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Effect of polyunsaturated fatty acid deficiency on dipole relaxation in the membrane interface of rat liver microsomes

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Abstract

The influence of a fat-free diet on the lipid dynamics of rat liver microsomes and liposomes of microsomal lipids was studied by using different fluorescence methods. Lifetime distribution and rotational diffusion of probes with different localization in the lipid bilayer were measured using multifrequency fluorometry. Lateral mobility was studied by measuring excimer formation of pyrenedodecanoic acid. Dipolar relaxation in the interfacial region was studied using 2-dimethylamino-6-lauroylnaphthalene (Laurdan). In spite of large changes in the fatty acid composition of microsomal lipids, polyunsaturated fatty acid deficiency showed no effect on the lifetime distribution and rotational mobility of 1,6-diphenyl-1,3,5-hexatriene (DPH), 1-(4-(trimethylamino)phenyl)-6-phenyl-1,3,5-hexatriene (TMA-DPH), 2-, 7- and 12-(9-anthroiloxy)stearic acids. The treatment did not change the lateral diffusion of pyrenedodecanoic acid, either. However, generalized polarization of Laurdan fluorescence was higher in polyunsaturated fatty acid deficient microsomes as compared to the polyunsaturated fatty acid sufficient ones. This effect was also observed in liposomes of the total microsomal lipids, indicating that the changes in fatty acid composition resulting from polyunsaturated fatty acid deficiency produced a small but significant decrease in the rate of dipolar relaxation in the region of the lipid polar groups of the bilayer. The absence of lipid gel phase domains in rat liver microsomes was also indicated by Laurdan fluorescence features.

Keywords: Essential fatty acid deficiency; Liver microsome; Lipid dynamics; Multifrequency fluorometry; Lifetime distribution; Rotational diffusion; Dipole relaxation; Lateral heterogeneity

1. Introduction

Although some authors have minimized the importance of polyunsaturaded fatty acids (PUFAs) for the structural properties of membranes [1], others have suggested that they could modify the conformational freedom of the acyl chains of lipid bilayers which

Abbreviations: Laurdan, 2-dimethylamino-6-lauroylnaphthalene; DPH, 1,6-diphenyl-1,3,5-hexatriene; TMA-DPH, 1-(4-(trimethylamino)phenyl]-6-phenyl-1,3,5-hexatriene; n-AS, *n*-(9-anthroiloxy)stearic acids; GP, generalized polarization; PUFA, polyunsaturated fatty acid.

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would be important for the lipid–protein interactions and membrane function [2,3]. The influence of unsaturation on the structural and dynamic properties of the bilayer is strongly dependent on the position of double bonds [4–6]. Since only PUFAs which derivates from essential fatty acids ($n-3$ and $n-6$ series) are able to place double bonds in the deep part of the membrane leaflet [7–9], their deficiency could result in altered transversal double bond distribution and physical properties of the membranes.

PUFA deficient diets modify the fatty acid composition of several membranes in different species [7–15]. In spite of its dependence on the particular membrane, tissue and species, $n-6$ and $n-3$ PUFA deficiency mainly decreases the proportion of 18:2($n-6$), 20:4($n-6$) and 22:6($n-3$) fatty acids. These $n-6$ and $n-3$ PUFAs are replaced by fatty acids from the de novo lipogenesis as saturated and mono- and polyunsaturated of the $n-9$ and $n-7$ series. Deficiency in $n-3$ PUFAs, with a sufficient supply of $n-6$ acids, is reflected on a decrease in 22:6($n-3$) which is compensated by an increase in 22:5($n-6$) [16]. On the contrary, if the specific deficiency is related to $n-6$ acids, the decrease in $n-6$ PUFAs is compensated by higher levels of $n-3$ PUFAs [17]. Appreciable amounts of 20:3($n-9$), a non essential PUFA which is not present in essential fatty acid sufficient animals, appear as a consequence of fat-free diets in rat [8,10,11] but not in guinea pig liver microsomes [12–15]. This $n-9$ PUFA is not detected in rats treated with diets deficient in $n-6$ and sufficient in $n-3$ fatty acids [17].

