Possible compensation of structural and viscotropic properties in hepatic microsomes and erythrocyte membranes of rats with essential fatty acid deficiency

Horacio A. Garda,[1] Ana M. Bernasconi, and Rodolfo R. Brenner[1]

Instituto de Investigaciones Bioquímicas de La Plata (INIBIOLP), UNLP-CONICET, Facultad de Ciencias Médicas, Calles 60 y 120, 1900-La Plata, Argentina

Abstract The effect of essential fatty acid deficiency on the structural and dynamic properties of the lipid matrix of rat liver microsomes and erythrocyte membranes was studied. The rate and range of the rotational mobility of 1,6-diphenyl-1,3,5-hexatriene and 2-, 7-, and 12-(9-anthroyloxy)stearate probes in the native membranes and in lipid vesicles prepared with the total lipid extracts of these membranes were evaluated by using differential polarized phase fluorometry. For the anthroyloxystearate probes, two modes of rotation (in and out of the plane of the aromatic anthracene ring) were partially resolved by measuring at different excitation wavelengths. The fat-free diet produces important changes in the fatty acid composition of the different glycerophospholipid classes without affecting the total double-bond number, the relative contents of cholesterol, phospholipid, and protein, and the glycerophospholipid class distribution. The principal changes, more pronounced in liver microsomes than in erythrocytes, are: an increase in nonessential monoene and triene (18:1n-9 and 20:3n-9) and a decrease in essential diene (18:2n-6) and tetraene (20:4n-6). These changes modify the double-bond distribution as a function of the distance from the interphase toward the bilayer interior, with a significant deficit (15% in erythrocytes and 30% in liver microsomes) in the double-bond density in the intermediate region of the membrane leaflet, corresponding to the carbon number 11-12 of an extended saturated acyl chain, and where the 12-anthroyloxystearate probe is located. In spite of the changes in fatty acid composition and double-bond distribution, with the only exception of a slight increase (about 15%) in the "out of the plane" rotation rate of the 7-(9-anthroyloxy) stearate probe in the erythrocyte lipid vesicles, no other significant change is observed. Thus, the changes in fatty acid composition would take place in such a way that at least the average structural and viscotropic properties of the lipid phase of the membrane, sensed by these probes, would be almost exactly compensated.—**Garda, H. A., A. M. Bernasconi, and R. R. Brenner.** Possible compensation of structural and viscotropic properties in hepatic microsomes and erythrocyte membranes of rats with essential fatty acid deficiency. *J. Lipid Res.* 1994. **35:** 1367-1377.

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Only a very small fraction of the total amount of essential fatty acids required by mammals can be explained by their role as precursors of biooxygenated derivatives (eicosanoids, docosanoids) (1). As the membrane phospholipids have a high content of polyunsaturated fatty acids that derive from essential fatty acids, a structurally specific role in the membranes has been attributed to these acids (2, 3). It was proposed (3, 4) that the major property of the polyunsaturated fatty acids that derive from essential fatty acids could be to place double bonds in the deep part of the membrane leaflet (see **Fig. 1**). This could modify the conformational freedom of the acyl chains in the deepest region of the lipid bilayer which could have an important physiological role by strengthening the lipid-protein interaction (5, 6). Nonetheless, Lee, East, and Froud (7) have minimized the importance of essential fatty acids over the structural properties of membranes.

Previous work from this laboratory has shown that essential fatty acid deficiency in rats (8, 9) and guinea pigs (10-14) modifies the fatty acid composition of total (8-12) and rough (13, 14) liver microsomes without affecting either the glycerophospholipid class distribution or the cholesterol, phospholipid, and protein relative content. Thus, essential fatty acid deficiency is a good tool to explore the effect of fatty acyl chain composition of membrane lipids on membrane properties. These studies have also shown that guinea pigs and rats respond differently to essential fatty acid deficiency. In liver microsomes of both animals, essential fatty acid deficiency produces a decrease in the proportion of 18:2n-6 and an increase in

Abbreviations: DPH, 1,6-diphenyl-1,3,5-hexatriene; n-AS, n-(9-anthroyloxy) stearic acids.

[1]Members of the Carrera del Investigador Cientifico, CONICET, Argentina.

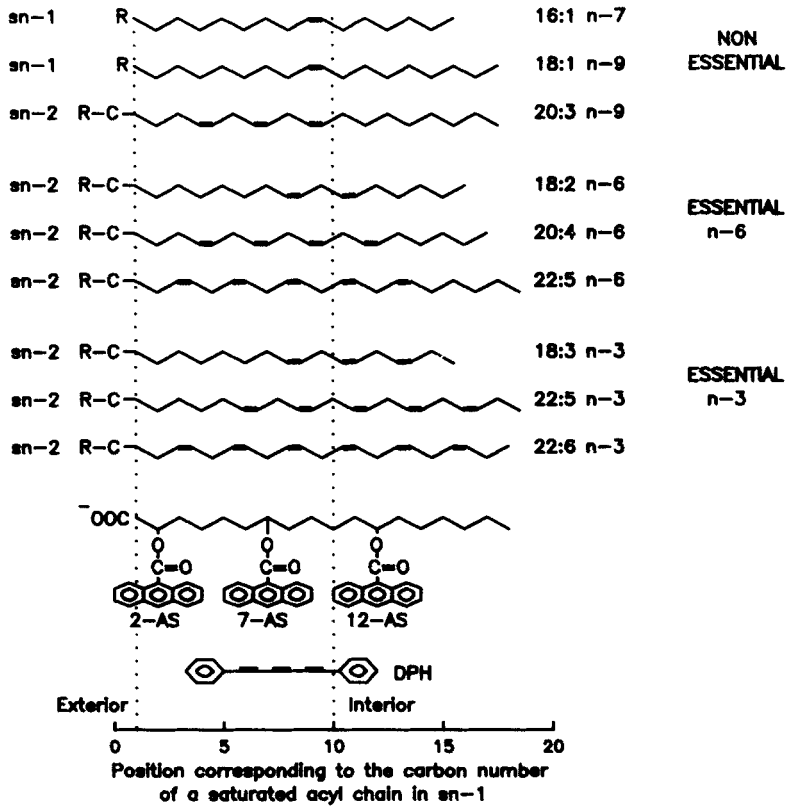

Fig. 1. Simplified representation of double-bond location of the main unsaturated fatty acids and localization of the fluorescent probes in the lipid bilayer. For the double-bond location, the bend of chains acylated in the *sn*-2 position of phospholipids (51, 52) was taken into account. It was also considered that each double bond introduces a shortening of the acyl chain by 50% of the single-bond axial length (3).

