of 12-SASL and ASL in normal and cholesterol-enriched microsomes and in dispersions of their extracted lipids at 4°C. While the two components are clearly seen in normal microsomes, in the cholesterol-enriched membranes their resolution is less clear in the spectra of 12-SASL and is essentially lost in the spectra of ASL, suggesting a greater mobility for the motionally restricted population. Nevertheless, spectral deconvolution provided clear evidence that both kinds of microsomes gave rise to two-component spectra in the whole range of temperature studied.

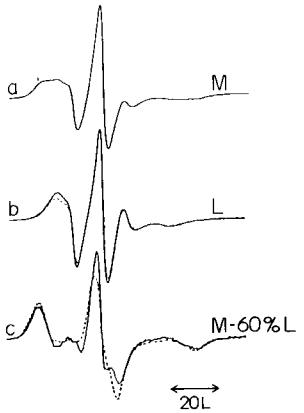

Fig. 6. Spectral deconvolution of 12-SASL EPR spectra in normal microsomes at 4°C. Normal microsomes (a, M). The full lines correspond to the extracted lipids (b, L) and to M minus 60% L (c). This subtraction yields the spectrum corresponding to the motionally restricted lipids. The spectrum of egg PC-50 mole% cholesterol at 28°C (dashed line, c) was chosen from a library to represent the motionally restricted component. Subtraction of this spectrum from M yields that corresponding to fluid bilayer lipids (dashed line, c). From Castuma et al. (1991) [47].

Spectral subtractions were performed according to procedures described in the literature [51,52]. Two procedures were used: either the spectrum due to the bulk lipid (at the appropriate (lower) temperature), or that of a motionally restricted system was subtracted from the microsome spectrum. In this latter case, a library was constructed with a variety of lipid systems and the spectra were chosen so as to match the outer extrema of the experimental spectrum. Fig. 6 illustrates the analysis of an experimental spectrum, using both subtraction procedures described above. The fraction f of the motionally restricted component was found to be 0.42 ± 0.05 for both 12-SASL and ASL, in both normal and cholesterol-enriched microsomes. In addition, f was temperature-independent.

The motionally restricted components obtained by spectral subtraction where analysed in terms of an effective order parameter, S_{eff} [47]. These values can only be considered as apparent, since their derivation assumes the fast motional limit, and the spectra obtained for the motionally restricted lipids probably contain slow molecular motions.

The temperature dependence of S_{eff} calculated for 12-SASL spectra of the bilayer and motionally restricted lipid of normal and cholesterol-enriched microsomes is presented in Fig. 7. While S_{eff} of the bulk lipids increases upon cholesterol enrichment, that of the immobilized population decreases. Arrhenius plots of the data in Fig. 7 (not shown) display no breaks, indicating the absence of a phase transition.

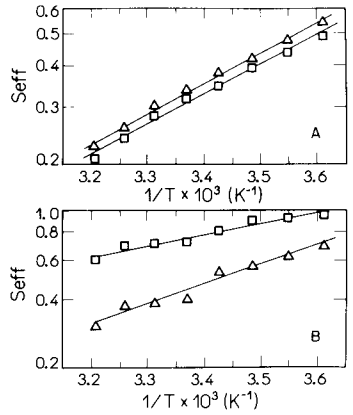

Fig. 7. Temperature dependence of S_{eff} values for (A) fluid and (B) motionally restricted spectral components displayed by 12-SASL in normal (squares) and cholesterol-enriched (triangles) microsomes. From Castuma et al. (1991) [47].

Effective correlation times, τ_{eff}, for the more immobilized population were calculated according to Freed [53], and for the bilayer lipid according to Schreier et al. [54]. The data were in agreement with the results obtained for S_{eff}. The motionally restricted lipids in the cholesterol-enriched membranes displayed small τ_{eff} values, in the limit of the applicability of the Freed equation.

