Early, A., McRobbie, S. J., Duffy, K. T., Jermyn, K. A., Tilly, R., Ceccarelli, A., & Williams, J. G. (1988) *Dev. Genet. (N.Y.) 9*, 383–402.
Giorda, R., & Ennis, H. L. (1987) *Mol. Cell. Biol. 6*, 2097–2103.
Giorda, R., Ohmachi, T., & Ennis, H. L. (1989) *J. Mol. Biol. 205*, 63–69.
Giri, J. G., & Ennis, H. L. (1978) *Dev. Biol. 67*, 189–201.
Hemmes, D. E., Kohma-Buddenhagen, E. S., & Hohl, H. (1972) *J. Ultrastruct. Res. 41*, 406–417.
Henikoff, S. (1984) *Gene 28*, 351–359.
Jaynes, J. B., & O'Farrell, P. H. (1988) *Nature 336*, 744–749.
Kelly, R., & Ennis, H. L. (1985) *Mol. Cell. Biol. 5*, 133–139.
Kelly, L. J., Kelly, R., & Ennis, H. L. (1983) *Mol. Cell. Biol. 3*, 1943–1948.
Kimmel, A. R., & Firtel, R. A. (1982) in *The Development of Dictyostelium discoideum* (Loomis, W. F., Ed) pp 233–324, Academic Press, Inc., New York.
Landschultz, W. H., Johnson, P. F., & McKnight, S. L. (1989) *Science 243*, 1681–1688.
Nellen, W., Silan, C., & Firtel, R. A. (1984) *Mol. Cell. Biol. 4*, 2890–2898.
Pearson, W. R., & Lipman, D. J. (1988) *Proc. Natl. Acad. Sci. U.S.A. 85*, 2444–2448.
Shaw, D. R., Richter, H., & Ennis, H. L. (1986) *Dev. Biol. 117*, 636–643.
Shi, Y., Son, H. J., Shahan, K., Rodriguez, M., Constantini, F., & Derman, E. (1989) *Proc. Natl. Acad. Sci. U.S.A. 86*, 4584–4588.
Sussman, M., & Brackenbury, R. (1976) *Annu. Rev. Plant Physiol. 2*, 229–265.
Tucker, M. L., Durbin, M. L., Clegg, M. T., & Lewis, L. N. (1987) *Plant Mol. Biol. 9*, 197–203.
Turner, R., & Tjian, R. (1989) *Science 243*, 1689–1694.
Warrick, H. M., & Spudich, J. A. (1988) *Nucleic Acids Res. 16*, 6617–6635.
White, T. J., Arnheim, N., & Erlich, H. A. (1989) *Trends Genet. 5*, 185–189.
Yanisch-Perron, C., Vieira, J., & Messing, J. (1985) *Gene 33*, 103–119.

Evidence for a Structurally Specific Role of Essential Polyunsaturated Fatty Acids Depending on Their Peculiar Double-Bond Distribution in Biomembranes†

C. L. Lêger,*,‡ D. Daveloose,§ R. Christon,‡ and J. Viret§
Station de Recherches de Nutrition, Centre de Recherches de Jouy-en-Josas, INRA, 78350 Jouy-en-Josas, France, and Département de Biophysique, CRSSA, B.P. 87, 38702 La Tronche Cedex, France
Received July 10, 1989; Revised Manuscript Received March 20, 1990

ABSTRACT: ESR spectrometry with 5-, 7-, 10-, and 12-doxylstearate probes and a combined index considering separately the double-bond numbers of essential and nonessential fatty acids were used to investigate the structural role of the double bonds of polyunsaturated fatty esters in membrane phosphoglycerides. Purified brush border membrane vesicles were prepared from the jejunum of piglets receiving either high (HLA) or low (LLA) dietary levels of linoleic acid (18:2 *n*–6). In the LLA as compared to the HLA group, there were no significant modifications of (a) the relative contents of cholesterol, phospholipid, and protein and of (b) the phosphoglyceride class distribution, contrasting with very large changes in the fatty acid compositions of each phosphoglyceride. These changes were characterized by an increase in nonessential monoene and triene (18:1 *n*–9 and 20:3 *n*–9) and a decrease in essential diene (18:2 *n*–6) in LLA- as compared to HLA-fed piglets. The essential tetraene 20:4 *n*–6 remained rather constant despite an overall nonsignificant increase in the LLA group. The total double-bond number (TDBn) was not significantly affected, contrasting with the variations in the double-bond numbers of essential and nonessential fatty acids (DBn_{EFA} and DBn_{nonEFA}, respectively). The combined DBn_{EFA}/DBn_{nonEFA} index was 1.7–3.3 times lower in LLA than in HLA membrane phospholipids. It was concluded that the diet was able to affect the double-bond distribution in the upper and inner half-parts of the membrane leaflet without changing the total number of double bonds. Concomitantly, besides a general decrease in the order parameter with the lipid matrix depth (the order profile), a shape change in the order profile was observed in a comparison of LLA to HLA piglet membranes. Therefore, it was tempting to consider these modified profile shapes as organizational consequences of changes in the double-bond depth-directed distribution. This supports the idea that the position of double bonds in the membrane depth could play a major structural role, providing the essential polyunsaturated fatty acids (EPUFA) with specific features due to their peculiar double-bond distribution and thus emphasizing the "non-eicosanoid" EPUFA function(s).

The most common conformation of lipids in biomembranes is known to be the bilayer structure (Luzzatti, 1968). In the outer faces of the bilayer, cohesion mainly originates both in the covalent anchorage of chains on the glycerol backbone and in the hydrophilic interactions of the head groups. Deeper in the interior of membranes, it is brought about by the hydrophobic interactions between the closest hydrocarbon chains (Lenaz & Castelli, 1985). These interactions near the surface and deeper have mutual stabilizing effects. They are modulated by chemically functional different groups of the phospholipid molecular structure, i.e., the nature of the polar head

† Partially supported by grants from the National Institute for Agricultural Research (AIP-INRA 87/4613) and the Interdisciplinary Environment Research Programme "Food-Health-Environment" (PIREN-CNRS).
* To whom correspondence should be addressed.
‡ INRA.
§ CRSSA.