the proportion of monoenes 16:1 and 18:1. However, a decrease in the content of 20:4n-6 occurs, which is compensated by an increase in the nonessential triene 20:3n-9 in rats, but no compensation occurs in guinea pigs. Also in rats, the total double-bond number is decreased at short times of treatment but is recovered at longer times (9), in contrast to a continuous decrease observed in guinea pigs (10). Moreover, an increase in the steady-state fluorescence anisotropy of 1,6-diphenyl-1,3,5-hexatriene (DPH) is produced by the essential fatty acid deficiency in guinea pigs (10–14), but no change in the physical properties of the membrane was detected in rats by using electron spin resonance with 5- and 12-doxylstearates (9). These results suggest that the changes in guinea pig membrane properties could be due to a change in the total double-bond number and that the nonessential 20:3n-9 could replace the essential derivate 20:4n-6 for its structural role in the membrane. However, more recently, Léger et al. (3) reported that in brush border membranes from piglet jejunum, essential fatty acid deficiency produces a general decrease in the order parameter and a shape change in the order profile sensed by 5-, 7-, 10-, and 12-doxylstearate probes which was attributed to changes in the double-bond transverse distribution and localization, as there is no change in the total double-bond number.

The aim of this work was to examine in more detail the effect produced by changing the double-bond transverse distribution on the physical properties of the membranes. For this reason, we studied the effect of a 35-day treatment of essential fatty acid deficiency on the rat. Differential polarized phase fluorometry, a method introduced by Weber (15) and applied to lipid systems by Lakowicz, Prendergast, and Hogen (16), was used in order to measure the rate and range of the rotational mobility of the following probes: *a)* DPH, which senses the whole lipid matrix, and *b)* a set of n-(9-anthroyloxy)stearates (n-AS, where n = 2, 7, and 12) which senses the physical properties progressively from the interphase to the deepest region of the membrane (see Fig. 1). Differential polarized phase measurements allowed us to distinguish between structural constraint and viscous resistance to probe rotation which are undistinguishable in steady-state anisotropy. For the n-AS probes, two rotational modes (in and out of the plane of the aromatic anthracene ring) were partially resolved by measuring at different excitation wavelengths (17).

We have previously observed (18) that heterogeneity in

fluorophore microenvironments exists for these probes in rat liver microsomal and erythrocyte membranes but, within the experimental error and limitation of two-frequency phase-modulation measurements, homogeneity is observed in protein-free liposomes prepared with the membrane total lipid extracts. As heterogeneity limits the interpretation of differential polarized phase data (18, 19), the effect of essential fatty acid deficiency was studied in liposomes from the erythrocyte and microsomal total lipid extracts in addition to the native membranes.

Data presented in this study do not support the hypothesis regarding a specific structural role of essential fatty acid derivatives, such as 20:4 n-6, in the membranes, suggesting that its replacement by the nonessential 20:3 n-9 would not greatly modify the average structural and dynamical properties of the membrane sensed by these probes.

EXPERIMENTAL

Materials

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

Animals and diets

After weaning, male Wistar rats were divided into two groups. The control group (5 animals) was fed a semisynthetic diet: 55% starch, 20% casein, 25% sunflower oil plus minerals and vitamins (20). The fat-deficient group (7 animals) was fed 70% starch, 30% fat-free casein, minerals, and vitamins. After 35 days, rats were killed by decapitation.

Liver microsomes and erythrocyte membrane separation

Livers were excised and homogenized in 0.25 M sucrose, 1 mM EDTA, pH 7.0. The microsomal membranes were prepared by centrifugation as previously described (21). Erythrocyte membrane preparation was also as described (18).

Protein content of the membrane preparations was measured by the method of Lowry et al. (22), and preparations were stored at −80°C.

Extraction and analysis of lipids

Total lipids were extracted according to the procedure of Folch, Lees, and Sloane Stanley (23) with chloroform–methanol 2:1 (v/v). Glycerophospholipid classes were separated by one-dimensional thin-layer chromatography on 20 × 20 cm plates with a mixture of silica gel H-fluorisil 9:1 (w/w) with a double development solvent system (24): chloroform–methanol–30% ammonium–water 140:50:7:3 (by vol) in the first run and chloroform–methanol–acetic acid–water 320:40:8:3 (by vol) in the second run. The extreme ends of the plates were sprayed with an iodine solution to visualize the spots. The unexposed part of the bands was scraped off and extracted with methanol. For fatty acid analysis, the total lipid extract and the different phospholipid fractions were dried and transesterified by heating at 64°C for 3 h in 14% boron trifluoride in methanol. The resulting methyl esters were separated on a column of SP 2330 on 100–200 mesh Chromosorb WAW using a Hewlett-Packard 5840 A gas–liquid chromatograph. The fatty acids were identified by comparing their relative retention times with those of reference standards.

Labeling of membranes and liposomes

Dispersions of the fluorescence probes (3.0 μM final concentration) in 20 mM sodium phosphate buffer, pH 7.0, were prepared by adding the corresponding volume of the stock solution to the buffer and vortexing. Then, the appropriate volume of membrane preparation was added to give a final concentration of 100 μg protein/ml. The measurements were carried out after agitation and a minimal 20-min (time required to reach the maximal fluorescence intensity) incubation time at room temperature in darkness.

Liposomes of the total lipid extracts (0.3 mg lipid/ml) were prepared in 20 mM sodium phosphate, pH 7.0, by sonication under N_2 as previously described (25). They were mixed with the same volume of a 6.0 μM dispersion of the probes in the same buffer, agitated, and incubated at room temperature for 20 min. Final concentrations were approximately 0.3 mM in lipids and 3.0 μM in probe. Unlabeled blanks with the same concentrations of liposomes or membranes as the samples were also prepared.