Several studies using spectroscopical methods as fluorescence [12,14,15] or electron spin resonance [3,7,13] have indicated that PUFA deficiency affects the physical properties of different membranes as guinea pig liver microsomes [12–15] or piglet jejunum brush border membranes [3,7]. This is attributed to those changes in fatty acid composition of membrane lipids, since no changes are observed either in the phospholipid class distribution or in the cholesterol, phospholipid and protein relative content. In some membranes, however, as in the case of rat liver microsomes [8,11] and erythrocyte membranes [8], in spite of large changes in fatty acid composition, the membrane lipid order and dynamics remain almost unaltered, suggesting that some homeoviscous compensation could occur [8]. The replacement of 18:0/20:4($n-6$) by 16:0/18:1 in the major phosphatidylcholine and phosphatidylethanolamine fractions of phospholipids (Garda et al., unpublished data) could be very important for this compensation. We have previously reported [8,18] that fluorescence lifetime heterogeneity is observed for the fluorescent probes DPH and n-AS in rat liver microsomes using two-frequency phase-modulation measurements. As lifetime heterogeneity can not be easily resolved using two-frequency data, we have used here multifrequency phase-modulation measurements [19,20] to calculate the fluorescence lifetime distribution and to obtain further information on the rotational mobility of these probes in liver microsomes of fat sufficient and fat deficient rats. The influence of PUFA deficiency on the membrane lipid lateral mobility was also studied by measuring the excimer formation of pyrenyl derivatives and on the rate of dipolar relaxation in the interfacial region of the membrane using Laurdan [21,22]. Laurdan has been reported to be located with the lauric acid tail anchored in the phospholipid acyl chain region and the fluorescent naphthalene ring at the hydrophilic/hydrophobic interface of the bilayer in the region of the glycerol backbone of the membrane glycerophospholipids [23,24]. Their fluorescence excitation and emission spectra are very sensitive to the environment polarity and dynamics [25]. Information on the lateral heterogeneity of the membrane can also be obtained from the Laurdan fluorescence [26].

2. Materials and methods

2.1. Materials

DPH was obtained from Aldrich (Milwaukee, WI) and a stock solution 2.0 mM in tetrahydrofuran was prepared. TMA-DPH, pyrenedodecanoic acid, Laurdan, 2-, 7-, and 12-AS were purchased from Molecular Probes (Junction City, OR); stock solutions 2.0 mM in methanol were prepared.

2.2. Animals and diets

After weaning, male Wistar rats were fed either a sunflower oil-containing diet: 55% starch, 20% casein, 25% sunflower oil plus minerals and vitamins

[27]; or a fat-free diet: 70% starch, 30% casein, minerals and vitamins. After 35 days rats were killed by decapitation and livers excised.

2.3. Microsomes and microsomal lipid-liposome preparation

Livers were homogenized in 0.25 M sucrose, 1 mM EDTA, pH 7.0. Microsomes were obtained by centrifugation as previously described [28] and stored at −80°C. Total microsomal lipids were extracted according to the procedure of Folch et al. [29] with chloroform/methanol 2:1 (v/v); liposomes of the total lipid extracts (0.3 mg of lipid/ml) were prepared in 20 mM sodium phosphate pH 7.0 by sonication under N_2 as previously described [30].

2.4. Analytical methods

Protein content of microsomal preparations was measured by the method of Lowry et al. [31]. Fatty acid composition was analyzed as described [8] in the total microsomal lipid extracts.

2.5. Multifrequency fluorescence measurements

Microsomes (0.1 mg of protein/ml) and liposomes (0.3 mg of lipid/ml) were labelled with 3 μM DPH or TMA-DPH; or 6 μM of 2-, 7- or 12-AS as previously described [8].

Measurements were made at 20°C with an ISS Greg-200 multifrequency phase shift and modulation spectrofluorometer interfaced with a personal computer for data acquisition and analysis. Excitation wavelength was 325 nm from a 40420NB He-Cd laser from Liconix at 20°C. Phase shift and modulation were measured at multiple frequencies between 5 and 100 Mhz using dimethyl-POPOP as reference (lifetime 1.45 ns). Data of differential polarized phase and modulation ratio were obtained at the same frequencies using glan Thompson prism polarizers in both excitating and emitting beams.