τ_{eff} values vary between 4×10^{-9} and 1×10^{-9} s for the fluid component, whereas for the motionally restricted one, τ_{eff} values lie between 10^{-7}–10^{-8} s and $(15\text{–}3) \times 10^{-9}$ s, for the native and cholesterol-enriched systems, respectively, in the temperature range examined. The subtracted spectra obtained at higher temperatures are probably affected by the slow exchange between the two label populations [51, 52, 55].

Since spectral subtraction of 5-SASL spectra involves a high degree of inaccuracy due to the similarity of lineshapes for the total and the bilayer lipid spectrum, the spectra of this probe were analysed in terms of the separation D between the outer extrema. As expected, D decreased in going from microsomes to extracted lipids and, for each system, it decreased with increasing temperature. In addition, D values were higher for cholesterol-enriched microsomes and lipid extracts than for their normal counter-parts, indicating that the spectrum due to bulk lipid predominates over that of the motionally restricted population. This is in agreement with the fraction of this population determined from spectral deconvolution.

Although two-component spectra were obtained for 16-SASL, no satisfactory subtractions could be performed. The spectra were analysed making use of the empirical ratio h_{+1}/h_0 where h_{+1} and h_0 represent the peak-to-peak heights of the low and mid-field resonances, respectively. When normal and cholesterol-

enriched membranes were compared to the lipid extracts, an increase in h_{+1}/h_0 was observed for the latter, indicating an increase in mobility. As with previous results, Arrhenius plots of these data gave no indication of significant breaks.

4.2. *Significance of spin label data*

While most EPR studies have been performed with purified enzymes reconstituted with lipids of defined composition [48] only a few papers have dealt with heterogeneous natural membranes [56, 57].

The above results provide evidence for cholesterol modulation of lipid–protein interactions in microsomal membranes. It is seen that cholesterol enrichment does not affect the fraction of motionally restricted lipid, suggesting that the energies involving phospholipid–protein and phospholipid–cholesterol interactions are of similar magnitude. Moreover, in contrast with the well-known rigidifying effect of the sterol on liquid crystalline bilayers, the molecule induces an enhancement of the mobility of the less mobile label population.

The fact that the fraction of constrained lipids remains constant over a wide temperature range, and the occurrence of exchange between the two label populations seen by EPR (as suggested by the need to subtract spectra due to bilayer lipid at lower temperatures, Fig. 5) seem to indicate that the spectra due to motionally restricted lipid correspond to annular lipids and not to lipid trapped between proteins [58]. Furthermore, the data are not suggestive of temperature-induced protein aggregation.

Further analysis of the data is done in section 5.

5. *Possible models for the effect of cholesterol and acyl chain unsaturation on lipid–protein interaction*

A fundamental question about the organization of membrane components refers to how the hydrophobic transmembrane segments of proteins are interfaced with bilayer lipids. A general view regards the protein as surrounded by a somewhat spatially disordered lipid arrangement, superimposed with an on-off exchange between boundary lipid and the usually liquid crystalline lipid bilayer [48,49].

In a large number of cases where membrane-bound enzymes were investigated, the enzyme activity has been found to depend on lipid composition [59]. Whether the observed effects were due to changes in the bulk bilayer properties or whether they were a consequence of specific changes in the immediate microenvironment of the protein, or both, is not always clear.

In the case of the microsomal membrane, UDPGT was found to have its kinetic properties altered by modifying either the cholesterol content

(sections 2.1, 2.2), or the fatty acid degree of unsaturation (section 2.3). The latter finding is in contrast with reports where a correlation between degree of unsaturation and enzyme activity has not been found [60].

A direct examination of the influence of acyl chain degree of unsaturation on lipid–protein interactions was not performed. However, the data on enzyme kinetics (Hill coefficients and temperature effects; Fig. 2, section 2.3), in conjunction with the fluorescence studies of bulk bilayer properties (Figs. 3, 4) strongly suggest that, in fat-deficient microsomes, while the bilayer undergoes a rigidifying process, the enzyme behaves as if it had been placed in a less tightly packed environment.