0006-2960/90/0429-7269$02.50/0

at the outer face of membrane and the double bonds of hydrocarbon chains in the inner part of it. The cholesterol molecule is also known to influence the structural properties of membrane lipids, but this is not the subject of this paper.

In this paper attention will be focused on the role of double bonds in polyunsaturated fatty acids almost exclusively esterified on the *sn*-2 positions of membrane phosphoglycerides. Concerning the spatial organization of the lipid bilayer, we shall examine the peculiar features of double bonds of non de novo synthetized fatty acids (i.e., essential polyunsaturated fatty acids or EPUFA), well-known to be distributed according to particular rules in the acyl chains. To that end, physical and biochemical information was obtained by studying (i) the variations in the order parameter S estimated from ESR spectra and (ii) the variations in a combined index indicating the distribution of double bonds toward the lipid matrix depth. Together with modifications of the membrane physical properties, it was of great importance to observe the coincidence between the specific chemical structure and the essentiality of chains, i.e., the metabolic origin and the specific physiological role.

Need for clarification of the role of double bonds is more marked today because of a more comprehensive understanding of the PUFA function as biooxygenated derivative precursors. Interestingly, only a very small fraction of the EPUFA requirements in mammals and humans can probably be explained by this precursor role (Hansen, 1983). Accordingly, questions concerning the size and nature of the membrane phospholipid pool providing the immediate precursors (i.e., PUFA) of oxygenated derivatives (eicosanoids and docosanoids) as well as the "non-eicosanoid" function(s) of EPUFA (Mead et al., 1986a,b) are insistently raised nowadays.

We shall examine whether new data are in favor of a physiological and specific role of EPUFA as structural elements of the biological membranes. Attention will be focused on the possibility of specific changes in the organizational properties of the lipid matrix—i.e., average spatial configuration (order) of structural elements—after EPUFA incorporation into the lipid matrix. This is fully justified by the nonrandom spatial distribution of the double bonds of the hydrocarbon chain in biomembranes due to (i) the asymmetric (transverse and lateral) distribution of the EPUFA-containing phospholipids [Szamel et al., 1987; see also discussions and references cited in Kleinfeld (1987) and Longmuir (1987)] and (ii) the multitude of molecular species of these phospholipids, differing by their fatty acid composition and distribution in their *sn*-1 and *sn*-2 positions (ether phospholipids are also to be mentioned).

As we know, food is generally able to modify the lipid composition of biomembranes (Spector, 1985; Stubbs & Smith, 1984; McMurchie, 1988). This phenomenon is used here to specifically and physiologically change the nature of the phospholipid hydrocarbon chains in plasma membranes. Under these conditions we will examine the role of the membrane fatty acid unsaturation and more particularly the effect of change in the double-bond distribution caused by a decrease in the linoleic acid (a *n*–6 fatty acid) and an increase in oleic and eicosatrienoic acids (*n*–9 fatty acids) on the order (or packing) of the membrane hydrophobic part located at the C5–C12 matrix depth.

Materials and Methods

Animals and feed used in the present experiment have been described elsewhere (Léger et al., 1989; Christon et al., 1989). Briefly, after being weaned (35 days of age) animals were fed for 6 weeks a diet containing 7% of either corn oil or hydrogenated coconut oil for the high (HLA) and low dietary linoleic acid group (LLA), respectively. The dietary levels of linoleic acid were 3.4% and 0.3% (in weight), respectively, for the two groups.

Purified brush border membrane vesicles (PBMV) were obtained from jejunum fragments by the slightly modified (Brot-Laroche & Alvarado, 1984) Mg^{2+}/EGTA precipitation method (Hauser et al., 1978). Storage was at −80 °C after freezing in liquid nitrogen. Neutral lipids, glycolipids, and phospholipids from the total lipid extract (Folch et al., 1957) were separated by a Sep-Pak procedure (Juaneda & Rocquelin, 1985). Protein and phospholipid quantitative determinations were carried out according to Lowry et al. (1951) and Bartlett (1959), respectively. Cholesterol was determined with an enzymatic procedure (Biochemica test combination, Boehringer). Phospholipid classes were separated by a technique developed in the laboratory (Christon et al., 1989). Their purity was checked (Gilfillan et al., 1983), and a particular attention was paid on their transmethylation conditions.

Fatty acid composition of phospholipid classes was assessed as already reported (Léger et al., 1989) on a CP WAX 52 CB bonded fused silica capillary column (50 m × 0.2 mm).

ESR measurements were carried out according to Léger et al. (1989). Membrane lipid spin-labeling used the 4,4′-dimethyl-3-oxazolidinyloxy (called doxyl) group introduced in the following molecular forms: 5-, 7-, 10-, or 12-doxylstearic acid (purchased from Molecular Probes). The spin-labels were incorporated into PBMV according to Hubbel and McConnell (1971). Spectra were interpreted by measuring the order parameter S, as already reported (Seelig, 1970; Hubbel & McConnell, 1971).

Results

When purified brush border membrane vesicles (PBMV) from HLA and LLA animals (HLABMV and LLABMV, respectively) were compared, it was previously observed (Christon et al., 1989) that there were no significant modifications of the relative concentrations of membrane proteins, cholesterol, and phospholipids, leading to the same cholesterol/phospholipid molar ratio. The relative amounts were found to be 0.01 ± 0.01 and 0.08 ± 0.00 for the cholesterol/protein ratio (w/w), 0.34 ± 0.01 and 0.29 ± 0.07 for the phospholipid/protein ratio (w/w), and 0.65 ± 0.05 and 0.62 ± 0.08 for the molar cholesterol/phospholipid ratio in HLABMV and LLABMV, respectively. In the same way, no phospholipid compositional changes were observed (see Table I).