2.6. Steady-state fluorescence measurements

Microsomes (0.1 mg of protein/ml) or liposomes (0.1 mg of lipid/ml) were labelled with 1 μM Laurdan or 50 μM pyrenedodecanoic acid and incubated at room temperature for 30 min.

Fluorescence measurements were made with a Spex, fluorolog photon counting spectrofluorometer interfaced to a personal computer. Softwares from ISS were used for data collection and analysis. A xenon arc lamp was utilized as light source and excitation wavelength was set through the excitation monochromator. The diffusion controlled rate of pyrenedodecanoic acid excimer formation was evaluated at 20°C through the excimer to monomer fluorescence intensity ratio I'/I. Excitation wavelength was set at 342 nm. Fluorescence intensities were measured at 374 nm for I and 480 nm for I'. Values were corrected for contribution of monomer emission at excimer maximum and excimer emission at monomer maximum. Laurdan spectral shifts were evaluated using the generalized polarization (GP) concept defined as $GP = (I_B - I_R)/(I_B + I\mathrm{dR})$ where I_B and I_R are the intensities observed at the blue and red maxima, respectively. For the calculation of excitation GP (exGP), the intensities at the emission wavelengths 435 and 490 nm were chosen. For the calculation of emission GP (emGP), the intensities for the excitation at 410 and 340 nm were chosen, corresponding to the edges of the two excitation bands.

3. Results

Table 1 shows the effect of a fat-free diet administration on the fatty acid composition of rat liver microsomes, in comparison with a diet containing sunflower oil. It is to note that the sunflower oil-containing diet is overloaded in $n-6$ with respect to $n-3$ fatty acids, and it is reflected in the relatively high proportion of 22:5($n-6$). The fat-free diet evoked the typical changes of PUFA deficiency [8,11]. A decrease in the $n-6$ acids 18:2 and 20:4 was compensated by an increase in the non-essential $n-9$ 18:1 and 20:3.

Centers and widths of the lorentzian lifetime distributions obtained from phase and modulation data obtained between 5 and 100 MHz for DPH, TMA-DPH, 2-, 7-, and 12-AS in microsomes and in liposomes of extracted microsomal lipids of fat-sufficient and fat-deficient rat livers are shown in Table 2. For the neutral probe DPH, a wider distribution centered at a somewhat higher lifetime was obtained in micro-

Table 1
Influence of a fat deficient diet on the fatty acid composition of rat liver microsomes

Fatty acid	Fat-sufficient (mol% ± S.D.)	Fat-deficient (mol% ± S.D.)
16:0	20.3 ± 1.1	22.3 ± 0.8 **
16:1($n-7$)	0.8 ± 0.4	3.3 ± 0.4 ***
18:0	22.3 ± 1.0	20.9 ± 0.8 *
18:1($n-9$)	6.2 ± 0.5	14.4 ± 1.0 ***
18:2($n-6$)	14.4 ± 0.6	5.9 ± 0.6 ***
20:3($n-6$)	0.5 ± 0.1	——– ***
20:3($n-9$)	——–	4.3 ± 1.3 ***
20:4($n-6$)	26.4 ± 1.6	20.3 ± 1.4 ***
22:4($n-6$)	1.7 ± 0.3	0.7 ± 0.1 ***
22:5($n-6$)	4.6 ± 0.4	2.8 ± 0.3 ***
22:5($n-3$)	0.6 ± 0.3	0.7 ± 0.2 ns
22:6($n-3$)	2.2 ± 0.4	4.4 ± 0.4 ***

ns Not significant difference.
* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

somes as compared with protein-free liposomes. As expected, a narrower distribution centered at a lower lifetime was obtained for the amphipatic TMA-DPH compared to the neutral DPH. However, no appreciable influence of the fat-deficient diet was observed on the lifetime distribution of DPH or TMA-DPH in both microsomes and protein-free liposomes. The center of the lifetime distribution increased in the order 2-AS < 7-AS < 12-AS for microsomes and protein-free liposomes, but no effect of the fat-free diet was observed for these probes.

