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INFLUENCE OF pH AND CHOLESTEROL ON THE STRUCTURE OF PHOSPHATIDYLETHANOLAMINE MULTIBILAYERS

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SUMMARY

1. The effect of pH and cholesterol on the structure of multibilayers of phosphatidylethanolamine was studied by the use of a rigid spin label 3-spiro-[2'-(*N*-oxyl-4',4'-dimethyloxazolidine)]-cholestane as a molecular probe.

2. The spin label was found to orient only in the hydrated multibilayer. Cholesterol decreased the rate of isotropic tumbling in the dry film and improved the orientation of the spin label continuously up to 50 mole % in hydrated films.

3. A slight decrease in orientation occurred from pH 2.0–8.3 in bilayers of phosphatidylethanolamine alone and with 50 mole % cholesterol. From pH 8.3–10.4 there was a sharp decrease in orientation in bilayers of phosphatidylethanolamine. The decrease was not as great when cholesterol was present. There was almost no pH effect on orientation in egg lecithin–cholesterol bilayers.

4. The results suggest that there is an intramolecular neutralization of the negative phosphate group by the positively charged ammonium group which contributes to the orientation in the bilayer by preventing repulsion of the polar head groups.

5. The orienting effect of cholesterol is attributed to restriction of the freedom of motion of the hydrocarbon chains and electrostatic interaction between the hydroxyl and phosphate groups.

INTRODUCTION

Investigations on oriented phospholipid–water systems by electron spin resonance (ESR) spin labelling[1,12,13] and X-ray diffraction techniques[2] have shown that the lipid hydrocarbon chains are uniformly packed in bilayers with their free ends near the centre. Incorporation of cholesterol into the multibilayers increases the rigidity and orientation of egg lecithin bilayers by restricting the freedom of motion of the hydrocarbon chains[3]. ESR studies with various derivatives of cholesterol showed that the β-hydroxyl at the 3-position is necessary for the optimum bilayer ordering effect of cholesterol, thus indicating that there may be an electrostatic interaction between the hydroxyl group of cholesterol and the polar head groups of the phospholipids which reduces the mobility of the polar head groups (ref. 3 and J. C. Hsia, R. A. Long, F. E. Hruska and H. D. Gesser, unpublished).

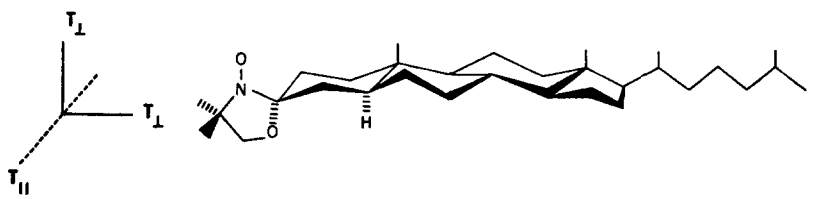
However, measurements of surface potentials of mixed monolayers of chol-

esterol with phosphatidylcholine[4] and phosphatidylethanolamine[5] indicate that there is no ion–dipole interaction between the hydroxyl group of cholesterol and the polar head group of the phospholipid while such an interaction was found between dicetyl phosphate and cholesterol and between phosphatidic acid and cholesterol[4]. It was suggested that the anionic phosphate group is neutralized by the cationic ammonium group by an intramolecular interaction. The choline and ethanolamine groups are believed to exist in the gauche conformation in the solid state rather than in the extended *trans* form, due to stabilization by such an intramolecular electrostatic interaction[6]. However, it is not known if this configuration also exists in the presence of water.

The purpose of this study was to investigate the pH effect on bilayer structure of phosphatidylethanolamine alone and with 50 mole % cholesterol and thereby provide information about the effect of electrostatic interactions at the bilayer–water interface.

A nitroxide-labelled probe is incorporated into a lipid film formed in a thin flat quartz cell which can be oriented in the magnetic field. Films of egg lecithin and dipalmitoyl lecithin in both the dry and hydrated states formed by this method have been shown to consist of ordered multibilayers which orient the spin label with its long axis nearly perpendicular to the plane of the film[1,7]. The ESR spectrum of the spin label depends on its orientation in the magnetic field and on its motion and thus reflects the degree of orientation and rigidity of the bilayer lattice components.