As for cholesterol, the spin label studies (section 4) provide a clear evidence for a role of the sterol on lipid–protein interactions in the microsomal membrane. The trends are similar to those observed for fatty acid unsaturation, namely, while increasing bilayer packing, cholesterol increases the mobility of boundary lipid (Figs. 5–7). These results are in agreement with those found by fluorescence measurements (sections 2.1, 2.2, Table 7).

Whether cholesterol participates directly in lipid–protein interactions is a controversial matter. There have been conflicting results in the literature regarding the involvement of cholesterol in lipid–protein interactions [61–63], and particularly in membrane protein functions (for a review, see Yeagle [44]). The sterol has been found to stimulate, inhibit, or not affect a number of protein-mediated activities.

Several hypotheses could be proposed to explain the present results. (1) Cholesterol might segregate unsaturated PC's towards the UDGPT micro-environment. This would be a consequence of the greater affinity of the sterol for saturated PC's. That this is the case in lipid bilayers has been demonstrated in several reports [64–67]. (2) Cholesterol might increase the exchange rate between boundary and bulk bilayer lipid [52]. Spectral simulations were performed considering this possibility [51,55]. No match with the experimentally obtained EPR spectra could be achieved, indicating the unlikelihood of this possibility. (3) A third possibility would be based on cholesterol being able to participate in lipid–protein interactions. If the protein environment consisted of gel phase-like lipids, as has been proposed [26], an increase in cholesterol content could lead to an increase in boundary lipid mobility, in agreement with the well-known effect of cholesterol on gel phase phospholipids [68].

6. *Concluding remarks*

The results found in the work with the microsomal membrane seem to indicate that manipulation of the lipid composition, whether by altering cholesterol content or by changing the degree of acyl chain unsaturation, affects not only

the motional and organizational properties of the bulk bilayer lipids, but also leads to changes in the composition of boundary lipids.This change would be responsible for the observed mobility changes of those lipids, which, in turn, would exert a modulating effect upon protein function.

In both cases where lipid composition was manipulated, procedures that led to an increase in bulk bilayer lipid packing, brought about an opposite effect on the boundary lipid, namely, an increase in the mobility of lipids in the protein microenvironment. The reasons for this pattern are still unknown. Although the exchange between the two lipid environments is slow in the EPR time scale, it is conceivable that the interplay between them leads to an averaging of their properties, giving rise to a range of possible packing states adequate for enzyme functioning.

It is noteworthy that, in spite of the complexity inherent to biological membranes, a considerable amount of information can be obtained at a molecular level about lipid–protein interactions in these membranes.

The increasing knowledge of the detailed architecture of protein transmembrane fragments points at the possibility of examining specific interactions between lipids and individual aminoacids which will help in the recognition of hydrophobic faces of those fragments, and, more specifically, those in contact with lipid components.

Acknowledgements

The financial support of CNPq, FAPESP, FINEP (Brazil) and CONICET (Argentina) is gratefully acknowledged. We thank Ms. Elisety de Andrade Silva and Mrs. Augusta Paes for typing the manuscript.

References

[1] Silvius, J.R., McMillen, D.A., Saley, N.D., Jost, P. C. and Griffith, O.H. (1984) Biochemistry 23, 538–547.
[2] Madden, T.D., Chapman, D. and Quinn, P.J. (1979) Nature 279, 538–540.
[3] Jones, O.T. and McNamee, M.G. (1988) Biochemistry 27, 2364–2374.
[4] Fong, T.M. and McNamee, M.G. (1986) Biochemistry 25, 830–840.
[5] Garda, H.A. and Brenner, R.R. (1985) Biochim. Biophys. Acta 819, 45–54.
[6] Leikin, A.I. and Brenner, R.R. (1987) Biochim. Biophys. Acta 922, 294–303.
[7] Leikin, A.I. and Brenner, R.R. (1988) Biochim. Biophys. Acta 963, 311–319.
[8] Leikin, A.I. and Brenner, R.R. (1989) Biochim. Biophys. Acta 1005, 187–191.
[9] Castuma, C.E. and Brenner, R.R. (1983) Biochim. Biophys. Acta 729, 9–16.
[10] Castuma, C.E. and Brenner, R.R. (1986) Biochim. Biophys. Acta 855, 231–242.
[11] Castuma, C.E. and Brenner, R.R. (1986) Biochemistry 25, 4733–4738.
[12] Castuma, C.E. and Brenner, R.R. (1989) Biochem. J. 258, 723–731.

