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THE EFFECT OF 5-(*n*-ALK(EN)YL) RESORCINOLS FROM RYE ON MEMBRANE STRUCTURE

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Summary

The increased membrane permeability for K^+, glycerol and erythritol, and membrane lysis induced by alkyl and alkenyl resorcinols, respectively, might be due to the interaction with membrane proteins and the formation of reversed micelles.

The 5-(*n*-alk(en)yl) resorcinols show a very high stability at the air/water interface. The molecular area is 0.28 and 0.37 nm^2 (at 30 mN/m) for alkyl and alkenyl resorcinols from rye, respectively.

Differential scanning calorimetry experiments show a miscibility of alk(en)yl resorcinols with phosphatidylcholines. Only for alkenyl resorcinols is a small reduction found in the free energy of dipalmitoyl phosphatidylcholine. Electron microscopy studies show protein patching in erythrocyte membranes after the addition of resorcinols. The resorcinol-induced K^+ release is not influenced by the presence of proteolytic enzymes, but strongly reduced by bovine serum albumin and glycophorin. ^{31}P-NMR measurements show the occurrence of an isotropic and hexagonal signal in egg phosphatidylcholine in the presence of about 30 mol% alk(en)yl resorcinol.

Introduction

The long-chain resorcinols (1.3-dihydroxy-5-(*n*-alk(en)yl) benzenes) with an odd number of carbon atoms in the aliphatic chain occur in various plant species [1—14]. In *Anacardiaceae*, *Gymnospermae*, *Proteaceae* and *Myrisinaceae* [1—9], resorcinols are found mainly with up to 15 carbon atoms in the aliphatic chain and varying degrees of unsaturation. Resorcinols with longer

aliphatic chains (15—31 carbon atoms) were found in cereal grains (*Gramineae*) [10—12]. The amount of these compounds in cereal grains varies from 3000 ppm dry wt. in rye to less than 300 ppm in oat and corn [13—15]. In rye the presence of homologs with alkenyl side chains was noted [11,16].

The 5-(*n*-alk(en)yl) resorcinols are evidently toxic, at least the homologs with chain lengths of up to 15 carbon atoms [6,17—26]. The 5-(*n*-alk(en)yl) resorcinols from rye are deleterious when fed to young rats, swine [11,27] and chicken [28]. In a previous paper, we have shown that 5-(*n*-alk(en)yl) resorcinols from rye can induce significant changes in the permeability of liposomes and erythrocytes [29]. 5-(*n*-Pentadecyl)resorcinol caused a specific permeability increase for small solutes such as K^+, erythritol and glucose but not for haemoglobin. The alkyl resorcinols from rye also cause some haemoglobin release. The alkenyl resorcinols, on the other hand, are very lytic and cause a complete release of haemoglobin. The mechanism of the action of alk(en)yl resorcinols is not known. They hardly induce changes in proton permeability. This might indicate that the translateral mobility is very low. On the other hand, it seems very unlikely that resorcinols will induce the formation of pores as has been demonstrated for some polyene antibiotics [30] and gramicidin [31].

In this paper, we will report on the properties of acyl resorcinols at the air/water interface and their effect on the organization of membranes as studied by differential scanning calorimetry (DSC), freeze-etch electron microscopy and ^{31}P-NMR.

Materials and Methods

Egg phosphatidylcholine was prepared by using the method of Pangborn [32]; phosphatidic acid was prepared from egg phosphatidylcholine according to the method of de Gier et al. [33]. Synthetic lipids were prepared as described before [34]. 5-Methylresorcinol (orcinol) and 5-(*n*-pentadecyl)-resorcinol were obtained from Aldrich (Milwaukee, WI, U.S.A.) and further purified by column chromatography on silica [35]. 5-(*n*-Pentyl)resorcinol (olivetol) was obtained from ICN and K&K Laboratories (Plainview, NY, U.S.A.). The total alkyl resorcinols from rye were extracted with acetone and purified by preparative thin-layer chromatography (TLC) [35]. The alkyl resorcinols (saturated) and alkenyl resorcinols (unsaturated) were fractionated by pentane extraction and preparative TLC on silica, and on $AgNO_3$-impregnated silica gel [29,35]. Other chemicals used were of analytical grade.