Fluorescence measurements

Steady-state fluorescence anisotropy (r_s), fluorescence lifetimes (τ), and differential polarized phase shift (Δ) were measured with an SLM 4800 C phase-modulation spectrofluorometer (SLM Instruments, Inc., Urbana, IL) essentially as described by Lakowicz et al. (16, 19) with some modifications to apply the background correction described previously (18). Excitation wavelength was 361 nm for DPH, and 318 nm and 388 nm for 2-, 7-, and 12-AS. The emitted light was passed through a sharp cut-off filter (Schott KV 389 for DPH or KV 418 for 2-, 7-, and 12-AS).

Lifetime measurements were obtained with the exciting light amplitude-modulated at 18 and 30 MHz by a Debye-Sears modulator. After the filter, the emission light was passed through a Glan-Thompson polarizer oriented 55° to the vertical to eliminate the effects of Brownian motion (26). The phase shift and demodulation of the emitted

light relative to a reference of known fluorescence lifetime were determined and used to compute the phase lifetime (τ_p) and the modulation lifetime (τ_m) of the sample. POPOP (1,4-bis(5-phenyloxazol-2-yl)benzene) in ethanol was used as the standard. It has a fluorescent lifetime of 1.35 ns (19).

The differential polarized phase shift (Δ) was determined according to Lakowicz et al. (16, 19) by exciting with vertically polarized light modulated at 18 and 30 MHz and by measuring the phase difference between the parallel and perpendicular components of the emitted light. It was corrected for the background contribution as described previously (18).

Steady-state anisotropies (r_s) were measured with the Debye-Sears modulator turned off. Fundamental anisotropy values (r_o) were previously estimated (18) to be: 0.390 for DPH, and 0.102 and 0.332 for the n-AS at 318 and 388 nm excitation wavelength, respectively. For r_o = 0.1, the angle between absorption and emission dipoles is 45° and only the rotation "out of the plane" of the anthracene ring depolarizes the emitted light, while for r_o = 0.332 absorption dipole is about 20° to the emission dipole and both rotational modes, "in" and "out" of the anthracene ring plane, are observed (17, 18, 27).

Analysis of fluorescence data

When a fluorescent molecule dissolved in an isotropic solvent is excited by a brief pulse of polarized light, the fluorescence anisotropy decays exponentially approaching zero. When the rotation of the fluorophore is hindered by the anisotropic environment, the anisotropy decays to a finite value (r_∞). This was taken into account in a modified Perrin's equation (19).

$$r_s = r_\infty + ((r_o - r_\infty)/(1 + \tau/\tau_r)) \qquad Eq.1)$$

where τ_r is the correlation time for the probe rotation, which is inversely related to the rate of rotation (R): τ_r = 1/(6 R).

τ_r and r_∞ can be considered to reflect different limitations on probe rotation. τ_r depends primarily on the local viscous resistance to rotation; r_∞, the infinite time anisotropy, reflects principally the limitation imposed by the local environment of the fluorophore on the extent or range of probe rotation.

To calculate τ_r and r_∞ separately, we used the method described by Lakowicz et al. (16, 19) as described in detail (18), in which R or τ_r is estimated from measurements of r_s, τ, and the differential polarized phase shift Δ according to the theory developed by Weber (15). The τ_r values obtained in this way were used in equation 1 which was rearranged in order to calculate r_∞.

RESULTS

Effect of fat-free diet on the fatty acid composition of liver microsomes and erythrocyte membranes

Table 1 shows the fatty acid composition of liver microsomes and erythrocyte membranes. The total acyl group distribution from normal and fat-deficient liver microsomes is in agreement with our previous finding (9). Fat deprivation resulted in a highly significant decrease in the n-6 acids linoleic and arachidonic. The total amount of n-3 acids, which are found in a lesser proportion, was not changed but a significant redistribution was produced with a decline in 22:3 and 22:4 and an increase in

TABLE 1. Effect of essential fatty acid deficiency on acyl group composition of membrane lipids of liver microsome and erythrocytes

	Microsomes		Erythrocytes	
Fatty Acid	Controls	Fat-Deficient	Controls	Fat-Deficient
	mol % ± SD		*mol % ± SD*	
16:0	20.3 ± 1.1	22.3 ± 0.8[a]	27.9 ± 0.9	29.8 ± 0.8[a]
16:1 n-7	0.8 ± 0.4	3.3 ± 0.4[b]	1.3 ± 0.4	2.1 ± 0.5[c]
18:0	22.3 ± 1.0	20.9 ± 0.8[c]	14.7 ± 0.4	11.8 ± 0.6[b]
18:1 n-9	6.2 ± 0.5	14.4 ± 1.0[b]	10.0 ± 0.7	14.6 ± 0.6[b]
18:2 n-6	14.4 ± 0.6	5.9 ± 0.6[b]	10.9 ± 0.4	3.7 ± 0.4[b]
20:3[d]	0.5 ± 0.1	4.3 ± 1.3[b]	1.1 ± 0.4	3.6 ± 0.7[b]
20:4 n-6	26.4 ± 1.6	20.3 ± 1.4[b]	25.3 ± 0.9	24.1 ± 0.7[b]
22:3 n-3	1.7 ± 0.3	0.7 ± 0.1[b]	3.6 ± 0.7	3.3 ± 0.5[c]
22:4 n-3	4.6 ± 0.4	2.8 ± 0.3[b]	2.6 ± 0.4	3.1 ± 0.6[c]
22:5 n-3	0.6 ± 0.3	0.7 ± 0.2[c]	0.9 ± 0.1	1.6 ± 0.2[b]
22:6 n-3	2.2 ± 0.4	4.4 ± 0.4[b]	1.7 ± 0.1	2.4 ± 0.2[b]
Double bonds/acyl chain	1.83 ± 0.15	1.67 ± 0.17[c]	1.73 ± 0.12	1.76 ± 0.12[c]

[a] $P < 0.01$.
[b] $P < 0.001$.
[c] $P < 0.05$.
[d] Corresponds to 20:3 n-6 for the control group and to 20:3 n-9 for the fat-deficient group.
[e] Nonsignificant difference.