In contrast, highly different fatty acid distributions appeared in the four studied phosphoglyceride classes when LLABMV were compared to HLABMV, except for arachidonic acid (AA, 20:4 *n*–6) which remained rather constant in spite of a general nonsignificant increase in LLABMV. The most important differences concerned almost exclusively the proportions of oleic acid (OA, 18:1 *n*–9), linoleic acid (LA, 18:2 *n*–6), and eicosatrienoic acid (ETA, 20:3 *n*–9). Especially, the LA/OA ratio was 3–6 times lower in LLABMV than in HLABMV whereas no significant change in the values of the total double-bond number (TDBn) and the TDBn/total saturated fatty acids (TDBn/TSFA) ratio was obtained. Moreover, the decreasing values of the double-bond number of the essential fatty acids (DBn_{EFA}) and the increasing values of double-bond number of the nonessential fatty acids (DBn_{nonEFA}) balanced each other, producing highly modified values for the DBn_{EFA}/DBn_{nonEFA} ratio (R) in LLABMV as compared to HLABMV. Biochemically, this was to a large extent due to the nonessential ETA present in LLABMV.

Table I: Simplified Fatty Acid Composition of Glycerophospholipids (GPL) of Enterocyte BBM[a]

	choline GPL		ethanolamine GPL		serine GPL		inositol GPL	
	HLA	LLa	HLA	LLA	HLA	LLA	HLA	LLA
% GPL	32.6[b]		32.5		10.1		7.8	
18:1 *n*-9	15.9 ± 3.6	28.8 ± 3.6	17.9 ± 1.8	28.1 ± 0.9	15.1 ± 5.6	24.5 ± 3.3	12.9 ± 0.5	22.8 ± 1.2
20:3 *n*-9		1.6 ± 0.4	0.1 ± 0.0	4.3 ± 0.4		1.1 ± 0.3		4.6 ± 1.5
18:2 *n*-6	27.4 ± 2.0	8.1 ± 2.3	26.3 ± 0.6	8.0 ± 2.0	13.4 ± 0.1	7.3 ± 1.8	18.6 ± 6.1	5.8 ± 2.2
20:4 *n*-6	3.3 ± 0.1	4.0 ± 0.9	10.0 ± 0.4	10.5 ± 0.5	2.0 ± 0.5	4.0 ± 0.8	9.7 ± 0.3	11.1 ± 0.0
TDBn	141.6 ± 0.1	132.1 ± 7.0	182.0 ± 2.2	163.9 ± 5.5	155.9 ± 11.0	135.2 ± 3.6	159.1 ± 1.0	178.1 ± 5.4
TDBn/TSFA	2.96 ± 0.1	2.90 ± 0.4	5.64 ± 0.5	5.42 ± 0.4	3.33 ± 0.8	2.69 ± 0.2	3.69 ± 0.2	4.40 ± 0.3
DBn_{EFA}	78.4 ± 0.1	55.7 ± 4.3	131.4 ± 1.3	95.3 ± 3.8	92.9 ± 8.5	59.5 ± 2.5	104.4 ± 0.8	105.7 ± 4.1
DBn_{nonEFA}	18.6 ± 0.1	44.0 ± 5.6	22.0 ± 0.9	51.6 ± 3.2	27.7 ± 7.1	33.7 ± 1.5	23.2 ± 0.6	41.5 ± 2.5
R[c]	4.22	1.26	5.97	1.85	3.36	1.77	4.5	2.55

[a] Only biochemically and physiologically more significant fatty acids are presented here, in mol % of total fatty acids (means ±SEM for three membrane preparations per dietary group: HLA or LLA). DB (i.e., TDBn, TDBn/TSFA, ...) take into account the complete fatty acid composition (not shown here). [b] The indistinguishable values for HLA and LLA led to them being pooled. [c] $R = DBn_{EFA}/DBn_{nonEFA}$.

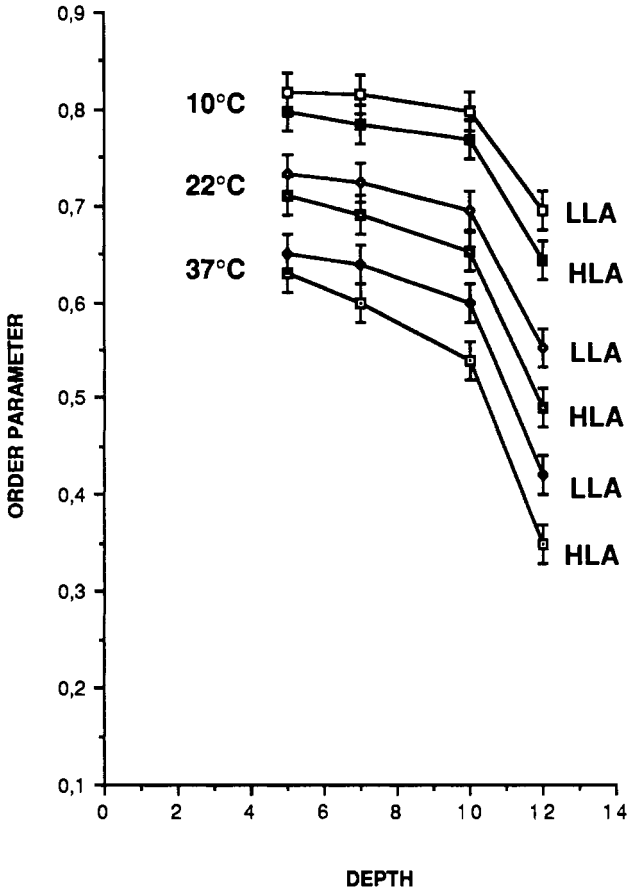

FIGURE 1: Evolution of the order parameter, S, with the depth in the lipid bilayer. LLA and HLA indicate the origin of the purified brush border membranes of enterocytes: LLABMV and HLABMV, respectively (see text). The 5-, 7-, 10-, and 12-doxylstearate probes are used for assessing the order parameters at C5, C7, C10, and C12 depths of the lipid matrix, respectively. Assay temperatures are indicated. Each point with vertical bars represents the mean ± SE.