Monolayer experiments. Force-area curves were measured on a Teflon trough (32.2 X 17.2 cm). The trough was filled with double-distilled water/10 mM Tris-HCl (pH 7.0). The resorcinols were spread from $CHCl_3$ solution. The compression rate was 74.9 cm^2/min and the surface tension was recorded by the Wilhelmy plate technique, using a Beckman L.M.C. 500 electrobalance.

Electron microscopy. Erythrocytes (100 μl suspension of 50% haematocrit) were incubated for 15 min at 37°C with 30 μM 5-(*n*-alk(en)yl) resorcinol. The samples were centrifuged at 20 000 rev./min for 30 min. The freeze-fracture electon microscopy of erythrocyte membranes was performed as described by Ververgaert et al. [36].

Calorimetric experiments. The experiments were performed on a Perkin-Elmer DSC-2B apparatus operating at a heating rate of 5°C/min at range 1.0 as described before [37]. 0.5 ml of a $CHCl_3$ solution containing 5 μmol of phosphatidylcholine with or without alk(en)yl resorcinol was evaporated in a test tube to dryness. Residual solvent was removed by storing the tube overnight under high vacuum. 50 μl of 10 mM Tris-HCl buffer (pH 7.0) with or without 25% of ethylene glycol were added. The lipids were dispersed by agitating on a vortex mixer for at least 1 min at a temperature above the transition temperature of the lipids. About 15 μl of the dispersion were sealed in an aluminium pan. Each sample was scanned at least five times to show the complete reversibility of the transitions and mixing of the components. The variations of the transition temperature between various scans were 1°C or less. After the calorimetric scans the amount of phospholipid present in the pan was determined by a phosphorus determination [38].

^{31}P-NMR measurements. Liposomes were prepared from mixtures of egg phosphatidylcholine and different resorcinols dissolved in $CHCl_3$. 50 mg of phospholipid were transferred to the vial and the $CHCl_3$ was evaporated under N_2. The samples were stored under high vacuum overnight and hydrated by exhaustive vortex mixing in 1 ml of 25% ^2H_2O/10 mM Tris-HCl buffer (pH 7.0)/2 mM EDTA. The spectra were obtained on a Bruker WH-90 Fourier Transform NMR spectrometer operating at 36.4 MHz for ^{31}P as described before [39].

K^+ release from erythrocytes in the presence of protein. Erythrocytes were prepared from fresh rabbit blood as described before [29]. To 10 ml of 100 mM $CaCl_2$/10 mM Tris-HCl buffer (pH 7.0), subsequently were added 50 μl erythrocyte suspension (haematocrit 50%), protein solutions and 1—5 μl of an ethanolic solution of alk(en)yl resorcinol. Potassium leak was measured with a potassium-sensitive glass electrode as described before [40]. The results of potassium measurements were corrected for small electrode changes induced by the presence of protein.

Results

In order to determine the interfacial properties of the 5-(*n*-alk(en)yl) resorcinols, their force-area curves were measured (Fig. 1). The resorcinols with an alkyl chain of 15 or more carbon atoms form very stable monolayers

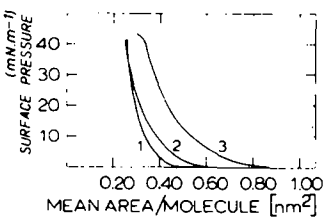


Fig. 1. Force-area curves of 5-(*n*-alk(en)yl) resorcinols measured in 10 mM Tris-HCl, pH 7.0, at 37°C. (1) 5-(*n*-Alkyl) resorcinols from rye, (2) 5-(*n*-pentadecyl)resorcinol, (3) 5-(*n*-alkenyl) resorcinols from rye.

