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Nonelectrolyte permeability of liposomes of hydroxyfatty acid-containing phosphatidylcholines

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Two phosphatidylcholines containing hydroxylated fatty acids, 1-palmitoyl-2-[5-hydroxy-6,8,11,14-eicosatetraenoyl]-*sn*-glycero-3-phosphocholine (1-palm-2-5HETE PC) and 1-palmitoyl-2-[15(*S*)-hydroxy-5,8,11,13-eicosatetraenoyl]-*sn*-glycero-3-phosphocholine (1-palm-2-15HETE PC), and one phosphatidylcholine containing nonhydroxylated fatty acids, 1-palmitoyl-2-arachidonyl-*sn*-glycero-3-phosphocholine (1-palm-2-arach PC) were synthesized. Permeation of small nonelectrolytes (glycerol, 1,2-propanediol, urea, methylurea, propionamide and dimethylformamide) was assessed in multilamellar liposomes containing these synthetic PCs plus egg yolk phosphatidycholine (EPC) in the presence and absence of cholesterol. In liposomes containing 23% cholesterol, 69.3% EPC and 7.7% of either 1-palm-2-5HETE PC or 1-palm-2-15HETE PC the permeability to small nonelectrolytes was 60 to 400% greater than in liposomes containing 23% cholesterol and 77% EPC. The HETE-containing PCs also increased permeability in liposomes without cholesterol but the effects were less striking. Addition of the synthetic PCs did not affect the energy of activation of permeation.

Introduction

Hydroxylated fatty acids are formed non-enzymatically by chemical oxidation and enzymatically through lipoxygenase pathways. 5(*S*)-Hydroxy-6*Z*,8*E*,11*E*,14*E*-eicosatetraenoic acid (5-HETE) is a major product of the 5-lipoxygenase pathway in neutrophils [1]. 5-HETE is chemotactic and chemokinetic for neutrophils and induces neutrophil degranulation, hexose uptake and calcium flux [2–4]. 15(*S*)-Hydroxy-5*E*,8*E*,11*E*,13*Z*-eicosatetraenoic acid (15-HETE) is a product of neutrophils, mast cells, eosinophils and reticulocytes [5,6]. 15-HETE regulates lymphocyte mitogenesis, promotes neovascularization and stimulates migration of capillary endothelial cells [7,8]. Neutrophils, macrophages, and other cell types are capable of esterifying hydroxylated fatty acids into membrane phospholipids [9–11]. Whether the biologic effects of 5-HETE and 15-HETE are results of their incorporation into membrane phospholipids is not known. However, there is no evidence for specific membrane receptors for these compounds and, thus, it is assumed that their biologic effects relate to their uptake and esterification by the responding cell. Esterification of hydroxylated fatty acids into phospholipids results in the incorporation of a hydrophilic hydroxyl group into the hydrophobic portion of the phospholipid bilayer. The incorporation of a hydrophilic moiety into the hydrophobic portion of the phospholipid bilayer of a biological membrane would be expected to affect the biophysical and functional properties of the membrane including permeability of the membrane to small non-electrolytes.

One model system for studying the effects of changes in lipid composition on permeability is the multilamellar liposome. The property of multilamellar liposomes of acting as almost ideal osmometers allows the measurement of permeability of small nonelectrolytes by assessing the osmotic swelling of the liposomes in isotonic solutions of the penetrating permeant [12]. Previous studies using this technique have demonstrated that addition of cholestrol to phospholipid liposomes decreases their permeability [13]. Liposomes composed of

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Abbreviations: 1-palm-2-5HETE PC, 1-palmitoyl-2-[5-hydroxy-6,8,11,14-eicosatetraenoyl]-*sn*-glycero-3-phosphocholine; 1-palm-2-15HETE PC, 1-palmitoyl-2-[15(*S*)-hydroxy-5,8,11,13-eicosatetraenoyl]-*sn*-glycero-3-phosphocholine; 1-palm-2-arach PC, 1-palmitoyl-2-arachidonyl-*sn*-glycero-3-phosphocholine; EPC, egg yolk phosphatidylcholine; PC, phosphatidylcholine.

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phospholipids with unsaturated acyl groups are more permeable than those composed of phospholipids with saturated acyl groups, the greater the degree of unsaturation the more permeable the liposome [13]. Decreasing the carbon chain length of phospholipid fatty acids increases permeability [13]. Liposomes containing dialkyl phosphatidylcholine (PC) are somewhat more permeable to glycerol and urea than liposomes containing diester or diether PC [14].