Differential polarized phase fluorescence measurements between 5 and 100 MHz were used to calculate the rotational correlation times amd limiting anisotropy of DPH, TMA-DPH and n-AS in microsomes and microsomal lipid liposomes of fat-sufficient and fat-deficient rat livers. These data are shown in Table 2. The limiting anisotropy was higher for the amphipatic TMA-DPH than for the neutral DPH, indicating a more restricted wobbling motion for the first probe. For both, DPH and TMA-DPH probes, a higher limiting anisotropy was obtained in the total microsomal membranes in comparison with protein-free liposomes of the extracted microsomal lipids. Though no large influence of the fat free diet on the rotational mobility of these probes was observed. For the n-AS probes, measurements at 325 nm excitation wavelength would preferentially report the rotation out of the plane of the aromatic anthroyl ring [32,33], around the normal to the membrane surface [18]. The low limiting anisotropy values obtained in protein-free liposomes indicate that this motion was practically unhindered in these protein-free lipid bilayers, but a relatively high degree of hindering was observed in the total microsomal membranes, which decreased along with the depth of the anthroyl group from 2- to 12-AS. The rate of the unhindered 'out of the plane' rotation in protein-free liposomes was minimal for 7-AS and it increased for the external 2-AS and the internal 12-AS. The fat-free diet showed no effect on the rotational mobility of the n-AS probes.

Excimer to monomer fluorescence intensity ratios, I'/I, of pyrenedodecanoic acid incorporated into microsomes of fat-sufficient and fat-deficient rat livers

Table 2
Influence of a fat deficient diet on the lifetime distribution and rotational mobility of several fluorescent probes in rat liver microsomes

Sample [a]	Probe	Lifetime distribution [b] (ns)		Rotational correlation time (ns) [c]	Limiting anisotropy [c]
		Center	Width		
MS	DPH	7.4	4.2	3.6	0.189
MD	DPH	7.8	4.0	4.0	0.186
LS	DPH	8.6	0.9	2.9	0.168
LD	DPH	8.9	0.9	2.9	0.172
MS	TMA-DPH	4.2	1.0	1.6	0.277
MD	TMA-DPH	4.1	1.2	1.8	0.280
LS	TMA-DPH	4.2	0.6	2.0	0.224
LD	TMA-DPH	4.3	0.9	2.3	0.222
MS	2-AS	7.3	2.7	5.0	0.051
MD	2-AS	7.6	3.3	4.8	0.053
LS	2-AS	6.9	1.2	8.1	0.005
LD	2-AS	7.7	1.6	8.3	0.004
MS	7-AS	8.3	1.7	6.5	0.037
MD	7-AS	7.9	2.3	6.6	0.035
LS	7-AS	8.7	3.0	12.1	0.003
LD	7-AS	8.9	3.4	11.8	0.002
MS	12-AS	9.7	3.9	6.5	0.029
MD	12-AS	9.3	5.2	6.4	0.028
LS	12-AS	11.2	4.1	6.1	0.008
LD	12-AS	11.9	3.3	6.1	0.011

[a] MS, fat-sufficient microsomes; MD, fat-deficient microsomes; LS, liposomes from fat-sufficient microsomal lipids; LD, liposomes from fat-deficient microsomal lipids.
[b] Lorentzian distributions obtained from phase and modulation data at several frequencies between 5 and 100 MHz.
[c] Standard deviations are about 0.2 ns for the rotational correlation times and 0.01 for the limiting anisotropy.

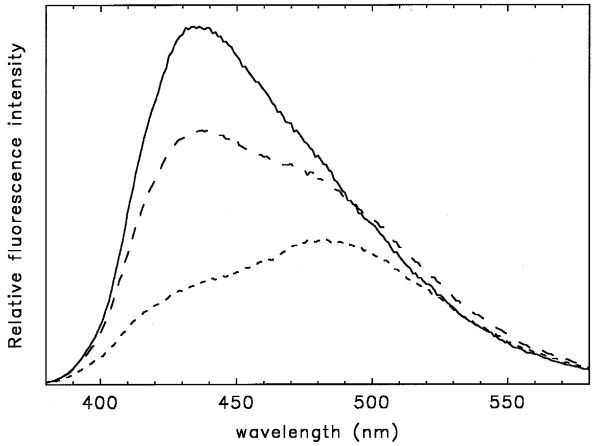

Fig. 1. Fluorescence emission spectra of Laurdan in rat liver microsomes at 5.4 (————), 19.3 (— — — —), and 52.3 (- - -) °C.

were 0.12 ± 0.02 and 0.13 ± 0.03, respectively. These results that represent mean values of seven samples in each condition indicated no significant differences in the membrane translational mobility between both conditions.