with collapse pressures higher than 40 mN/m. The alkyl resorcinols occupy 0.28 nm^2/molecule and the alkenyl resorcinols 0.37 nm^2/molecule at a pressure of 30 mN/m. These results indicate that the alkyl resorcinols have a stability and orientation at the interface similar to that of phospholipids. The presence of double bonds causes a drastic increase in molecular area. At low surface pressures, pentadecylresorcinols occupy a greater molecular area than the longer chain saturated analogs.

Resorcinols have been shown to have a striking effect on the barrier properties of membranes [29]. The effect of alk(en)yl resorcinols on the phase transition of phosphatidylcholines was studied to determine if resorcinols could increase membrane permeability by affecting the hydrophobic core of membrane lipids. The pure resorcinols showed a main transition at 68°C for pentadecyl- and alkyl resorcinols (Fig. 2A and B). The alkenyl resorcinols showed a broad transition from 19 to 43°C with a main transition at 39°C (Fig. 2C). In mixtures of pentadecylresorcinols and dipalmitoyl phosphatidylcholine or dielaidoyl phosphatidylcholine, a gradual shift of the phase transition temperature to intermediate values is observed. This indicates mixing of the phosphatidylcholine with pentadecylresorcinol. The occurrence of a second peak, e.g., at a molar ratio of dipalmitoyl phosphatidylcholine to pentadecylresorcinol of 1 : 1 and of dielaidoyl phosphatidylcholine to pentadecylresorcinol of 5 : 1 (Fig. 2A) indicates that the mixing is not complete at all molar ratios and a partial phase separation occurs. Similar results are found for mixtures of the saturated resorcinols from rye and dipalmitoyl or dielaidoyl phosphatidylcholine (Fig. 2B). The effect of the phospholipid fatty acid chains on the miscibility is indicated by the occurrence of the partial phase separation at higher molar ratios of phosphatidylcholine to saturated resorcinol for dipalmitoyl than for dielaidoyl phosphatidylcholine (Fig. 2B). Mixtures of unsaturated resorcinols from rye and dipalmitoyl or dielaidoyl phosphatidylcholine show a gradual shift of the phase transition temperature to intermediate values with increasing molar ratios (Fig. 2C). There is no strong indication for a phase separation of the two compounds. The saturated resorcinols from rye and pentadecylresorcinol show practically no reduction of the heat content of dipalmitoyl phosphatidylcholine (Fig. 3). The unsaturated resorcinols from rye reduce the enthalpy from dipalmitoyl phosphatidylcholine from 9.5 to 6 kcal/mol at a molar ratio of 1 : 1.

The effect of the surface active resorcinols on the structure of membranes was studied by freeze-etch electron microscopy. 5-(*n*-Pentadecyl)resorcinol induces a specific permeability increase for small solutes. Practically all K^+ is released from erythrocytes and from the outer shells of liposomes. Freeze-etch electron microscopy shows some particle aggregation in erythrocyte membranes treated with pentadecylresorcinol (Fig. 4A). Also, the etch surface seems to be changed. The alkyl resorcinols from rye show a partial release of K^+ from erythrocytes but also cause some lysis of cells. The freeze-etch picture of erythrocytes treated with alkyl resorcinols shows no significant change with respect to the distribution of protein particles (Fig. 4B). The most drastic effect on membranes was caused by alkenyl resorcinols. These compounds induce a complete K^+ and haemoglobin release. The freeze-etch pictures show a nearly complete destruction of membrane structures and a