22:6n-3. The decrease in n-6 acids was counterbalanced by a rise in the nonessential monoenoic 16:1 and 18:1 and the trienoic 20:3n-9, in such a way that the total double-bond number was not significantly changed. Also, a small increase in 16:0 compensated for a decline in 18:0 and no net change in the total saturated acid content was produced.

In erythrocyte membranes, fat deprivation also resulted in a large decrease in linoleic acid content but in a less marked decline of arachidonic acid. Unlike liver microsomes, in erythrocyte membranes the fat-free diet produced an increase in the n-3 acids 22:5 and 22:6 without any change in the 22:3 and 22:4 of the same series. As in liver microsomes, a rise in the nonessential 16:1, 18:1 and 20:3 n-9 was produced with no change in the total double-bond number.

Fatty acids were also analyzed in the three major glycerophospholipid classes (28) of liver microsomes, as shown in **Table 2.** The changes in fatty acid composition produced by the fat-free diet are different in each phospholipid class. The decrease in linoleic acid is produced in all three phospholipid classes but there was no decrease in arachidonic acid in the phosphatidylethanolamine fraction as a result of fat deprivation. The highest increase in monoenoic acids was in the phosphatidylcholine fraction. In the phosphatidylinositol fraction, which contains the largest amount of arachidonic acid but the smallest amount of linoleic acid, the fat-free diet produced the largest increase in the proportion of the nonessential trienoic 20:3n-9 but no increase in monoenoic acids.

From the fatty acid composition we calculated the transverse double-bond distribution from the interphase to the deep interior of the membrane lipid bilayer (**Fig. 2**). Fat deprivation produced a significant decrease in the double-bond density in the intermediate region of the membrane bilayer corresponding to the carbon number 11-12 of an extended saturated acyl chain. This is the region where the 12-AS probe is approximately located. The reduction in the number of double-bonds produced by the fat-free diet in this region was more pronounced in liver microsomes (about 30%) than in erythrocyte membranes (about 15%).

Effect of fat-free diet on the viscotropic properties of the membranes

In a recent study (18), we reported lifetime heterogeneity for DPH and n-AS in liver microsomes and erythrocytes from normal rats; with two-frequency measurements, lifetime homogeneity was observed in the protein-free liposomes from the total lipid extracts of these membranes. Lifetime heterogeneity in the native membranes can be due not only to the influence of membrane proteins but also to the presence of lateral as well as transbilayer lipid domains that could be scrambled in liposomes of extracted lipids. The same was observed here for the fat-deficient animals, and no significant effect of the fat-free diet was observed on the lifetimes of these probes either in erythrocyte or in microsomal lipid vesicles or whole native membranes (data not shown). As lifetime heterogeneity limits the interpretation of the differential polarized phase data, as was discussed in the already-mentioned report (18), we studied the effect of fat-free diet on the rotational properties of these probes in protein-free liposomal preparations in addition to native membranes.

TABLE 2. Effect of essential fatty acid deficiency on acyl group composition of the main glycerophospholipid classes of liver microsomes

	Phosphatidylcholine		Phosphatidylethanolamine		Phosphatidylinositol	
Fatty Acid	Controls	Fat-Deficient	Controls	Fat-Deficient	Controls	Fat-Deficient
	mol % ± SD		*mol % ± SD*		*mol % ± SD*	
16:0	26.2 ± 1.7	27.3 ± 2.2 [a]	19.1 ± 1.9	20.4 ± 3.2[a]	14.5 ± 5.0	9.4 ± 1.3[b]
16:1 n-7	0.7 ± 0.3	3.4 ± 0.3[c]	0.8 ± 0.6	2.0 ± 1.0[b]	1.3 ± 0.6	1.0 ± 0.4[a]
18:0	21.6 ± 1.5	17.9 ± 1.0[d]	28.0 ± 3.4	24.7 ± 2.0[a]	39.7 ± 6.3	45.5 ± 0.5[b]
18:1 n-9	5.3 ± 0.6	14.8 ± 0.9[c]	6.4 ± 1.9	8.8 ± 0.9[b]	5.5 ± 2.0	4.0 ± 0.3[a]
18:2 n-6	13.9 ± 2.1	7.8 ± 1.2[c]	10.8 ± 2.4	4.9 ± 0.9[c]	5.1 ± 3.2	1.1 ± 0.2[d]
20:3 n-9		3.5 ± 1.5[c]		3.0 ± 1.2[c]		13.3 ± 3.8[c]
20:3 n-6	0.2 ± 0.1	1.5 ± 0.6[d]	0.4 ± 0.4	0.7 ± 0.5[a]	0.6 ± 0.5	0.3 ± 0.5[a]
20:4 n-6	25.6 ± 1.9	18.2 ± 1.9[c]	22.3 ± 1.7	23.6 ± 1.4[a]	28.3 ± 4.5	22.6 ± 3.7[b]
22:3 n-3	0.9 ± 0.3	0.6 ± 0.7[a]	2.8 ± 1.3	0.8 ± 0.6[d]	1.4 ± 0.9	0.5 ± 0.2[b]
22:4 n-3	3.5 ± 0.6	2.0 ± 0.6[d]	6.1 ± 1.0	3.5 ± 1.2[d]	2.5 ± 1.3	1.1 ± 0.4[b]
22:5 n-3	0.5 ± 0.2	0.5 ± 0.2[a]	0.7 ± 0.3	1.1 ± 0.5[a]	0.4 ± 0.3	0.4 ± 0.2[a]
22:6 n-3	1.5 ± 0.4	2.6 ± 0.4[d]	2.6 ± 0.5	6.5 ± 1.4[c]	0.6 ± 0.5	0.9 ± 0.5[a]

[a] Nonsignificant difference.
[b] $P < 0.05$.
[c] $P < 0.001$.
[d] $P < 0.01$.