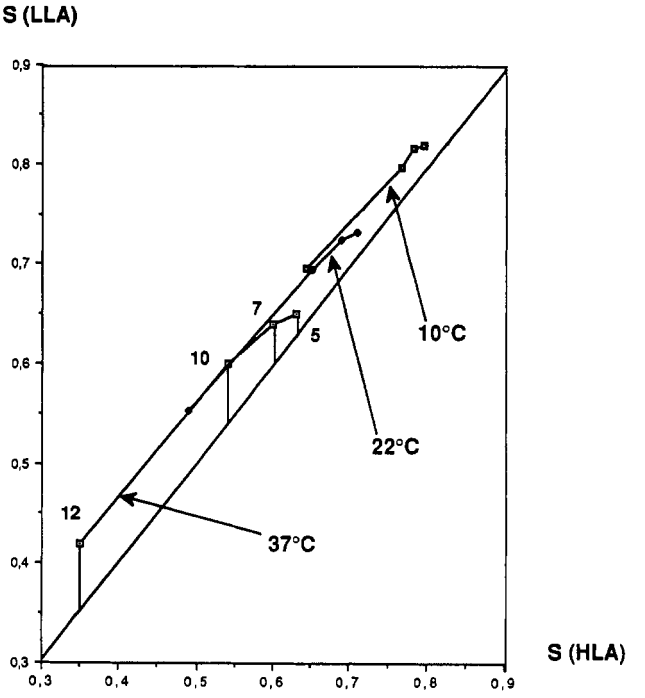

FIGURE 2: Direct comparisons of the order parameters, for each depth indicated near the curve, between LLA and HLA membranes at 37, 22, and 10 °C. The vertical lines joining each point to the diagonal represent the differences $S_{LLA} - S_{HLA}$ by the graphic construction.

Table II: Order Parameter by C–C Unit of Depth[a]

	membranes	
depth	HLA	LLA
Upp[b]	0.018 ± 0.005	0.010 ± 0.005
Inn[b]	0.095 ± 0.014	0.090 ± 0.014
Inn/Upp	5.3[c]	9.0[d]

[a] Obtained by dividing the variation of S at 37 °C by the number of C–C sections of the hydrocarbon chain between C5 and C10 and between C10 and C12. [b] Upp and Inn correspond to C5–C10 and C10–C12, respectively. [c] 10.7 at 10 °C. [d] 11.9 at 10 °C.

Figure 1 shows the order profiles of PBMV at 37, 22, and 10 °C. HLABMV were clearly less ordered than LLABMV, regardless of temperatures and studied depths. However, beyond this general observation, it is possible to collect more information on the membrane-ordering diet-dependent changes. Figure 2 shows at 37 °C the following: (i) the four points corresponding to the four depths explored (C5, C7, C10, and C12) were located above the diagonal, which was a consequence of the more ordered structure of LLABMV as compared to HLABMV at each depth; (ii) at the same depth, the difference between the order parameters ($S_{LLA} - S_{HLA}$) progressively increased from C5 to C10 and reached a constant value from C10 to C12; (iii) as shown by the curve slope variation, decreases in S_{LLA} and S_{HLA} were very similar between C10 and C12, but more marked for S_{HLA} than for S_{LLA} between C5 and C10. These differences were greatly reduced at 22 and 10 °C.

Data from Table II illustrate the order parameter variation per unit of depth—expressed as a C–C section of the hydrocarbon chain[1]—in the C5–C10 (or upper) and C10–C12 (or inner) regions. It clearly shows that the gradient of disorder was much higher in HLABMV than in LLABMV in the upper

[1] This form of expression corresponds to an order gradient in the cross section of the lipid matrix.

region, while it was identical for both membrane preparations in the inner region. As a consequence, the values of the inner/upper (Inn/Upp) gradient ratios were 5.3 and 9.0, respectively, in HLABMV and LLABMV, indicating a much greater contrast between these two regions with LLABMV than with HLABMV. The decreasing temperatures enhanced this contrast in both types of membranes. However, this was more pronounced in HLABMV, resulting in an equal contrast at 10 °C in HLABMV and LLABMV.

Discussion

The relative amounts of cholesterol, phospholipids, and proteins were similar to those obtained in the pig (Christiansen & Carlsen, 1981) but rather different from those of the rat (Chapelle & Gilles-Baillien, 1983; Brasitus et al., 1984, 1988; Meddings, 1989) brush border membrane, essentially due to a higher phospholipid/protein ratio in pig PBMV. Thus, these data seem to largely depend on the animal species. It is of interest to notice that (i) the diet appears not to be able to modify these ratios [see discussion in Christon et al. (1989a)] and (ii) recent findings allow us to assess the complete stability of the PBMV–protein electrophoretic profile in similar dietary conditions (Christon et al., 1989b).

Concerning the fatty acid composition, little is known on the PBMV phospholipid classes. However, it appears that pig PBMV have higher LA and lower AA levels than the rat PBMV (Brasitus et al., 1984; Meddings, 1989). A marked influence of the diet was also observed on the levels of OA, LA, and ETA, which increased, decreased, and appeared, respectively, with the *n*–6 fatty acid depressed regimen, whereas AA remained rather constant, although a slight nonsignificant increase in the level of this *n*–6 fatty acid could be observed. Such a result could be compared with that obtained by Lefkowith et al. (1985) for ethanolamine, serine, and choline phosphoglycerides in the heart and kidney cortex membranes of mouse. This paradoxical response of phosphoglyceride AA to the EPUFA deficiency is most likely a rather general response of extrahepatic membranes without excluding a tissue specificity.