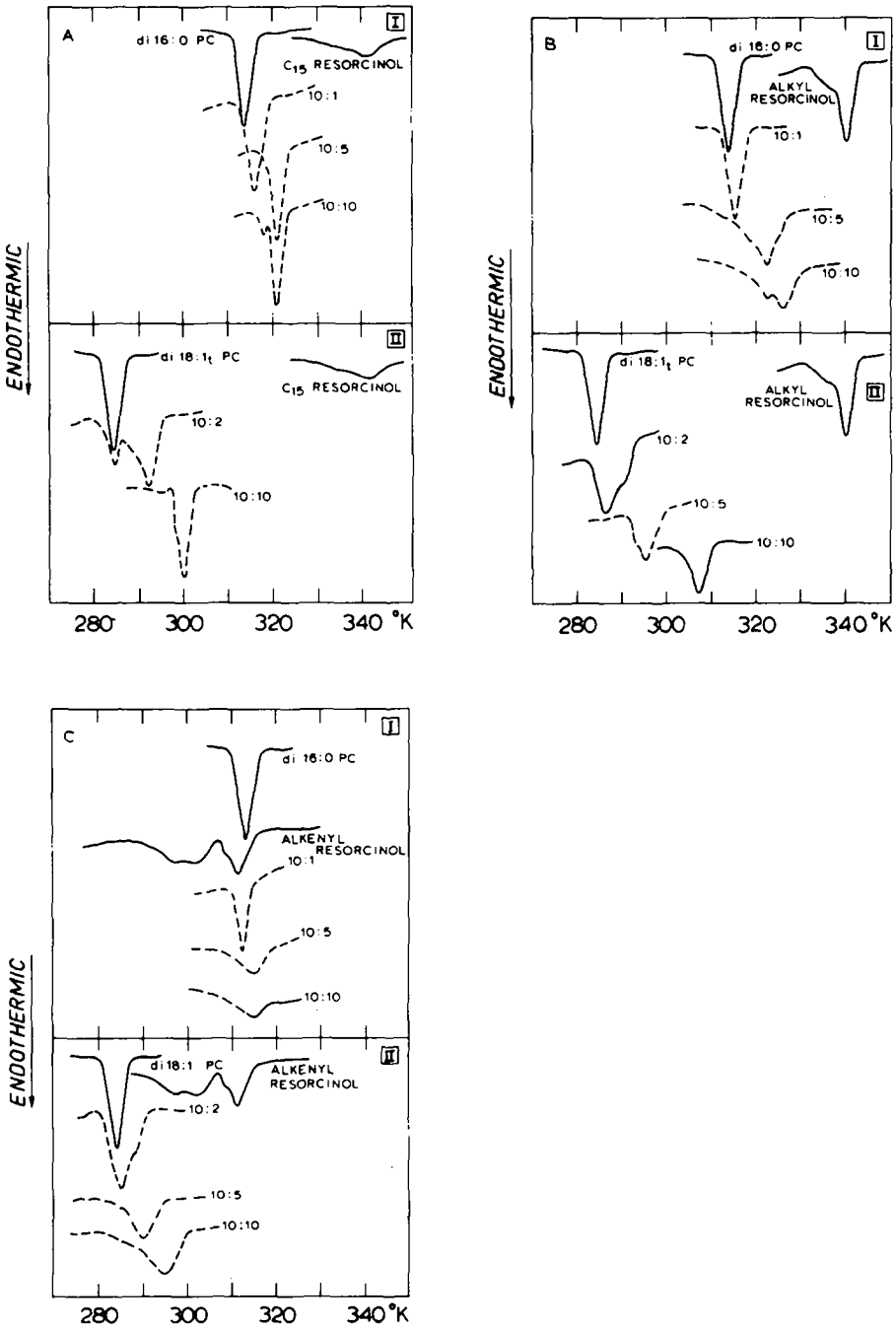


Fig. 2. (A) Calorimetric scans of: (I) mixture of dipalmitoyl phosphatidylcholine (di 16 : 0 PC) and pentadecylresorcinol at different molar ratios, (II) mixture of dielaidoyl phosphatidylcholine (di 18 : 1_t PC) and pentadecylresorcinol at different molar ratios. (B) Calorimetric scans of: (I) mixtures of dipalmitoyl phosphatidylcholine and alkyl resorcinols from rye at different molar ratios, (II) mixtures of dielaidoyl phosphatidylcholine and alkyl resorcinols from rye at different molar ratios. (C) Calorimetric scans of: (I) mixtures of dipalmitoyl phosphatidylcholine and alkenyl resorcinols from rye at different molar ratios, (II) mixtures of dielaidoyl phosphatidylcholine and alkenyl resorcinols from rye at different molar ratios.

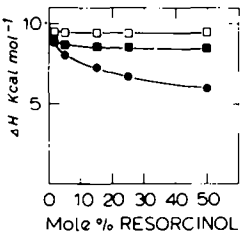


Fig. 3. Effect of 5-(*n*-alk(en)yl) resorcinols on the energy content of the phase transition in mixtures with dipalmitoyl phosphatidylcholine. ■, 5-(*n*-pentadecyl)resorcinol; □, 5-(*n*-alkyl) resorcinols from rye; ●, 5-(*n*-alkenyl) resorcinols from rye.