A rigid spin label, 3-spiro-[2′-(*N*-oxyl-4′,4′-dimethyloxazolidine)]-cholestane (I) was used to detect changes in the order of the bilayer structure. The interpretation of the spectra of this spin label in different environments has been described previously[1,12].



I

METHODS

Materials

Egg lecithin was purchased from Serdary Research Laboratory. Phosphatidylethanolamine was a gift from Dr D. O. Tinker, University of Toronto. 3-Spiro-[2′-(*N*-oxyl-4′,4′-dimethyloxazolidine)]-cholestane was prepared by the method of Keana *et al.*[9]. Solvents and chemicals used were reagent grade.

Preparation of lipid film

Thin lipid films containing a spin label to phospholipid mole ratio of 1:150 were prepared by evaporating, under a reduced pressure, a chloroform–methanol solution of the lipids in a flat quartz ESR cell (4 cm × 1 cm × 0.25 mm) thus forming a flat continuous film. The dry films were left under vacuum throughout measurement of spectra. For the investigation of cholesterol effect, hydration was carried out by

introducing a 0.02 M phosphate buffer containing 0.15 M NaCl at pH 7.4. For the investigation of pH effect, different buffers at pH 2.0–10.4 containing 0.1 M NaCl and at a constant ionic strength of 0.15 M[10] were continuously pumped through the cell at a rate of 1 ml/min by a polystaltic pump from Buchler Instruments. The position of the film and ESR spectrometer settings were not changed during the series of measurements at different pH values. Preliminary experiments were also carried out in which a new film was made for each buffer solution used. Similar results were obtained.

ESR measurement

All spectra were recorded at room temperature on a Varian E-6 X-band spectrometer. The magnetic field was calibrated with Fremy's salt, $a_n = 13.091$ gauss[11]. The spectra were recorded with the plane of the film parallel and perpendicular to the laboratory magnetic field and the spectra were recorded as the first derivative of absorption peaks.

RESULTS

The ESR spectrum of the spin label depends on its orientation in the magnetic field and on its motion. If the long axis of the spin label is oriented perpendicular to the plane of the lipid film, the hyperfine separation with the film perpendicular to the magnetic field, $a_\perp$, is approximately equal to $T_\perp \cong 6$ gauss and with the film parallel to the magnetic field $a_\parallel \cong T_\parallel \cong 32$ gauss. If the spin label rotates rapidly about its long axis $a_\parallel \cong (T_\perp + T_\parallel)/2 \cong 19$ gauss while $a_\perp \cong T_\perp \cong 6$ gauss*. If the spin label is not rotating perpendicular to the plane of the bilayer but is inclined at some angle, $a_\parallel$ decreases and $a_\perp$ increases. $a_\perp$ is proportional to the angle at which the spin label is tilted and is thus a measure of the orientation of the bilayer lattice components. If the spin label is isotropically distributed the spectra will not be angular dependent. The hyperfine splitting $a_\parallel \cong a_\perp \cong (T_\parallel + 2T_\perp)/3 \cong 15$ gauss for rapid isotropic motion. The ratio of the low field peak height to the centre peak height, M_{+1}/M_0 (see Fig. 3) decreases from 1.0 as the orientation of the bilayer decreases, or as the rigidity of the bilayer increases.

The ESR spectra of dry and hydrated films of phosphatidylethanolamine alone and with 50 mole % cholesterol with the plane of the film perpendicular and parallel to the magnetic field are shown in Figs 1 and 2. Dry films of phosphatidylethanolamine alone and with cholesterol were angular independent; hence only one orientation is shown in Fig. 1. $a_\parallel \cong a_\perp \cong 15.6$ gauss for dry films of phosphatidylethanolamine alone, indicating rapid isotropic tumbling of the spin label (correlation time $\tau_c = 4.9 \cdot 10^{-9}$ s, ref. 15), contrary to the behaviour of dry films of egg lecithin and dipalmitoyl lecithin[7] in which the spin label was oriented and immobilized. However, $2a_\parallel \cong 2a_\perp \cong 60$ gauss for dry films containing cholesterol indicating immobilization of the spin label. Hydration of films of phosphatidylethanolamine alone and with cholesterol produced orientation in a lamellar structure with rapid anisotropic ro-

* The geometric relationship of the tensor components (*i.e.* $T_\perp$ and $T_\parallel$) to the long axis of the steroid nucleus of the cholestane spin label is as shown. The values have been determined in a cholesteryl chloride crystal[16] and dry dipalmitoyl lecithin–cholesterol multibilayers[1]. $a_\perp$ and $a_\parallel$ are the hyperfine separations observed when the laboratory magnetic field is perpendicular or parallel to the quartz cell supporting the lipid film, measured as indicated in Figs 1 and 2.