[13] Burchell, B. and Coughtrie, M.W.H. (1989) Pharmac. Ther. 43, 261–289.
[14] Iyanagi, T., Haniu, M., Sogawa, K., Fujii-Kuriyama, Y., Watanabe, S., Shively, J.E. and Anan, K.F. (1986) J. Biol. Chem. 261, 15607–15614.
[15] Jackson, M.R., Fournel-Gigleux, S., Harding, D. and Burchell, B. (1988) Mol. Pharmacol. 34, 638–642.
[16] Vessey, D.A. and Zakim, D. (1971) J. Biol. Chem. 246, 4649–4656.
[17] Vessey, D.A. and Zakim, D. (1973) J. Biol. Chem. 253, 4652–4666.
[18] van Heusden, G.P.H. and Wirtz, K.W.A. (1984) J. Lipid Res. 25, 27–32.
[19] Pechey, D.T., Graham, A.B. and Wood, G.C. (1978) Biochem. J. 175, 115–124.
[20] Vessey, D.A. and Zakim, D. (1973) Biochim. Biophys. Acta 315, 43–48.
[21] Erikson, R.M., Zakim, D. and Vessey, D.A. (1978) Biochemistry 17, 3706–3711.
[22] Singh, O.M.P., Graham, A.B. and Wood, G.C. (1981) Eur. J. Biochem. 116, 311–316.
[23] Magdalou, J., Hochman, Y. and Zakim, D. (1982) J. Biol. Chem. 257, 13624–13629.
[24] Cremmings, J., Graham, A.B. and Wood, G.C. (1980) Biochem. J. 185, 521–526.
[25] Hochman, Y. and Zakim, D. (1983) J. Biol. Chem. 258, 11758–11762.
[26] Hochman, Y., Kelley, M., and Zakim, D. (1983) J. Biol. Chem. 258, 6509–6516.
[27] Morero, H., Siñeriz, F. and Farias, R.N. (1974) J. Biol. Chem. 249, 770l-7706.
[28] Bloj, B., Morero, R.D. and Farias, R.N. (1974) J. Nutr. 104, 1265–1271.
[29] Bloj, B. Morero, R.D., Farias, R.N. and Trucco, R.E. (1973) Biochim. Biophys Acta 311, 67–69.
[30] Goldemberg, A.L., Farias, R.N. and Trucco, R.E. (1972) J. Biol. Chem. 247, 4299–4304.
[31] Bloj, B., Morero, R.D. and Farias, R.N. (1973) FEBS Lett. 38, 101–105.
[32] Massa, E.M., Morero, R.D., Bloj, B. and Farias, R.N. (1975) Biochem. Biophys. Res. Commun. 66, 115–122.
[33] Wyman, J. (1963) Cold Spring Harbor Symp. Quant. Biol. 28, 483–488.
[34] Siñeriz, F., Farias, R.N. and Trucco, R.E. (1975) J. Theor. Biol. 52, 113–120.
[35] Danks, S.M. and Tribe, M.A. (1979) J. Therm. Biol. 4, 183–191.
[36] Houslay, M.D. and Palmer, R.W. (1978) Biochem. J. 174, 909–919.
[37] Brasitus, T.A. (1983) Biochim. Biophys. Acta 728, 20–30.
[38] Shinitzky, M. and Barenholz, Y. (1978) Biochim. Biophys. Acta 515, 367–394.
[39] Pownall, H.J. and Smith, L.C. (1973) J. Am. Chem. Soc. 95, 3136–3140.
[40] Sklar, L. (1980) Mol. Cell. Biochem. 32, 169–177.
[41] Williamson, P., Mattocks, K. and Schlegel, R.A. (1983) Biochim. Biophys. Acta 732, 387–393.
[42] Van Blitterswijk, N.J., van Hoeven, R.P., and van der Meer, B.W. (1981) Biochim. Biophys. Acta 644, 323–332.
[43] Pugh, E.L., Kates, M. and Szabo, A.G. (1980) Can. J. Biochem 58, 952–958.
[44] Yeagle, P.L. (1985) Biochem. Biophys. Acta 822, 267–287.
[45] Brenner, R.R. (1984) Prog. Lipid Res. 23, 69–96.
[46] Stubbs, C.D. and Smith, A.D. (1984) Biochim. Biophys. Acta 779, 89–137.
[47] Castuma, C.E., Brenner, R.R., De Lucca-Gattás, E.A., Schreier S. and Lamy-Freund, M.T. (1991) Biochemistry 30, 9492–9497.
[48] Marsh, D. and Watts, A. (1988) In: Lipid Domains and the Relationship to Membrane Function, pp. 163–200, Alan R. Liss, New York.
[49] Devaux, P.F. and Seigneuret, M. (1985) Biochim. Biophys. Acta 822, 63–125.
[50] Arion, W.J., Lange, A.J., and Walls, A.E. (1980) J. Biol.Chem. 255, 10387–10395.
[51] East, J.M., Melville, D. and Lee, A.G. (1985) Biochemistry 24, 2615–2623.
[52] Ryba, N.P.J., Horvath, L.I., Watts, A. and Marsh, D. (1987) Biochemistry 26, 3234–3240.
[53] Freed, J.H. (1976) in Spin Labeling, Theory and Applications (Berliner, L.j., Ed.) Vol. I, pp 53–132, Academic Press, New York.
[54] Schreier, S., Polnaszek, C.F. and Smith, I.C.P. (1978) Biochim. Biophys. Acta 515, 375–436.
[55] Davoust, G. and Devaux, P.F. (1982) J. Magn. Res. 48, 475–494.