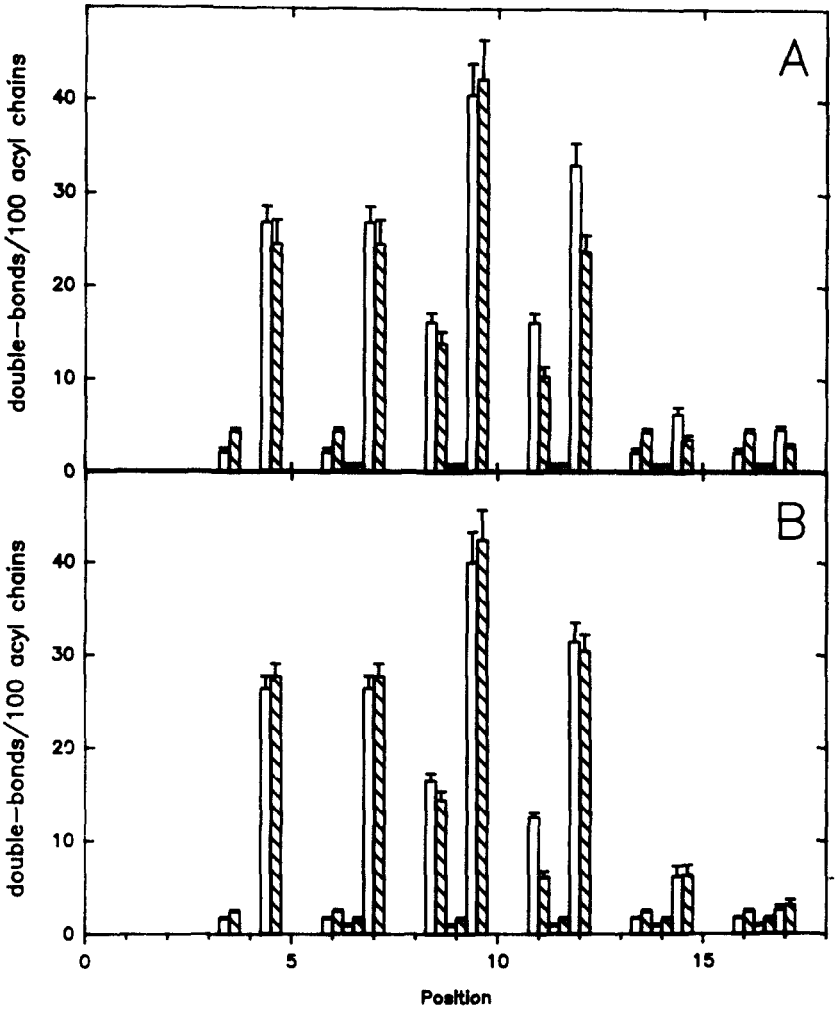

Fig. 2. Effect of essential fatty acid deficiency on the transverse double-bond distribution from the interphase to the deep interior of the lipid bilayer in liver microsomes (A) and in erythrocyte membranes (B). Position corresponds to the carbon number of an saturated acyl chain in *sn*-1. Polyunsaturated fatty acids were considered to be acylated to the phospholipid *sn*-2 position and chain bend (51, 52) was taken into account. Acyl chain shortening (50% of the single-bond axial length) for each double bond (3) was considered; (□) control group; (▩) fat-deficient group.

Only in the lipid vesicles can the anisotropic motion of these probes be resolved from the differential polarized phase data. In native membranes, only rough approximations to the average values of τ_r and r_∞ can be calculated (18). We report in this paper only those data obtained with the liposomal preparations as the same conclusions can be extracted with respect to the effect of fatty acid deficiency from the data obtained in the whole native membranes.

The steady-state anisotropy (r_s), the rotational correlation time (τ_r), and the limiting anisotropy (r_∞) for DPH in liposomes from normal and fat-deficient liver microsomes and erythrocyte membranes are shown in **Figs. 3 A, B, and C,** respectively; τ_r is slightly higher and r_∞ is lower indicating that the DPH environment is less ordered but somewhat more viscous in microsomal than in erythrocyte lipid vesicles as was reported (18). This is possibly due to the higher cholesterol content present in erythrocyte membranes as compared with liver microsomes (29). However, Fig. 3 shows that the fat-free diet had no significant effect on the rotational properties of DPH either in microsomal or in erythrocyte lipid vesicles. Similar results were obtained at 30 MHz excitation frequency (not shown). Measurements in the native microsomal and erythrocyte membranes (not shown), although more limited in their interpretation due to the lifetime heterogeneity, also show no significant differences between normal and fat-deficient animals. Thus, the change in the double-bond transverse distribution produced by the essential fatty acid deficiency seems to have no effect either on the ordering or on the viscous resistance for the DPH rotation.