We have devised a method for the synthesis of PCs containing hydroxylated fatty acids [15]. We have synthesized 1-palmitoyl-2-[5-hydroxy-6*Z*,8*E*,11*E*,14*E*-eicosatetraenoyl]-*sn*-glycero-3-phosphocholine (1-palm-2-5HETE PC) and 1-palmitoyl-2-[15(*S*)-hydroxy-5*E*,8*E*,11*E*,13*Z*-eicosatetraenoyl]-*sn*-glycero-3-phosphocholine (1-palm-2-15HETE PC). We have also synthesized 1-palmitoyl-2-arachidonyl-*sn*-glycero-3-phosphocholine (1-palm-2-arach PC) which does not contain a hydroxylated fatty acid. Liposomes containing various concentrations of 1-palm-2-15HETE PC or 1-palm-2-5HETE PC were formed and their molecular dynamics assessed by electron spin resonance spectroscopy. Results of this study demonstrate that the introduction of even small amounts of 1-palm-2-15HETE PC into PC liposomes substantially decreases the order parameter in the membrane interior [15].

In the present study permeation of small nonelectrolytes was assessed in liposomes containing various concentrations of 1-palm-2-5HETE PC, 1-palm-2-15HETE PC, 1-palm-2-arach PC and egg yolk phosphatidylcholine (EPC) in the presence and absence of cholesterol. Addition of small amounts 1-palm-2-arach PC to EPC increases the average degree of unsaturation of the PC fatty acids. Addition of 1-palm-2-5HETE PC or 1-palm-2-15HETE PC to EPC has the same effect on the degree of saturation as addition of 1-palm-2-arach PC and also introduces a hydroxy group into the hydrophobic portion of the membrane bilayer. Addition of 1-palm-2-5HETE PC or 1-palm-2-15HETE PC to EPC increases permeability even more than addition of 1-palm-2-arach PC. Thus, the results of this study demonstrate that increasing the degree of unsaturation of phosphatidylcholine fatty acids increases liposomal small nonelectrolyte permeation. The addition of a hydroxyl group to a PC fatty acid increases liposomal permeability even further.

Materials and Methods

Lipids. EPC and cholesterol were purchased from Sigma. 1-Palm-2-arach PC, 1-palm-2-5HETE PC, and 1-palm-2-15HETE PC were synthesized and characterized as recently described [15]. Briefly the synthesis was as follows: The hydroxyl groups on 5-HETE and 15-HETE were blocked by formation of *tert*-butyldimethysilyl ethers. The anhydrides of the blocked fatty acids were prepared with dicylohexylcarbodiimide. 1-Palmitoyllysophosphatidylcholine was prepared by treating dipalmitoyl phosphatidycholine with phospholipase A_2 (*Crotalus adamanteus*). Blocked fatty acid anhydride and lysophosphatidylcholine and pyrrolidinopyridine were stirred together to yield the blocked phosphatidylcholine. The blocked phosphatidylcholine was unblocked with acetic acid. 1-Palm-2-arach PC was synthesized by the same method but deleting the steps in which the hydroxyl group was blocked and unblocked.

Liposome production. Liposomes were prepared from chloroform solutions containing 10 μmol of PC and 0.44 μmol of dicetyl phosphate, or 10 μmol PC, 3 μmol cholesterol, and 0.44 μmol of dicetyl phosphate [14]. For the sake of simplicity the contribution of dicetyl phosphate to vesicle composition is not included in the descriptions of the vesicles in the 'Results' sections; however, all vesicles in this study contained 4 mol% dicetyl phosphate. Solutions were evaporated to dryness under a stream of N_2, and then further dried in a vacuum desiccator for 15 min at room temperature. Nitrogen was used to break the vacuum. Multilamellar liposomes were formed by vortexing the lipid in 1 ml of 0.15 M KCl.