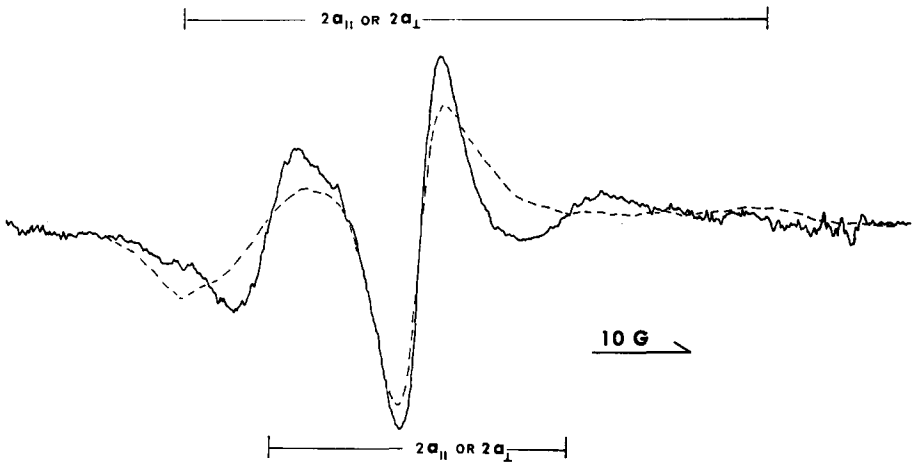


Fig. 1. ESR spectra of spin-labelled cholestane in dry films of phosphatidylethanolamine (———) and phosphatidylethanolamine, 50 mole %–cholesterol (– – – –). The spectrum recorded with the magnetic field parallel to the plane of the lipid film is similar to that recorded in the perpendicular orientation. $2a_{\|} \cong 2a_{\perp}$ for the dry film of phosphatidylethanolamine was measured between the midpoints of the low and high field peaks. $2a_{\|} \cong 2a_{\perp}$ for the dry film of phosphatidylethanolamine–cholesterol was measured as the maximum separation between the low and high field peaks.

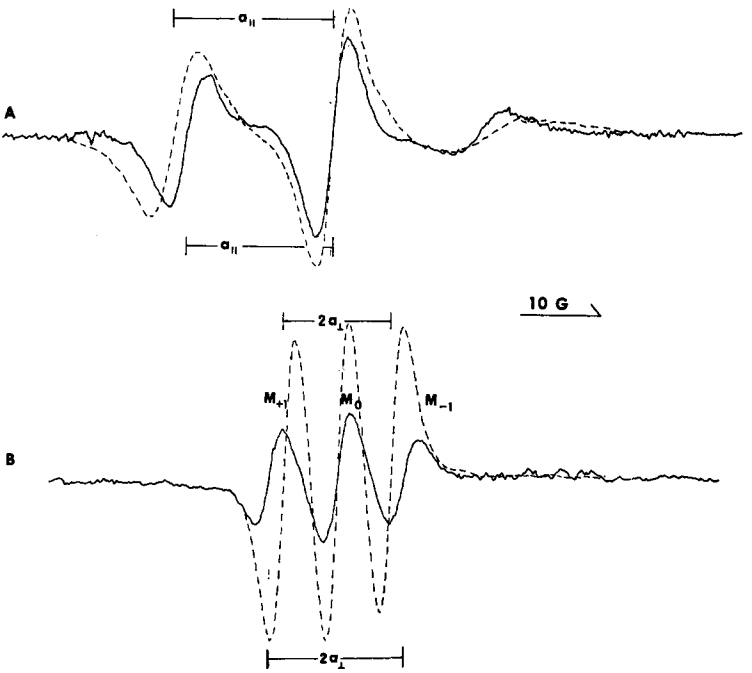


Fig. 2. ESR spectra of spin-labelled cholestane in hydrated films of phosphatidylethanolamine (———) and phosphatidylethanolamine, 50 mole %–cholesterol (– – – –) recorded when the plane of the lipid film is (A) parallel to the magnetic field and (B) perpendicular to the magnetic field. $a_{\|}$ was measured between the midpoints of the low field and centre peaks. $2a_{\perp}$ was measured between the midpoints of the low and high field peaks. The peaks in the perpendicular orientation are designated M_{+1}, M_0 and M_{-1}.