Fig. 1 shows the emission spectra of Laurdan in rat liver microsomes at different temperatures. The presence of two emission bands is evident. The relative intensity of the red band increased with the temperature at the expense of the blue band. Laurdan spectral features have been described by the generalized polarization (GP) concept [21]. It has been shown that the wavelength dependence of the GP value can be used to explore the coexistence of domains of different phase states in the membrane. An opposite wavelength dependence of the GP value is observed in pure and in mixed-phase lipid bilayers [22,26]. Excitation and emission GP spectra of Laurdan in microsomes and in protein-free microsomal lipid-liposomes of fat-sufficient rat livers at 20°C are shown in Fig. 2. For the excitation spectra, lower GP values were obtained in liposomes as compared with total microsomes which indicated that the presence of microsomal proteins decreased the rate of dipolar relaxation in the interfacial region where this probe was located. In both cases excitation GP decreased and emission GP increased along with the wavelength, indicating the absence of lipid gel-phase domains at this temperature. A similar wavelength dependence of GP was found either for microsomes or liposomes from fat-deficient rat livers (not shown). A continuous decrease in Laurdan GP when increasing temperature from 5 to 50°C (not shown data) was observed in

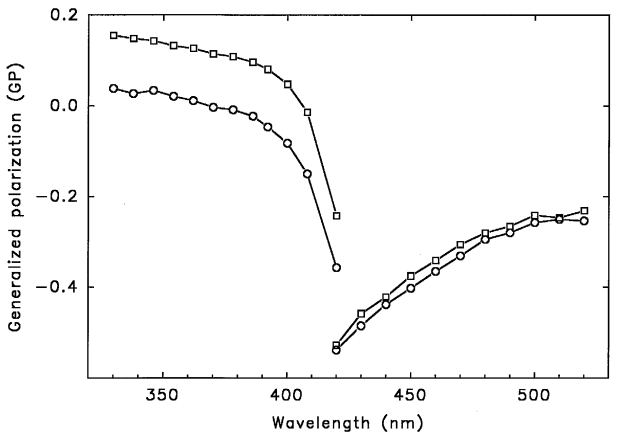

Fig. 2. Excitation and emission generalized polarization (GP) spectra of Laurdan fluorescence in microsomes (□) and in liposomes of microsomal lipids (○) of rat livers. Excitation GP spectra were calculated by $GP = (I435 - I490)/(I435 + I490)$, using excitation wavelength from 330 to 420 nm. Emission GP spectra were calculated by $GP = (I410 - I340)/(I410 + I340)$, using emission wavelengths from 420 to 520 nm.

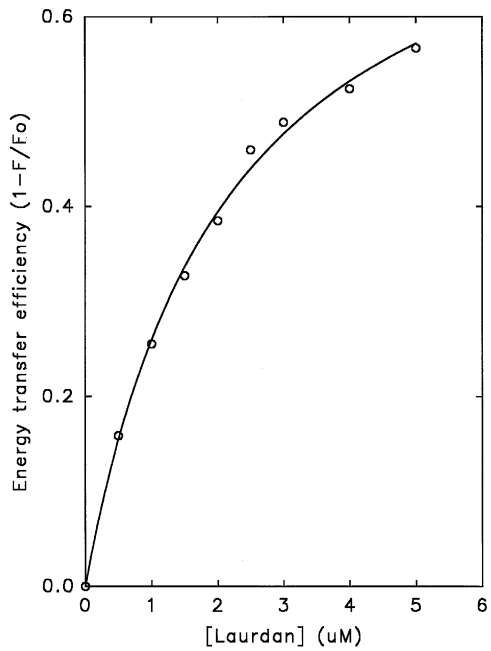

Fig. 3. Resonance energy transfer from tryptophan to Laurdan in rat liver microsomes. Energy transfer efficiency was calculated from $E = (1 - F/F_o)$, where F and F_o are the tryptophan fluorescence intensity in the presence and in the absence of Laurdan, respectively.