The above data on the PBMV composition show that there are differences between species. On the other hand, the present order parameters (0.63/0.65 at C5 and 0.35/0.42 at C12 for HLABMV/LLABMV) were similar to those obtained in the same way by Hauser et al. (1982) in the rabbit and those reported by Viret and Leterrier (1976) (except for the myelin membrane), who summarized the main results found in the literature on various species.

Introducing double bonds into the lipid matrix affects the physical organization of the lipid bilayer. Although the effect of the first double bond introduced is more marked than that of the subsequent ones (Stubbs et al., 1981; Evans & Tinoco, 1978; Ghosh & Tinoco, 1972), the opportunity for the setting of a complex of structurally different zones within the bilayers exists in the case of multiple double bonds. This leads to particular attention being needed to be paid to the shape of the order profile (i.e., the gradient of the order parameter in the depth direction). Moreover, it may be hypothesized that the successive local configurations (organization gradient) in the cross-sectional direction are of physiological relevance toward enzyme activities, transport, recognition, and signal transduction in the biomembranes. This reinforces the need for assessment of the local physical characteristics.

The order parameter, S, is widely used for describing the order profile (Gruen, 1980). The profile of decreasing order from the surface to the center of the biomembrane lipid bilayer has also been well established [see Seelig and Seelig (1980) and cited papers; Brenner, 1984]. Therefore, it could be interesting to directly compare the S gradient across two types of membranes only differing by the fatty acid composition, which is the case here. A good insight into the discrete profile variations is given by Figure 2.

Figure 3 is a simplified representation of fatty acids, which are the most usual phosphoglyceride constituents in the membrane bilayer. The purpose of this scheme is to show the relative positions of double bonds in the cross-sectional direction. To do this, the bend of chains acylated in the *sn*-2 position of phosphoglycerides (Seelig & Seelig, 1975; Hitchcock et al., 1974) has to be taken into account. For a simplified representation, we choosed the all-antiperiplanar ("trans") conformation of the saturated part of the chains and the all-anticoplanar conformation of the *cis,cis*-1,4-diene groups in unsaturated chains. This is a justified [see data in Mead et al. (1986a,b)] and useful representation. First, the most probable conformation for thermodynamical considerations is the "anti" array as maximalizing stabilizing intermolecular effects. It could also be promoted in the saturated part of chains by the proximity of the rigidifying glycerol backbone and the strong hydrophylic interactions between polar groups at the outer faces of the bilayer structure. Second, this type of representation makes the shortening by each double bond visible and facilitates comparisons between double bonds belonging to different fatty acid series. It is noteworthy that the shortening represents nearly 50% of the single-bond axial length (0.7 vs 1.28 Å) in the present conformation. Accordingly, the first double bonds starting from the hydrocarbon chain extremity close to the bilayer surface are not displaced, contrasting with the subsequent ones. It is clear that (i) there is no shift of the double-bond position (DBp) in 16:1 *n*–7 and 18:1 *n*–9, (ii) the first double bond starting from the methyl extremity is shifted by approximately one C–C bond in 20:3 *n*–9, resulting in a transfer of the double bond in the 11-position from the inner to the outer half-part of the bilayer (approximately delimited by the C10 depth), and (iii) none of the EPUFA are characterized by a similar methyl terminal double-bond shift relative to the C10 depth limit. Hence, the EPUFA are the only fatty acid structures capable of positioning a double bond(s) in the inner half-part of a leaflet of the lipid bilayer. Taking advantage of this feature, we will compare it to other organizational characteristics of the biomembrane matrix and to the contrasted results obtained with the combined index of R and TDBn, as the passage from HLABMV to LLABMV compositional states results in a considerable decrease in R and no change in TDBn.

Cohesion of the inner lipid part of the membrane leaflet is largely due to intermolecular interactions. However, the midregion is characterized by a progressively more marked loss of cohesion between the hydrocarbon chains of phospholipids in the depth direction. This loss of cohesion appears to be due to a progressive divergence in the general parallelism of the saturated hydrocarbon chains, amplified by the presence of double bonds (Seelig & Seelig, 1977; Karabatsos & Fenoglio, 1970; Galli et al., 1979). This divergent magnification is probably more than proportional to the number of double bonds because of the cooperative nature of intermolecular interactions. This could explain the particular shape of each order profile, characterized (see Figure 2) by a progressively more marked difference between S_{HLA} and S_{LLA} from C5 to C10. However, these differences are less marked at 22 and 10 °C than at 37 °C.

As regards more particularly the relationship between the fatty acid structure and spatial organization of the membrane