The most probable location of DPH is with its sym-

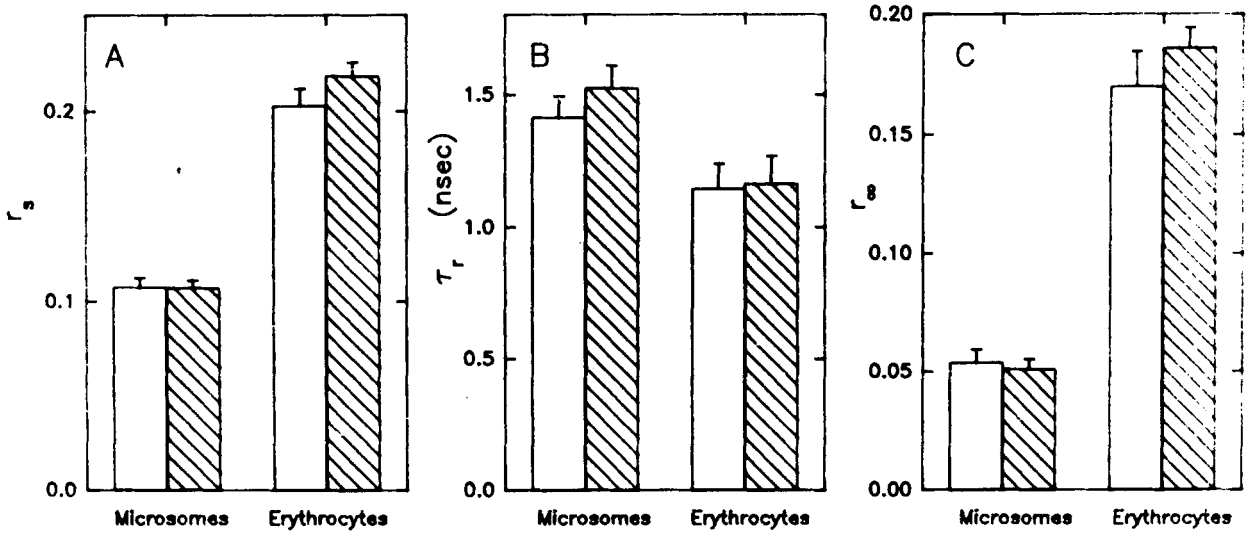

Fig. 3. Effect of essential fatty acid deficiency on DPH rotation in liposomes prepared with the total lipid extracts of liver microsomes and erythrocytes. A) Steady-state anisotropy (r_s); B) correlation time (τ_r); and C) limiting anisotropy (r_∞). τ_r and r_∞ were calculated as indicated in Experimental from differential phase shift measurements at 18 MHz; (□) control group; (▨) fat-deficient group.

metrical long axis normal to the plane of the membrane (30) (Fig. 1). This probe seems to be distributed in a quite wide range along the membrane normal axis as indicated by energy transfer studies (31) and suggested also by their wider lifetime distribution (32) as compared with DPH-phosphatidylcholine (33), and is likely to sense almost the total hydrophobic lipid matrix. Thus, DPH could be insensitive to the probably localized structural or viscotropic changes that could result from the altered double-bond transverse distribution produced by the essential fatty acid deficiency. For this reason, we have also used 2-, 7-, and 12-AS probes. They locate the fluorescent anthroyl moiety at different depths in the lipid bilayer as indicated by quenching experiments (34–36) and thus should be sensitive to localized alterations in the structural or dynamical properties of the membrane.

Figure 4 and **Figure 5** show the effect of fat-free diet on the rotational parameters of 2-, 7-, and 12-AS in microsomal lipid vesicles and in erythrocyte lipid vesicles, respectively. For the three n-AS in normal and fat-deficient microsomal and erythrocyte lipid vesicles, r_∞ values obtained at 318 nm excitation do not differ significantly from zero (Figs. 4C and 5C) indicating that the "out of the plane" mode of rotation is completely unhindered. It is in agreement with previous observations made by using this methodology (18) or nanosecond time-resolved fluorometry (17, 27) in protein-free lipid vesicles. As discussed (18), the major contribution to this unhindered "out of the plane" motion is the rotation about the axis aligned with the stearate acyl chain which would be located parallel to the phospholipid acyl chains. At 388 nm excitation, discrete r_∞ values were obtained (Figs. 4F and 5F) indicating a hindered movement. At 388 nm both rotational modes, "in" and "out" of the plane, contribute to the depolarization. As the "out of the plane" mode is completely unhindered, the "in the plane" mode should be hindered and the only contribution to the r_∞ value observed at 388 nm excitation. It was proposed (18) that the hindered "in the plane" motion corresponds mainly to the wobbling of the stearate acyl chain which would reproduce the wobbling of the phospholipid acyl chains.

In erythrocyte lipid vesicles, the range for 2-, 7-, and 12-AS wobbling is small indicating a more ordered structure as is evident from the higher r_∞ values obtained at 388 nm excitation wavelength (Fig. 5F) compared with microsomal lipid vesicles (Fig. 4F). Also, erythrocyte lipid vesicles have a more viscous microenvironment mainly in the region sensed by 7-AS for the unhindered rotation about the stearate acyl chain as can be seen by comparing the τ_r values in Figs. 4B and 5B. The higher cholesterol content of erythrocyte membranes in comparison with microsomes (20) could be the reason for these differences. However, only a minor change reaching the 5% level of significance by the Student's *t* test is produced by the essential fatty acid deficiency. This indicates a 15% reduction in the correlation time obtained at 318 nm excitation wavelength for the 7-AS probe in erythrocyte lipid vesicles (Fig. 5B). This is possibly due to a slight increase in the rate of the rotation about the stearate acyl chain. Figs. 4 and 5 show that, with the above mentioned exception, there are no significant differences between normal and fat-deficient rats for the parameters that describe the rotational dynamic of 2-, 7-, and 12-AS either in microsomal or in erythrocyte lipid vesicles. In native microsomes and erythrocyte membranes (data not shown), the approximately calculated τ_r and r_∞ values were also not