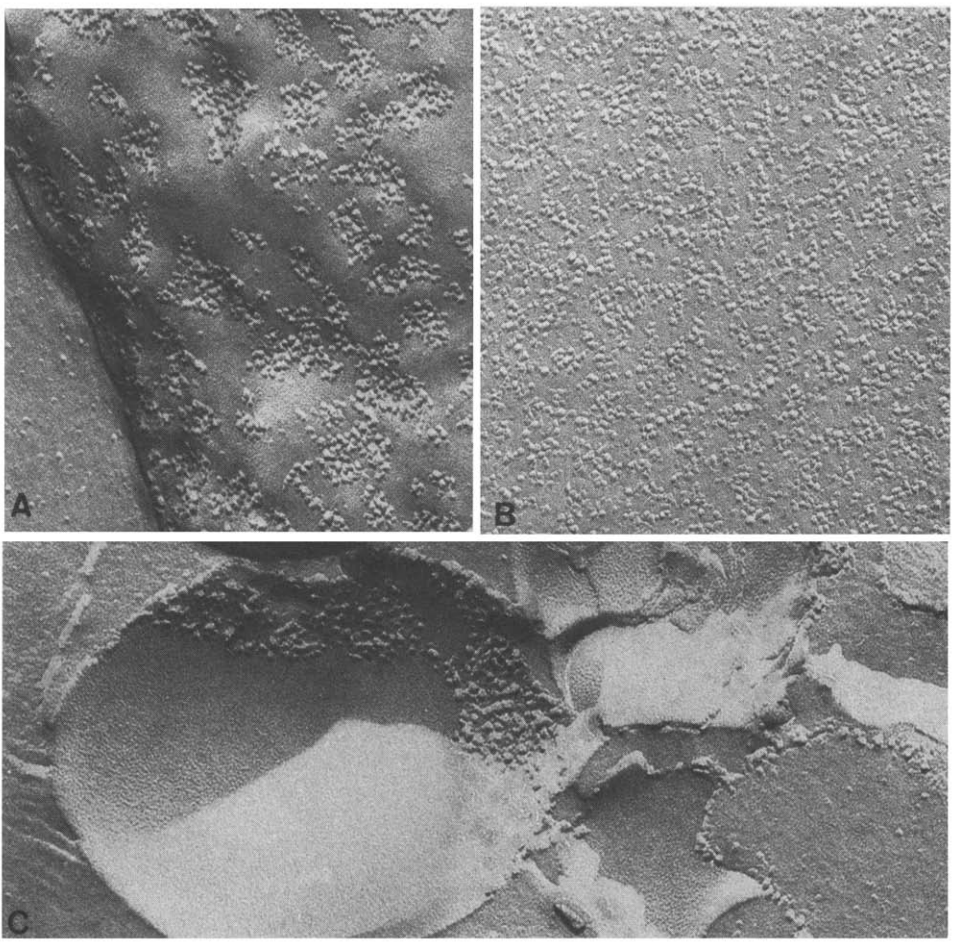


Fig. 4. Freeze-fracture micrographs of rabbit erythrocyte membranes after incubation with 5-(*n*-alk-(en)yl) resorcinols. (A) Pentadecylresorcinol, (B) alkyl resorcinols from rye, (C) alkenyl resorcinols from rye. Magnification, X100 000.

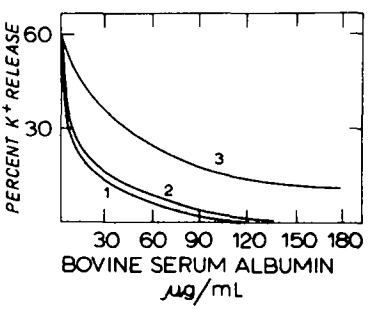
TABLE I

EFFECT OF PROTEINS ON THE RESORCINOL-INDUCED K^+ RELEASE FROM RABBIT ERYTHROCYTES

Protein concentration 25 μg/ml, resorcinol concentration 7.5 μM

Protein	% K^+ release	
	Pentadecylresorcinol	Alkenyl resorcinol
Trypsin	67	70
Chymotrypsin	61	56
Lysozyme	66	60
Polylysine	80	71
Bovine serum albumin	48	18
Glycophorin	24	36