tation. For phosphatidylethanolamine alone $a_{||} = 18.3$ gauss and $a_{\perp} = 8.3$ gauss, while for films containing cholesterol $a_{||} = 19.6$ gauss and $a_{\perp} = 6.7$ gauss. Thus hydration induces the formation of a lamellar liquid crystalline phase. Both hexagonal, H_I and H_{II}, and lamellar phases have been detected by electron microscopy in aqueous dispersions of phosphatidylethanolamine with the lamellar phase predominating[17]. However, at early stages of equilibration with water the lipid appeared to be mainly unstructured. Thus there is evidence that the particular phases observed may be dependent on the water content of the system. Cholesterol improves the orientation in hydrated films continuously up to 50 mole % as can be seen from the decrease in $a_{\perp}$ and increase in M_{+1}/M_0 with increase in cholesterol in Fig. 3. The data are summarized in Table I.

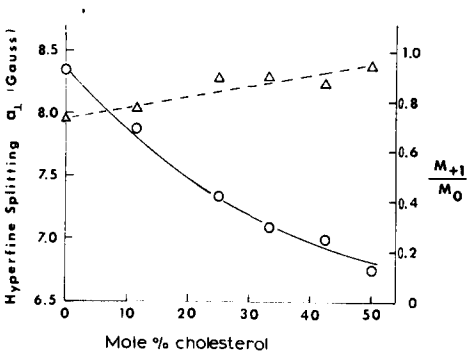


Fig. 3. Dependence of the hyperfine splitting $a_{\perp}$ (○—○), and the ratio of the low field peak height to centre peak height (M_{+1}/M_0) (△---△), on concentration of cholesterol in hydrated multibilayers of phosphatidylethanolamine.

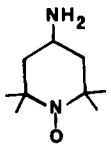
TABLE I

EFFECT OF CHOLESTEROL ON HYDRATED PHOSPHATIDYLETHANOLAMINE BILAYER STRUCTURE

Cholesterol (mole %)	$a_{||}$ *(gauss)*	$a_{\perp}$ *(gauss)*	M_{+1}/M_0
0	18.3	8.34	0.74
11.7	18.5	7.99	0.77
25.0	18.9	7.33	0.89
33.3	19.2	7.09	0.90
42.3	19.5	7.00	0.87
50.0	19.6	6.75	0.94

Hydrated films of phosphatidylethanolamine are better oriented than hydrated films of egg lecithin[1,7]. This is probably due to the greater space required by the larger choline group which keeps the hydrocarbon chains farther apart so that they have more freedom of motion. Monolayer studies have shown that the molecules in a completely condensed phosphatidylethanolamine film are more closely packed than those in an equivalent lecithin monolayer[6]. With cholesterol present, however, the degree of orientation is approximately the same in phosphatidylethanolamine and egg lecithin bilayers.

The effect of pH was measured on films of phosphatidylethanolamine alone and with 50 mole % cholesterol, and on egg lecithin with 33 mole % cholesterol (the concentration at which maximum orientation occurs[7]). Since the hyperfine splitting is known to depend on the polarity of the environment, the pH effect on a water-soluble spin label 1-oxyl-2,2,6,6-tetramethyl-4-amino-piperidine (II)



II

was determined by measuring the spectra of the spin label dissolved in the appropriate buffer. The effect of pH on the hyperfine splitting, $a_{\perp}$, and the ratio of the peak heights, M_{+1}/M_0, of the lipid films and the water-soluble spin label are shown in Fig. 4. The data are summarized in Table II. For phosphatidylethanolamine bilayers, $a_{\perp}$ increases slightly from pH 2.0–8.3 and then increases sharply from pH 8.3–10.4. There is a corresponding decrease in M_{+1}/M_0. New peaks in the spectra due to contributions from isotropically rotating spin label could be seen at pH 9.7 and 10.4. The pH effect on an equimolar phosphatidylethanolamine–cholesterol film is similar but there is not as large an increase in $a_{\perp}$. There is almost no pH effect on orientation in films of egg lecithin–cholesterol. The slight rise in $a_{\perp}$ at pH 10.4 is of the same magnitude as that for the water-soluble spin label and is probably due to stabilization of the negative charge on oxygen causing a greater spin density on nitrogen[14,15] which results in an increase in hyperfine splitting constants.