Liposome swelling. Osmotic swelling of liposomes was assessed by light scattering with a Beckman DU-50 spectrophotometer. Aqueous, degassed solutions of glycerol, urea, and other permeants were at a concentration of 0.3 M, making them isosmotic with the entrapped 0.15 M KCl [14]. These solutions, contained in glass test tubes, were maintained at a desired temperature by their partial immersion in the same temperature controlled circulating water bath that served to control the temperature of the cuvette holder. The dissolved permeant (1 ml) was placed into a cuvette in the spectrophotometer and 20 μl of a liposome suspension was injected. The spectrophotometer was programmed to take readings every second over a 30 s period, and registered the decreasing absorbance at 450 nm resulting from vesicle swelling. Absorbance was measured beginning 8 to 12 s after injection, and the decrease in adsorbance was linear during the 30 s when readings were taken. At least eight determinations were made for each data point in each experiment. The permeability coefficient and the change in volume of the liposomes are inversely proportional to the change in absorbance divided by the initial absorbance squared per second $(dA/A^2)(1/dt)$ [13].

Lipid analysis. Lipids were extracted from the liposomes by methanol-chloroform extraction. Fatty acid methyl esters were prepared by reaction of the extract with 14% boron trifluoride in methanol. Fatty acid methyl esters were separated on a Varian model 3700 gas chromatrograph with a capillary column utilizing

OV-101 as the stationary phase and quantified with a flame ionization detector.

Results

Table I presents the fatty acid composition of EPC and of 90% EPC plus 10% of each of the synthetic lipids. Addition of the synthetic lipids causes a slight increase in the average carbon chain length of the phospholipid fatty acids and a considerable increase in the average number of double bonds per fatty acid. All three of the synthetic fatty acids have the same effect on the carbon chain length and the number of double bonds.

Table II presents the optical density of liposomes containing either 100% PC or 77% PC plus 23% cholesterol. The PC component, in turn, is either all EPC or 90% EPC plus 10% of the designated synthetic PC. Optical density is assessed after suspending the liposomes in 0.15 M KCl. The liposomes contain the same solution in which they are suspended and thus should not shrink or swell. Absorption is proportional to the number of vesicles and inversely proportional to their size. In the 100% PC liposomes addition of any of the three synthetic phosphatidylcholines to EPC results in an increase in absorption at 450 nm and thus a decrease in vesicle size compared to EPC alone. The addition of PCs with a hydroxy group (1-palm-2-5HETE PC and 1-palm-2-15HETE PC) results in even smaller liposomes than the addition of 1-palm-2-arach PC. The addition of cholesterol to EPC or EPC + 1-palm-2-arach PC results in a decrease in vesicle size. However, addition of cholesterol to liposomes containing the hydroxy PC has little effect on vesicle size. Both for liposomes composed of EPC and liposomes composed of synthetic PCs it has been demonstrated previously that osmotic swelling is similar to an ideal osmometer

TABLE I

Fatty acid composition of phosphatidylcholine liposomes

EPC	EPC	EPC 90% + 10% 1-palm-2-arach PC	EPC 90% + 10% 1-palm-2-5HETE	EPC 90% + 10% 1-palm-2-15HETE
16:0	34.6	36.2	36.2	36.2
18:0	13.3	12.0	12.0	12.0
18:1	30.3	27.0	27.0	27.0
18:2	14.2	12.8	12.8	12.8
20:4	3.0	7.7	7.7	7.7
5-HETE	–	–	5.0	–
15-HETE	–	–	–	5.0
Average carbon chain length	17.3	17.5	17.5	17.5
Average number of double bonds per fatty acid	0.74	0.87	0.87	0.87

TABLE II

Optical density of dispersions of vesicles

Phosphatidylcholine	100% PC	77% PC + 23% cholesterol
EPC	0.134 ± 0.006 [a]	0.326 ± 0.017
EPC + 10% 1-palm-2-arach PC	0.144 ± 0.010	0.259 ± 0.008
EPC + 10% 1-palm-2-5HETE PC	0.209 ± 0.012	0.226 ± 0.007
EPC + 10% 1-palm-2-15HETE PC	0.334 ± 0.002	0.298 ± 0.031

[a] ± Standard deviation (S.D.).