[56] Bigelow, D.J., Squier, T.C. and Thomas, D.D. (1986) Biochemistry 25, 194–202.
[57] Li, G., Knowles, P.F., Murphy, D.J., Nishida, I. and Marsh, D. (1989) Biochemistry 28, 7446–7452.
[58] Hoffman, W., Pink, D.A., Restall, J. and Chapman, D. (1981) Eur. J. Biochem. 114, 585–589.
[59] Sandermann Jr., H. (1978) Biochim. Biophys. Acta 515, 209–237.
[60] Lee, A.G., East, J.M. and Froud, R.J. (1986) Prog. Lipid Res. 25, 41–46.
[61] Klapauf, E. and Schubert, D. (1977) FEBS Lett. 80, 423–425.
[62] Yeagle, P.L., Young, J. and Rice, D. (1988) Biochemistry 27, 6449–6452.
[63] Simmonds, A.C., Rooney, E.K. and Lee, A.G. (1984) Biochemistry 23, 1432–1441.
[64] Gruyer, W. and Bloch, K. (1983) Chem. Phys. Lipids 33, 313–322.
[65] Fugler, L., Clejan, S. and Bittman, R. (1985) J. Biol. Chem. 260, 4098–4102.
[66] Kusumi, A., Subczynski, W.K., Pasenkiewicz-Gierula, M., Hyde, J.S. and Mekrle, H. (1986) Biochim. Biophys. Acta 854, 307–317.
[67] Pasenkiewicz-Gierula, M., Subczynski, W. and Kusumi, A. (1990) Biochemistry 29, 4059–4069.
[68] Schreier-Muccillo, S., Marsh, D., Dugas, H., Schneider, H. and Smith, I.C.P., (1973) Chem. Phys. Lipids 10, 11–27.