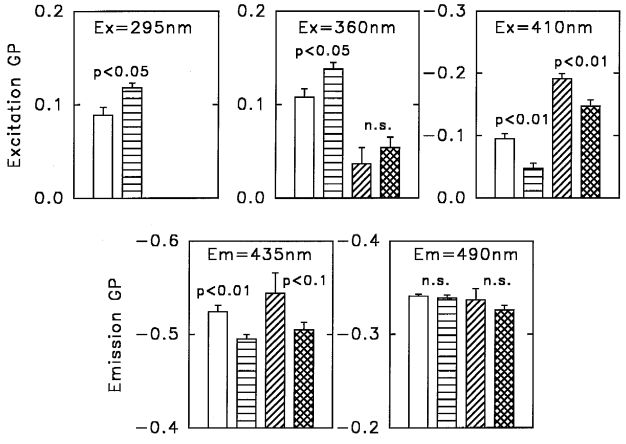

Fig. 4. Influence of essential fatty acid deficiency on Laurdan generalized polarization in: liver microsomes of fat-sufficient (empty box) and fat-deficient (horizontally lined box) rats, and in microsomal lipid liposomes of rats treated with a fat-sufficient (diagonally lined box) or a fat-deficient (cross-hatched box) diet. The mean values of 7 animals per group and the standard deviations (error bars) are given.

microsomal lipid-liposomes and in total microsomes of fat-sufficient and fat-deficient rat livers, using either 360 or 410 nm excitation wavelength to select preferentially the liquid-crystalline and the gel domains, respectively [25]. This fact indicates the absence of abrupt phase transition at this temperature range.

As shown in Fig. 3, Laurdan is a good energy transfer acceptor of the tryptophan fluorescence. Thus, the Laurdan GP obtained by exciting tryptophan at 295 nm would preferentially report the properties of the lipid region surrounding the microsomal proteins. A continuous decrease in GP was also obtained by exciting at 295 nm (not shown) indicating non detectable phase transitions in the lipid region surrounding the microsomal proteins.

The influence of PUFA deficiency on Laurdan GP in microsomal lipid-liposomes and total microsomes of rat liver is illustrated in Fig. 4. Regarding the protein-free liposomes of microsomal lipids, a significant increase in the negative exGP value obtained at 410 nm excitation was observed due to the PUFA deficiency. However, no significant change was observed in the exGP value by exciting at 360 nm. In a similar fashion, PUFA deficiency produced a significant increase on the Laurdan emGP in microsomal lipid-liposomes when the emission was observed at 435 nm. In contrast, no change was produced when the emission was observed at 490 nm. A similar influence of PUFA deficiency on the behaviour of Laurdan fluorescence was observed in total microsomes. In this case, opposite to the absence of effect in liposomes, a less pronounced but significant increase in the exGP obtained at 360 nm excitation was also produced by the fat-free diet. Then, these results indicate that PUFA deficiency increases Laurdan GP in both, microsomal lipid-liposomes and total microsomes; being the effect more noticeable by the exGP measurements in the red region of the absorption band and those of the emGP in the blue region of the emission band. Fig. 4 also shows the Laurdan exGP values obtained by exciting at 295 nm in microsomes of fat-deficient and fat-sufficient rat livers. The fat-free diet also produced a significant increase in Laurdan GP obtained via energy transfer from tryptophan, indicating that the effect of PUFA deficiency is also evoked in the lipid region near the microsomal proteins.

4. Discussion

Multifrequency phase and modulation measurements indicated that the changes in fatty acid composition produced by $n-6$ PUFA deficiency in the rat did not influence the lifetime distribution of probes with different localization in the lipid bilayer as DPH, TMA-DPH or 2-, 7- and 12-AS. Also, those changes in fatty acid composition evoked by the fat-deficient diet did not modify the rotational behaviour of the above probes. These results confirm previous observations from two-frequency measurements stating that microsomal proteins increase the lifetime heterogeneity [18], or as interpreted here, the width of the lifetime distribution of these fluorescent probes. Besides, these multi-frequency measurements show that the order and acyl chain dynamics of the hydrophobic region of the membrane are not largely modified by PUFA deficiency [8]. It is also shown that the lateral difussion of pyrenedodecanoic acid is not altered by changes in fatty acid composition resulting from $n-6$ PUFA deficiency in the rat. However, a small but significant increase in the generalized polarization of Laurdan fluorescence is produced by the fat-free diet.