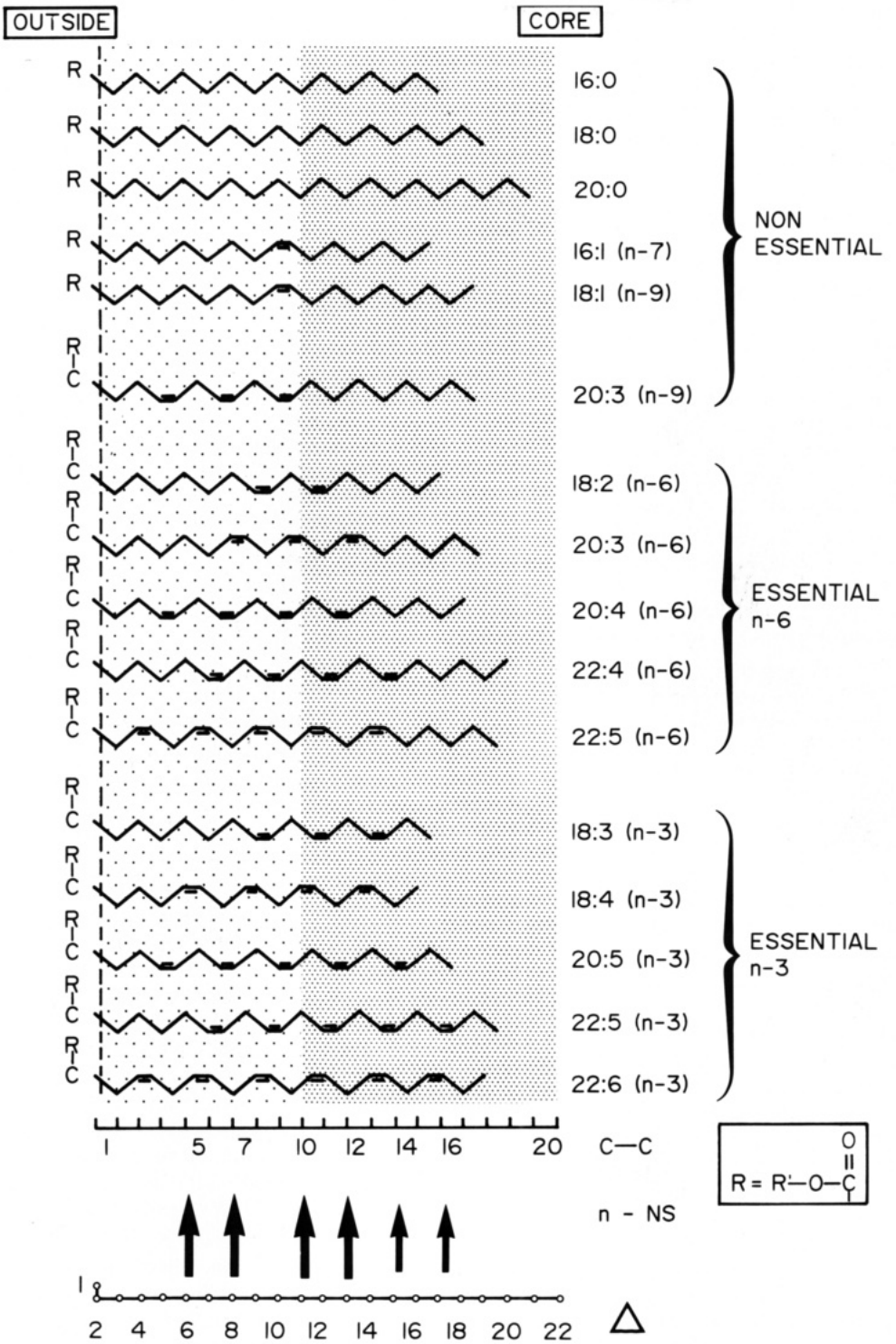

FIGURE 3: Scheme of the relative positions of hydrocarbon chain double bonds in a biomembrane monolayer (see Discussion).

lipid bilayer, the following may be concluded from the above considerations: first, a much more rapid progression of the conformational freedom from C5 to C10 with LA than with OA, and second, a similar progression of the conformational freedom from C10 to C12 with either fatty acids, which results in maintaining the higher conformational freedom with LA from C10 to the deeper region. It clearly appears that (i) the differences in the fluidity gradient caused by the diet are more pronounced near the surface of the membrane than in the core and (ii) the C10 depth probably plays an important role in the bilayer, from structurally and probably physiologically (see EPUFA double bonds, above) points of view. According to previous observations made by Orly and Schramm (1975) using free monoenoic fatty acids, the major property of the EPUFA could be to place double bonds in the vicinity of this depth—and at the minimum one double bond in the deeper half-part of the biomembrane leaflet. This could trigger chain spacing,[2] which in turn will be able to create a sufficient

[2] The notion of chain spacing is easy to use for a microscopic description of phenomena, but the notion of delocalization of chain segments [or methyl groups; see Gruen (1980)] or conformational freedom would be preferable for macroscopic description purposes (the biomembrane is not an inflatable balloon) as delocalization or conformation changes need much less energy in the core than near the surface of the lipid bilayer.

conformational freedom in the deeper region.

Recent observations on membrane-embedded proteins in membranes differing by their LA content (Léger et al., 1989; Christon et al., 1989a) allow us to conclude that the conformational freedom of the membrane lipids plays an important physiological role. Due to an increased hydrocarbon chain flexibility, such a conformational freedom could be responsible for a better lipid "coating" on the surface of the membrane proteins, leading to strenghthening the lipid–protein interactions in HLABMV as compared to LLABMV. Interestingly, stronger lipid–protein interactions could promote the optimally active conformation of intrinsic proteins. However, conformational freedom is not always related to increasing performances of biologically active membrane-bound intrinsic proteins [for a review, see McMurchie (1988)]. Consideration of specific and optimal conformational freedom is probably needed for each intrinsic protein environment. Thus, the effect of a given gain of conformational freedom depends on the original state of the protein environment relative to the optimal state required by the fully active complex. Therefore, this effect could be positive, negative, or null.

The "nature" of double bonds may also be involved. In terms of TDBn and TDBn/TSFA, it is clear that the increasing levels of OA + ETA (nonessential fatty acids) counterbalance the decreasing level of LA (an essential fatty acid). A probably fine metabolic fit leads to this mutual compensation. Therefore, the structural differences evidenced here across the membrane might not be attributed to the TDBn (if that was true, we could not detect any modification in the order profiles) but rather to the different distributions of double bonds on both sides of C10 characterized by the DBn_{EFA}/DBn_{nonEFA} ratio. Thus, a more complete explanation of the structural differences observed requires the notion of double-bond transverse localization and distribution. It is concluded that the quantitative notion of number of double bonds should be replaced or completed by a qualitative notion of double-bond position. All these results might be in favor of a specific structural role of EFA in biological membranes.