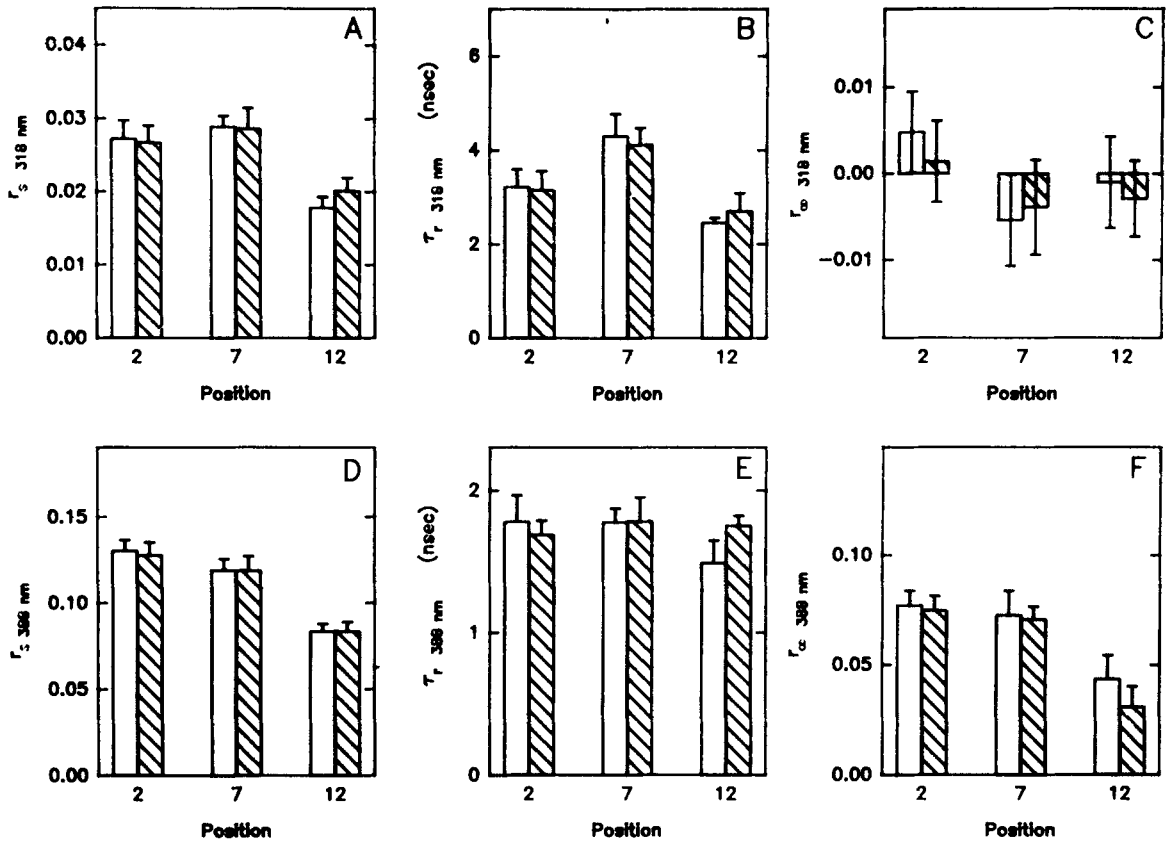

Fig. 4. Effect of essential fatty acid deficiency on the mobility of n-AS probes in microsomal lipid vesicles. A, B, and C) Parameters obtained at 318 nm excitation wavelength ("out of the plane" motion). D, E, and F) Parameters obtained at 388 nm excitation wavelength ("in and out of the plane" motion). A and D) Steady-state anisotropy (r_s). B and E) Correlation time (τ_r). C and F) Limiting anisotropy (r_∞). τ_r and r_∞ were calculated as indicated in Experimental from differential phase shift measurements at 18 MHz; (□) control group; (▨) fat-deficient group.

significantly different between normal and fat-deficient animals. By measuring at 30 MHz excitation frequency (data not shown), although somewhat different parameters were obtained in comparison with those at 18 MHz, no significant differences were observed between control and fat-deficient animals. This indicates that the altered transverse double-bond distribution produced by the fatty acid deficiency does not bring about great changes in the ordering and acyl chain dynamics in the regions sensed by these probes.

DISCUSSION

For many years, the relationship between fatty acid unsaturation and physical properties of membrane lipid phase has been, and still is, a controversial subject. Introduction of a *cis* double bond into the lipid matrix affects the physical organization of the lipid bilayer. It disrupts the cooperativity of the tightly packed saturated acyl chains by increasing the cross-sectional area per acyl chain as indicated by monolayer studies (37–39) (reviewed in references 40 and 41). It also results in an increase in both range and rate of wobbling of general probes as DPH (42). However, no simple relationship exists between double-bond number and lipid matrix properties. The effect of the first introduced double-bond is more notorious than that of subsequent ones (39–42). Less attention has been paid to the double-bond position, although in some cases it may have a more important influence than the actual double-bond number (41). Melting temperatures of 18:1 and 18:2 acids (43, 44) and phase transition temperatures of di18:1-phosphatidylcholines (45) indicate a maximal double-bond effect when it is located at the center of the acyl chain. Consequently, more attention should be paid to the transversal distribution of double bonds in the lipid matrix.

The fatty acids present in plant phospholipids are rich in double bonds near the terminal methyl group, i.e., in the internal deep region of the membrane bilayer. Mammals take up these fatty acids and modify them by introducing extra double bonds toward the carboxyl group, i.e., toward the external region of the membrane bilayer. In this respect, the biochemical phenomena are well