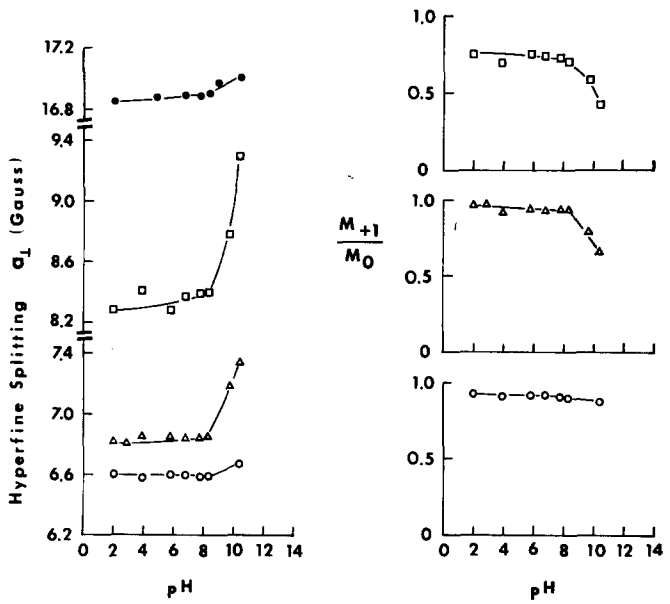


Fig. 4. Dependence of the hyperfine splitting, $a_{\perp}$, and the ratio of the peak heights (M_{+1}/M_0) on pH in hydrated multibilayers of phosphatidylethanolamine (□), phosphatidylethanolamine, 50 mole %–cholesterol (△), and egg lecithin, 33 mole %–cholesterol (○), and in a solution of a water-soluble spin label (●).

TABLE II

EFFECT OF pH ON PHOSPHOLIPID BILAYER STRUCTURE

Buffer	*pH*	*Phosphatidylethanolamine:cholesterol = 1:1*		*Phosphatidylethanolamine*		*Egg lecithin:cholesterol = 2:1*		*Spin label II* a_n *(gauss)*
		$a_\perp$ *(gauss)*	M_{+1}/M_0	$a_\perp$ *(gauss)*	M_{+1}/M_0	$a_\perp$ *(gauss)*	M_{+1}/M_0	
Glycine	2.0	6.82	0.97	8.28	0.75	6.60	0.93	16.85
	2.9	6.81	0.97	—		—		—
Acetate	3.9	6.85	0.92	8.41	0.70	6.58	0.91	—
	4.8	—		—		—		16.89
Phosphate	5.8	6.85	0.94	8.28	0.75	6.60	0.92	—
	6.8	6.84	0.93	8.37	0.74	6.60	0.92	16.89
	7.8	6.84	0.94	8.39	0.73	6.59	0.91	16.89
Ammonia	8.3	6.85	0.94	8.40	0.71	6.59	0.90	16.91
	9.0	—		—		—		16.98
	9.7	7.18	0.79	8.78	0.59	—		—
	10.4	7.34	0.66	9.29	0.42	6.67	0.87	17.02

DISCUSSION

Phosphatidylethanolamine has a net negative charge at pH 7.5 while phosphatidylcholine is a zwitterion at pH 3.5–10.0 (ref. 6). Therefore the decrease in orientation of phosphatidylethanolamine bilayers at high pH is probably caused by repulsion between the net negatively charged polar head groups thus giving more freedom of motion to the hydrocarbon chains. Monolayer studies have shown that at pH 4.0, films of phosphatidylethanolamine were more condensed than at pH 7.0, while lecithin films were identical at both pH values[8].

Cholesterol does not prevent the increase in disorder of the bilayer lattice structure at high pH but does limit it to some extent. Apparently the cholesterol restricts the freedom of motion of the hydrocarbon chains even in the expanded film. It may also reduce the repulsion between the negative phosphate groups through electrostatic interactions between the hydroxyl group and phosphate group.

These results suggest that neutralization of the negative phosphate group by the positively charged ammonium group contributes to the order of the bilayer by preventing repulsion of the polar head groups. However, in the presence of cholesterol there may also be some electrostatic interaction between the hydroxyl and phosphate groups which helps maintain order in the bilayer even at high pH when the polar head groups have a net negative charge.

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