The structural features of EPUFA are possibly able to deeply and specifically affect not only membrane microdomains of physiological importance (for example, the topological vicinity of enzymes, receptors, transport systems, or channels) but also the shape of the structural profile across the membrane. It remains to be established whether these structural (and like complementary) organizations are able to promote the optimal expression of membrane-bound intrinsic protein functions.

ACKNOWLEDGMENTS

We thank Kirsten Rerat for improving the English form of this paper. We also thank Jacqueline Thevenoux and A. Linard for help in providing experimental data.

Registry No. 16:0, 57-10-3; 18:0, 57-11-4; 20:0, 506-30-9; 16:1 (*n*-7), 373-49-9; 18:1 (*n*-9), 112-80-1; 20:3 (*n*-9), 20590-32-3; 18:2 (*n*-6), 60-33-3; 20:3 (*n*-6), 1783-84-2; 20:4 (*n*-6), 506-32-1; 22:4 (*n*-6), 99796-73-3; 22:5 (*n*-6), 25182-74-5; 18:3 (*n*-3), 463-40-1; 18:4 (*n*-3), 20290-75-9; 20:5 (*n*-3), 10417-94-4; 22:5 (*n*-3), 24880-45-3; 22:6 (*n*-3), 6217-54-5.

REFERENCES

Bartlett, G. R. (1959) *J. Biol. Chem. 234*, 466–468.

Brasitus, T. A., Kwo-Yih Yeh, Holt, P. R., & Schachter, D. (1984) *Biochim. Biophys. Acta 778*, 341–348.

Brasitus, T. A., Dahiya, R., Dudeja, P. K., & Bissionnette, B. M. (1988) *J. Biol. Chem. 263*, 8592–8597.

Brenner, R. R. (1984) *Prog. Lipid Res. 23*, 69–96.

Brot-Laroche, E., & Alvarado, F. (1984) *Biochim. Biophys. Acta 775*, 175–181.

Chapelle, S., & Gilles-Baillien, M. (1983) *Biochim. Biophys. Acta 753*, 269–271.

Christiansen, K., & Carlsen, J. (1981) *Biochim. Biophys. Acta 647*, 188–195.

Christon, R., Even, V., Daveloose, D., Léger, C. L., & Viret, J. (1989a) *Biochim. Biophys. Acta 980*, 77–84.

Christon, R., Daveloose, D., Viret, J., & Léger, C. L. (1989b) *Biomembranes and Nutrition* (Léger, C. L., & Bêrêziat, G., Eds.) Colloque de l'INSERM, Vol. 195, pp 207–217.

Folch, J., Lees, M., & Sloane-Stanley, G. H. (1957) *J. Biol. Chem. 226*, 497–509.

Gally, H. U., Pluschke, G., Overath, P., & Seelig, J. (1989) *Biochemistry 18*, 5605–5610.

Gilfillan, A. M., Chu, A. J., Smart, D. A., & Rooney, S. A. (1983) *J. Lipid Res. 24*, 1651–1656.

Gruen, D. W. R. (1980) *Biochim. Biophys. Acta 595*, 161–183.

Hansen, N. S. (1983) *World. Rev. Nutr. Diet. 42*, 102–134.

Hauser, H., Howell, K., Dawson, R. M. C., & Bowyer, D. E. (1980) *Biophys. Biochim. Acta 602*, 567–577.

Hauser, H., Gains, N., Semenza, G., & Spiess, M. (1982) *Biochemistry 21*, 5621–5628.

Hitchcock, P. B., Mason, R., Thomas, K. M., & Shipley, G. G. (1974) *Proc. Natl. Acad. Sci. U.S.A. 71*, 3036–3040.

Juaneda, P., & Rocquelin, G. (1985) *Lipids 20*, 40–41.

Kleinfeld, A. M. (1987) in *Current Topics in Membranes and Transport* (Bronner, F., Ed.) pp 1–27, Academic Press, Orlando, San Diego, New York, and Austin.

Lefkowith, J. B., Flippo, V., Sprecher, H., & Needleman, P. (1985) *J. Biol. Chem. 260*, 15736–15744.

Léger, C. L., Christon, R., Viret, J., Daveloose, D., Mitjavila, S., & Even, V. (1989) *Biochimie 71*, 159–165.

Lenaz, G., & Castelli, G. P. (1985) in *Structure and Properties of Cell Membranes* (Benga, G., Ed.) Vol. 1, pp 93–136, CRC Press, Boca Raton, FL.

Longmuir, K. J. (1987) in *Current Topics in Membranes and Transport* (Bronner, F., Ed.) pp 129–174, Academic Press, Orlando, San Diego, New York, and Austin.

Lowry, O. H., Rosebrough, N. J., Farr, A. L., & Randall, R. J. (1951) *J. Biol. Chem. 193*, 265–275.

Luzzatti, V. (1968) in *Biological Membranes* (Chapman, D., Ed.) pp 71–123, Academic Press, New York.

Mac Donald, P. M., Sykes, B. D., & Mac Elhaney, R. N. (1984) *Can. J. Biochem. 62*, 1134–1150.

McMurchie, E. J. (1988) in *Advances in Membrane Fluidity*, Vol. 3, Physiological Regulation of Membrane Fluidity (Aloia, R. C., Curtain, C. C., & Gordon, L. M., Eds.) pp 189–237, Alan R. Liss, New York.

Mead, J. F. (1984) *J. Lipid Res. 25*, 1517–1521.

Mead, J. F., Alfin-Slater, R. B., Howton, D. R., & Popjak, G. (1986a) in *Lipids, Chemistry, Biochemistry and Nutrition*, pp 23–48, Plenum Press, New York and London.