[12]. Fig. 1 presents the effects of the addition of various amounts of 1-palm-2-arach PC, 1-palm-2-5HETE PC and 1-palm-2-15HETE PC on the permeation of glycerol in EPC liposomes in the presence and absence of cholesterol. In the absence of cholesterol each of the three synthetic PCs increases the glycerol permeability of EPC liposomes in a dose dependent fashion. The dose response curves of EPC liposomes to the three synthetic phospholipids are effectively identical. Additions of the synthetic PCs in concentrations as low as 1% of the total phospholipid give appreciable increases in glycerol permeability. In all conditions liposomes containing 23% cholesterol are less permeable than corresponding vesicles without cholesterol. The permeability dose response curves for all three synthetic PCs in the presence of cholesterol are below and paral-

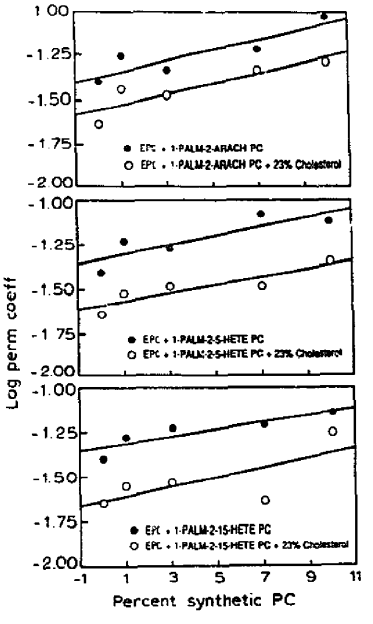

Fig. 1. Effect of the addition of synthetic PCs on the permeation at 37°C of glycerol in liposomes composed PCs alone or 77% PCs 23% cholesterol.

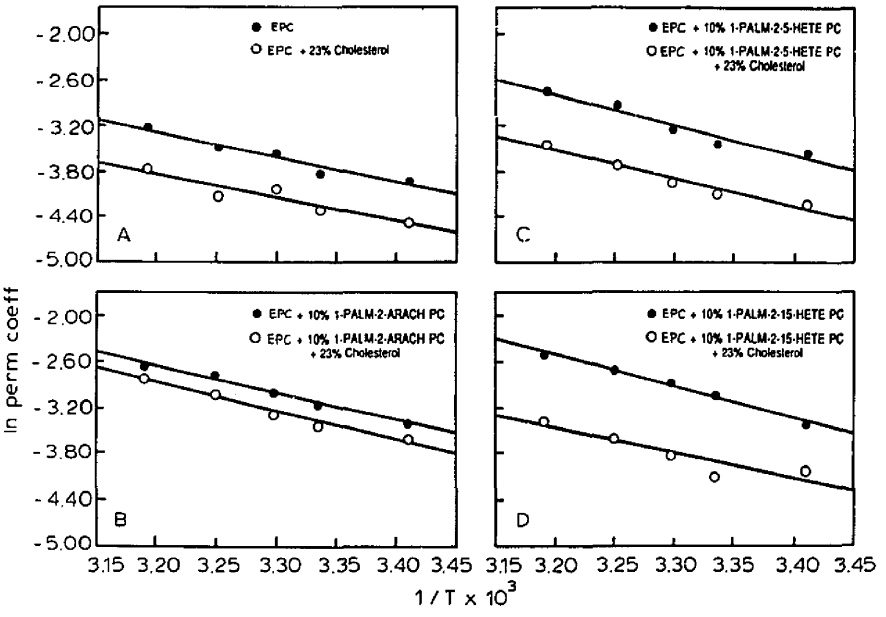

Fig. 2. Arrhenius plots of the permeation of glycerol in liposomes of various lipid composition.

lel to the dose response curves in the absence of cholesterol.

To address the question of whether the addition of permeants to suspensions of vesicles increases apparent vesicle size by inducing vesicle swelling through permeation or by inducing vesicle fusion we performed the following experiment. Vesicles were loaded with glycerol and then suspended in isosmotic KCl. The O.D. increased over time (data not shown) consistent with a decrease in vesicle size. The decrease in vesicle size was due to permeation of the glycerol out of the vesicle. This experiment unambiguously demonstrates that the effects of permeants on vesicle size are results of permeation rather than fusion.

Fig. 2 presents the Arrhenius plots of glycerol permeability for liposomes containing EPC or EPC plus one of the three synthetic PCs as 10% of the phospholipids in the presence and absence of cholesterol. The Arrhenius plots are used to determine energies of activation (Table III). The energies of activation for glycerol permeation in all of these liposomes are similar. The presence and absence of cholesterol has no effect on the energy of activation.