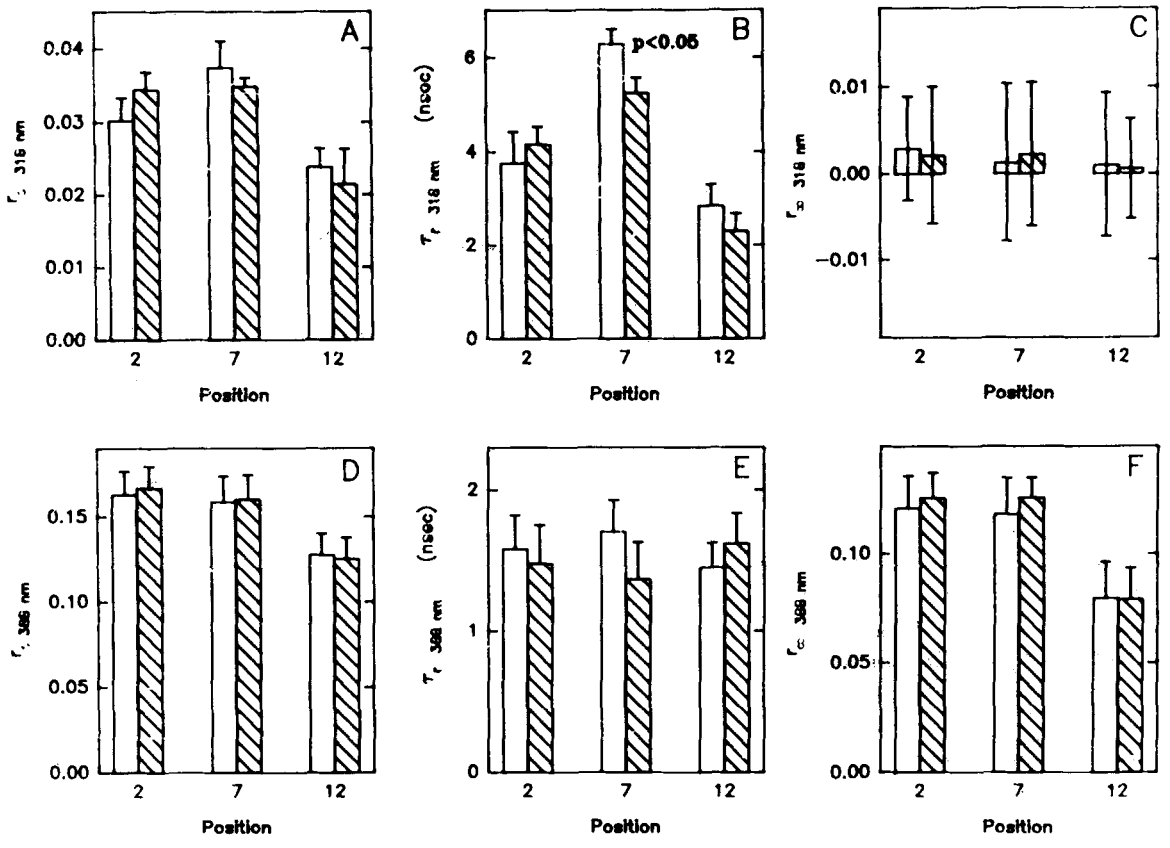

Fig. 5. Effect of essential fatty acid deficiency on the mobility of n-AS probes in erythrocyte membrane lipid vesicles. A, B, and C) Parameters obtained at 318 nm excitation wavelength ("out of the plane" motion). D, E, and F) Parameters obtained at 388 nm excitation wavelength ("in and out of the plane" motion). A and D) Steady-state anisotropy (r_s). B and E) Correlation time (τ_r). C and F) Limiting anisotropy (r_∞). τ_r and r_∞ were calculated as indicated in Experimental from differential phase shift measurements at 18 MHz; (□) control group; (▨) fat-deficient group.

known (46), but nothing is known concerning the importance of these double bonds for the physical properties and function of animal cellular membranes.

Essential fatty acid deficiency results in large changes in the fatty acyl pattern of membrane lipids as shown in Tables 1 and 2 and in many other reports (3, 8–14). However, it is necessary to remark that relatively high levels of essential n-3 and n-6 acids are still present even after long deprivation periods, suggesting the existence of mechanisms that preserve these essential fatty acids.

The results shown here suggest that essential fatty acid deficiency does not result in largely altered lipid matrix physical properties of microsomal and erythrocyte membranes, at least with the probes and the conditions that were used. It must be noted that the measurements in liposomes of extracted membrane lipids can be obscured because of the presence of transbilayer (47) as well as lateral (48) lipid domains in the native membranes that can be scrambled in liposomes of extracted lipids. Moreover, it should also be noted that Wang et al. (49), by using an orientational distribution analysis, found that the factor of DPH orientational distribution greatly impacts the rotational rate and rank order parameters. Consequently, equation 1 is limited and multifrequency measurements would be required to fully describe the rotational behavior. On the other hand, the results obtained in the native membranes are limited in their interpretation because of the lifetime heterogeneity and it is not possible to assess whether some differences between normal and fat-deficient membranes would not be observed by using more sensitive multifrequency phase-modulation measurements or by using probes localized in specific domains. However, very important changes in lipid matrix structure and/or dynamics would result in appreciably altered parameters such as steady state anisotropy, and/or differential polarized phase shift of these "bulk lipid probes" used here which are apparently distributed randomly in the lipid bilayer, although this was not observed.

Essential fatty acid deficiency results in an altered steady-state anisotropy of DPH in guinea pig liver microsomes (10–14), but in that case a decrease in the total degree of unsaturation could be the reason. The fact that fat deprivation does not produce an increase in 20:3n-9

in guinea pigs as in rats suggests that this nonessential trienoic acid could play a role in the observed compensation of the average physical properties of rat membranes. In piglet jejunum brush border membranes, essential fatty acid deficiency brings about a general decrease in the order parameter reported by a set of doxylstearate probes (3). In this case, the total double-bond number is not changed, suggesting that changes in physical properties are possibly due to an altered double-bond transverse distribution. The present results in liver microsomes and erythrocyte membranes, however, do not agree with those obtained by electron spin resonance in piglet intestinal brush border membranes. We do not know the reasons for such discrepancies, but some differences can be noted. In piglet brush border membranes (3), contrary to rat liver microsomes and erythrocytes, essential fatty acid deficiency does not reduce the arachidonic acid content, which is also found in low proportions. Besides, in piglet brush border membranes, the fatty acyl distribution is changed similarly in all the glycerophospholipid classes (3). On the other hand, it is shown here that changes in fatty acid composition produced by the fat-free diet are very different in each phospholipid fraction, and possibly this could play a role in the observed compensation of the membrane lipid matrix structural and dynamic properties. A surprising observation made here was the fact that a high proportion of 20:3n-9 substituted for 20:4n-6 in the phosphatidylinositol fraction in the fat-deficient microsomes.

On other hand, the reason for the existence of a large variety of lipid molecules found in biological membranes represents another problem not yet solved. The lipid and fatty acid composition is unique for each membrane type and to a great degree, it is independent of species (50), suggesting that lipid requirements for a fully functional membrane are highly specific. However, the opposite conclusion has also been drawn. It has been argued that the presence of a wide variety of lipid species in the membrane demonstrates that the exact lipid composition is unimportant and that within relatively broad limits any lipid mixture will do as well as any other (7). The results shown here indicate that in spite of large changes in the fatty acid composition of membrane phospholipids, the average structural and dynamic properties of the lipid phase are nearly invariably maintained. This finding minimizes on one hand the importance of an exact lipid composition, but it suggests on the other hand that fine metabolic regulations would maintain the lipid matrix physical properties within relatively narrow limits.

These results cannot disregard possible transbilayer or lateral rearrangements of lipids as a result of essential fatty acid deficiency, and this fact needs to be investigated in further experiments.

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