Mead, J. F., Alfin-Slater, R. B., Howton, D. R., & Popjak, G. (1986b) in *Lipids, Chemistry, Biochemistry and Nutrition*, pp 369–403, Plenum Press, New York, and London.

Meddings, J. B. (1989) *Biochim. Biophys. Acta 984*, 158–166.

Orly, J., & Schramm, M. (1975) *Proc. Natl. Acad. Sci. U.S.A. 72*, 3433–3437.

Seelig, A., & Seelig, J. (1975) *Biochim. Biophys. Acta 406*, 1–5.

Seelig, A., & Seelig, J. (1977) *Biochemistry 16*, 45–50.

Seelig, J., & Seelig, A. (1980) *Q. Rev. Biophys. 13*, 19–61.
Spector, A. A., & Yorek, M. A. (1985) *J. Lipid Res. 26*, 1015–1035.
Stubbs, C. D., & Smith, A. D. (1984) *Biochim. Biophys. Acta 779*, 89–137.
Stubbs, C. D., Kouyama, T., Kinosita, K., Jr., & Ikegami, A. (1981) *Biochemistry 20*, 4257–4262.
Szamel, M., Goppelt-Strübe, M., Bersler, W., Wiesmuller, K. H., & Resch, K. (1987) *Biochim. Biophys. Acta 899*, 247–257.
Viret, J., & Leterrier, F. (1976) *Biochim. Biophys. Acta 436*, 811–824.

Role of Membrane Lipids in the Interaction of Daunomycin with Plasma Membranes from Tumor Cells: Implications in Drug-Resistance Phenomena†

Pablo V. Escriba, Antonio V. Ferrer-Montiel, Jose A. Ferragut, and Jose M. Gonzalez-Ros*
Department of Neurochemistry, School of Medicine, University of Alicante, 03080 Alicante, Spain
Received December 6, 1989; Revised Manuscript Received March 26, 1990

ABSTRACT: Equilibrium binding studies on the interaction between the anthracycline daunomycin and plasma membrane fractions from daunomycin-sensitive and -resistant murine leukemia P-388 cells are presented. Drug binding constants (K_S) are 15 000 and 9800 M^{-1} for plasma membranes from drug-sensitive and drug-resistant cells, respectively. Drug binding to the membranes is not affected by either (i) thermal denaturation of membrane proteins or (ii) proteolytic treatment with trypsin, thus suggesting that the protein components of the membranes do not have a major role in determining the observed drug binding. Also, fluorescence resonance energy transfer between tryptophan and daunomycin in the membranes indicates that interaction of protein components with the drug should not be responsible for the observed differences in drug binding exhibited by plasma membranes from drug-sensitive and -resistant cells. Plasma membranes from drug-sensitive cells contain more phosphatidylserine and slightly less cholesterol than membranes from drug-resistant cells. Differences in the content of the acidic phospholipid between the two plasma membranes seem to produce a different ionic environment at membrane surface domains, as indicated by titration of a membrane-incorporated, pH-sensitive fluorescence probe. The possible role of membrane lipids in modulating drug binding to the membranes was tested in equilibrium binding studies using model lipid vesicles made from phosphatidylcholine, phosphatidylserine, and cholesterol in different proportions. The presence of phosphatidylserine greatly increases both the affinity and the stoichiometry of daunomycin binding to model lipid vesicles. The similarity between the effects of phosphatidylserine and other negatively charged compounds such as dicetyl phosphate, cardiolipin, or phosphatidic acid suggests that electrostatic interactions are important in the observed binding of the drug. The presence of cholesterol in the model lipid vesicles causes a decrease in both drug binding affinity and stoichiometry. Furthermore, differences observed in drug binding to lipid vesicles containing phosphatidylserine and cholesterol at molar fractions resembling those found in plasma membranes from drug-sensitive and -resistant cells are similar to those observed with entire plasma membranes. On the basis of these observations, we conclude that the lipid components of plasma membranes from the drug-sensitive and -resistant P-388 cells are important in determining the extent of daunomycin binding to the membranes and in establishing the observed resistance-related differences in drug binding.

Anthracycline antibiotics such as daunomycin (DNM)[1] are potent cytotoxic drugs currently used as antitumor agents. The classical model to explain anthracycline cytotoxicity (Arcamone, 1981; Waring, 1981) has been based upon interference with nucleic acid function. However, because of the cytotoxicity demonstrated for nonpenetrating, polymer-immobilized drug (Tritton & Yee, 1982), effects at the level of the cell membrane have also been proposed.

Clinical use of anthracyclines is partly limited by the appearance of drug-resistant tumor cells during treatment. Furthermore, cell lines that develop resistance to these drugs become cross-resistant to many other chemotherapy agents (Myers et al., 1987). This acquired cross-resistance, referred to as "multidrug-resistance" (MDR), constitutes a major obstacle to the success of chemotherapy programs, and, thus, the elucidation of its molecular basis is of interest.

MDR cell lines have often been shown to exhibit a reduced net accumulation of drug relative to the parental drug-sensitive line (Myers et al., 1987; Bradley et al., 1988). Most authors

† This work has been supported by grants from CICYT and FISSS of Spain. A.V.F.-M. and P.V.E. are recipients of predoctoral fellowships from "Fondo de Investigaciones Sanitarias de la Seguridad Social" and "Programa de Formacion de Personal Investigador", respectively.
* Author to whom correspondence should be addressed.

[1] Abbreviations: DNM, daunomycin (daunorubicin); MDR, multiple drug resistance; P388/S, wild (drug-sensitive) P388 murine leukemia cells; P388/R, multidrug-resistant P388 murine leukemia cells selected for resistance to daunomycin; P-170, high molecular weight (~170K) glycoprotein; PC, phosphatidylcholine; PS, phosphatidylserine; FRET, fluorescence resonance energy transfer; Tris, tris(hydroxymethyl)-aminomethane; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid.

0006-2960/90/0429-7275$02.50/0