Table IV presents the permeability of liposomes at 37°C to six small nonelectrolytes: two alcohols, two ureas, and two amides. The 100% PC liposomes contain either 100% EPC or 90% EPC and 10% of the designated synthetic lipid. Liposomes designated as '+chol' contain 23% cholesterol and 77% PC. For every phospholipid combination the addition of cholesterol decreases permeability to all the permeants. Cholesterol results in the greatest decrease in permeability when it is incorporated in liposomes containing EPC alone, the addition of cholesterol has lesser effects on permeability when incorporated into liposomes containing 1-palm-2-arach PC and even less when incorporated into liposomes containing 1-palm-2-5HETE PC or 1-palm-2-15HETE PC.

The addition of synthetic PCs has the most consistent effect on permeability in liposomes that also contain cholesterol. In the presence of cholesterol the addition of 10% 1-palm-2-arach PC to EPC results in a consistent increase in permeability for all the permeants. The permeability increases by 25–50% for most permeants. In the presence of cholesterol the addition of 10% 1-palm-2-5HETE PC or 1-palm-2-15HETE causes an even greater increase in permeability. Addition of the HETE containing PCs increases permeability by from 45% to 300% compared to EPC alone. The size of the increase varies with the permeant. In assess-

TABLE III

Activation energies (Δ E kcal/mol) of the permeation of glycerol

Lipid	Activation energy	
	lipid	+23% cholesterol
EPC	16.2±2.1 [a]	15.5±1.2
EPC+10% 1-palm-2-arach PC	16.8±1.5	17.2±1.9
EPC+10% 1-palm-2-5HETE PC	18.3±2.5	16.2±0.9
EPC+10% 1-palm-2-15HETE PC	19.3±0.8	14.4±2.1

[a] ± S.D.

TABLE IV

Permeability of liposomes

Data expressed as (d A/A^2)·s^{-1}. Liposomes without cholesterol (−chol) contain EPC alone or EPC + 10 mol% of the designated synthetic PC. Liposomes with cholesterol (+chol) contain 30 mol% cholesterol and 70 mol% phospholipid.

Permeant	Lipid composition of liposome							
	EPC		EPC + 1-palm-2-arach PC		EPC + 1-palm-2-5HETE PC		EPC + 1-palm-2-15HETE PC	
	−chol	+chol	−chol	+chol	−chol	+chol	−chol	+chol
Glycerol	0.042	0.025	0.099	0.052	0.096	0.074	0.115	0.101
1,2-Propanediol	0.172	0.101	0.150	0.139	0.170	0.146	0.183	0.164
Urea	0.075	0.021	0.080	0.033	0.092	0.064	0.110	0.097
Methylurea	0.144	0.072	0.163	0.092	0.154	0.116	0.161	0.153
Dimethylformamide	0.167	0.104	0.185	0.130	0.172	0.163	0.172	0.162
Propionamide	0.127	0.090	0.115	0.115	0.154	0.134	0.144	0.141

ing the effect of the HETE hydroxy group on permeability the most relevant comparison is that of EPC + 10% 1-palm-2-arach PC with EPC + 10% 1-palm-2-5HETE PC or EPC + 10% 1-palm-2-15HETE PC. In the presence of cholesterol the liposomes with the HETE containing PCs are more permeable than those containing 1-palm-2-arach PC for every permeant, the increases range from a few percent for 1,2-propanediol to a 100% for urea. For the most part addition of 1-palm-2-15HETE increases permeability more than addition of 1-palm-2-5HETE.

In the absence of cholesterol the effects of the synthetic phospholipids on permeability are less marked and less consistent. In the absence of cholesterol the addition of synthetic lipids to EPC either has no effect on permeability or increases it by 10 to 20%.

Discussion

The major new finding of this study is that addition of a hydroxy group to one acyl chain in 10% of PC molecules increases the permeability of PC-cholesterol liposomes to small nonelectrolytes by 60–400%. In comparison the addition of one double bond to one acyl group in 100% of PC molecule increases permeability by 100% and reduction of the carbon chain length by two carbons in both acyl groups of 100% of PC molecules increases permeability by 100% [13]. Similarly, substitution of diacyl PC for dialkyl PC increases permeability by 100% [14]. Thus, on a molar basis, the addition of a hydroxy group is 6–40-times more potent in increasing permeability than the acyl alterations previously studied.