The same effect is observed in liposomes of the total microsomal lipids.

Spectral shifts and GP of Laurdan in lipid bilayers were attributed to dipolar relaxation of a few water molecules present at the hydrophilic/hydrophobic interface where the fluorescent moiety of Laurdan is located [22,25]. Changes in the acyl chain packing in the lipid bilayer modify the water content and the dynamics of water molecules in the interfacial region. However, since changes in the 'static' dielectric constant due to increased water penetration are not enough to explain the spectral shifts, they are attributed to changes in the relaxation rate of water molecules [22]. It was previously shown [28] that fat-deficient diets do not alter the phospholipid class composition of liver microsomes. Also, Laurdan fluorescence is unsensitive to the type and charge of the phospholipid polar group [22]. Thus, the observed increase in Laurdan GP evoked by PUFA deficiency can only be due to changes in the fatty acid composition resulting in an increased lipid packing and decreased water mobility in the membrane interface. This is apparently hard to reconcile with the lack of effect on the rotational behaviour of the hydrocarbon region probes. However, it is possible that small changes in lipid packing which do not alter appreciably the rotational behaviour of relatively large molecules as DPH and n-AS probes could change the dynamics of small molecules like water. Thus, these results show the high sensitivity of Laurdan to detect small changes in membrane dynamical properties as those produced by PUFA deficiency in rat liver microsomes.

Measurements of Laurdan GP via energy transfer from tryptophan offering an insight into the lipid region surrounding the microsomal proteins, indicate that the effect on the dipole relaxation in the bilayer interface produced by PUFA deficiency is more intense near the proteins. PUFA deficiency produces a somewhat larger increase in the Laurdan GP by excitation at 295 nm via energy transfer from tryptophan (35%) as compared with direct excitation at 360 nm (29%). This fact would indicate a specific effect on the protein/lipid interface which could be important in membrane physiology.

Laurdan fluorescence can also provide valuable information on the lateral heterogeneity of the membrane [25]. The emission spectra of Laurdan in rat liver microsomes show the presence of two bands (Fig. 3). Two bands in the emission spectrum of Laurdan have been found in mixed-phase bilayers with coexisting domains, such as those composed of mixtures of dipalmitoylphosphatidylcholine and dilauroylphosphatidylcholine, which were attributed to the gel and liquid-crystalline phases [34]. However, the wavelength dependence of the excitation and emission GP values in rat liver microsomes does not correspond to that observed in gel/liquid-crystalline mixed-phase bilayers. In dipalmitoylphosphatidylcholine/dilauroylphosphatidylcholine bilayers, the excitation GP increases whereas the emission GP decreases with the wavelength [25,26]. On the contrary, as shown here, in rat liver microsomes as well as in liposomes of microsomal lipids, the excitation GP decreases whereas the emission GP increases with the wavelength (Fig. 4). This behaviour of the Laurdan GP is typical of pure liquid-crystalline bilayers [25,26]. Thus, although the emission spectra suggest the presence of lateral heterogeneity, the wavelength dependence of GP discards the possibility of coexisting gel and liquid-crystalline domains. A possible explanation for these observations should be the coexistence of liquid-crystalline domains with different dynamic properties. Such a liquid-liquid immiscibility has been described for bilayers composed of mixtures of dipalmitoylphosphatidylethanolamine and dielaidoylphosphatidylcholine [35]. Further studies will be necessary either to assess or discard this possibility in rat liver microsomal membrane.

The influence of PUFA deficiency on Laurdan GP is more evident on the red edge of the excitation band and on the blue edge of the emission band. Since these spectral regions would preferentially select the least 'fluid' domains [25], this observation could indicate a selective effect of PUFA deficiency on these domains.

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