Of particular interest is the interaction of hydroxy-containing PC with cholesterol. Cholesterol decreases the permeability of liposomes by increasing the packing of phospholipid acyl groups. The data presented here could be interpreted to indicate that the addition of cholesterol to liposomes containing nonhydroxylated PC increases acyl packing and, as a result, the liposomes become smaller and less permeant. However, the addition of cholesterol to liposomes containing even a small mol% of a hydroxylated PC does not increase acyl packing. As a result, the liposomes do not become smaller or less permeant. Perhaps the presence of hydroxy groups in PCs competes with cholesterol hydroxy groups for hydrogen bonding with PC carboxyl groups [14].

The cell membrane separates the intracellular space from the extracellular space and contributes to the compartmentalization of cellular organelles. These functions require that the free diffusion of physiological solutes across the membrane be hindered. The observation that hydrophobic permeants, such as hydrocarbons and ethers, traverse membranes much more rapidly than electrolytes has lead to the concept that the hydrophobic portion of the membrane represents the barrier to diffusion. The theory governing the permeation of hydrophilic solutes across lipid bilayers has been dealt with in considerable detail [16–19]. According to generally accepted concepts, the process can be envisioned as a series of reactions. In the first reaction, the permeant approaches and penetrates the membrane. In subsequent reactions the permeant traverses the membrane by passing along a series of free energy maxima and minima. In general the free energy changes associated with this movement are less than that of the initial penetration reaction. In the last step the permeant is released from the membrane interior and falls to a free energy minimum. Since the first step of the process represents the greatest positive free energy change it is considered the rate-determining step and puts an upper limit on the permeation rate [16].

According to this theory, softening of the hydrophobic permeability barrier by the inclusion of fixed hydrophilic groups in the membrane interior might be expected to increase the rate of permeation of a hydrophilic solute through a membrane. The results presented in this paper confirm this supposition. Incorporation of

even small amounts (1 mol%) of hydroxylated PC into PC-cholesterol vesicles leads to significant increases in the permeability of the vesicles to a series of hydrophilic permeants. This increase is dose dependent, higher mole percents give higher permeability coefficients. The increase in permeability is due to the hydroxy group and not to changes in the overall saturation index of the PC as shown by comparison of the effects of hydroxy-PC incorporation to the effects of arachidonyl-PC incorporation. These PCs have an identical number of double bonds, yet the hydroxylated PC-containing vesicles show a higher permeability.

Not only the presence of a hydroxy group but also the position of the hydroxy group affects permeation. For most of the conditions tested the presence of a hydroxy group on the 15 carbon (15-HETE) resulted in a greater increase in permeation than the presence of a hydroxy group on the 5 carbon (5-HETE). Similarly in an earlier study of electron spin resonance in vesicles containing hydroxylated-PCs it was found that 1-palm-2-15HETE PC induced a greater decrease in the order parameter than did 1-palm-2-5HETE PC [15]. It may be that because the hydroxy group in 1-palm-2-15HETE PC is located deeper in the hydrophobic portion of the bilayer it has greater effects on both permeation and electron spin resonance.

The mechanism whereby hydroxy-PC incorporation increases permeability is not clear. The slopes of the Arrhenius plots were the same for all membrane compositions, indicating that the addition of the hydroxy groups had no effect on the energy of activation. Thus, the enthalpy of the penetration of the permeant into the membrane interior is the same for all the tested membrane compositions. However, another factor, the entropy of activation, also affects the activation free energy of the permeation process. It is possible that the addition of hydroxylated PCs changes the entropy of activation in a manner that enhances permeation. Arrhenius plots of the permeability of liposomes containing added double bonds or hydroxyl groups are shifted but still parallel to those of liposomes composed of EPC alone. This is because the initial energy barrier, determined largely by the initial breaking of hydrogen bonds between solvent and solute, is not changed; but, the contribution of the effective entropy differs.

Whatever the mechanism, the studies presented in this paper show that hydroxylated PCs, when incorporated at low concentration into phospholipid bilayers cause remarkable increases in the permeation of small nonelectrolytes. Whether the changes documented in this work are relevant to the biological properties of hydroxylated fatty acids remains to be determined. The permeability effects demonstrated here are relatively large and it is possible that under physiologic conditions incorporation of hydroxylated fatty acids into phospholipids may result in locally high levels of hydroxylated phospholipids. These changes may affect the permeability of certain regions of cellular membranes to important permeants and in so doing evoke a biologic response.

Acknowledgements

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