complete aggregation of protein particles (Fig. 4C). To see whether the protein aggregation in erythrocytes could be due also to an interaction of the resorcinols with proteins, the effect of several proteins on the acyl resorcinol-induced K^+ release was tested (Table I). Water-soluble proteins and proteo-



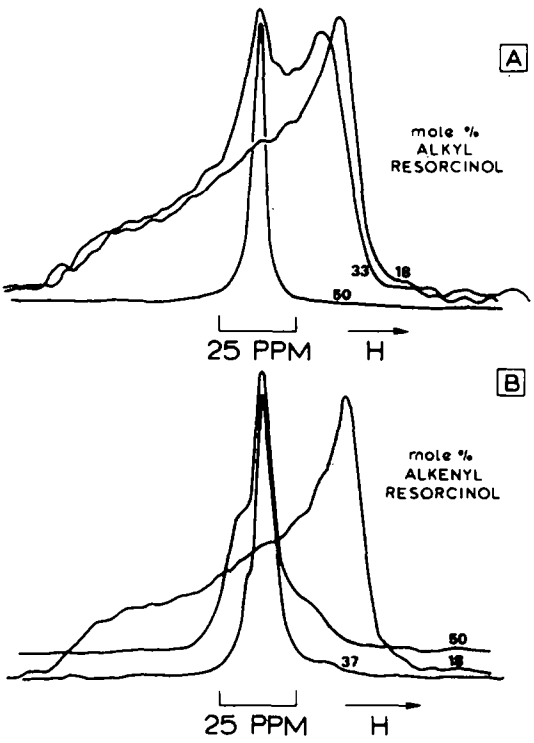


Fig. 5. Effect of bovine serum albumin on the resorcinol-induced K^+ release from rabbit erythrocytes. (1) Alkyl resorcinol, (2) pentadecylresorcinol, (3) alkenyl resorcinol. Resorcinol concentration 7.5 μM.

Fig. 6. 36.4 MHz ^{31}P-NMR spectra of aqueous dispersions of egg phosphatidylcholine and (A) alkyl resorcinol or (B) alkenyl resorcinol from rye in 10 mM Tris-HCl (pH 7.0)/2 mM EDTA at 36°C.

lytic enzymes had no effect or even showed some stimulation of the effect of pentadecyl- and unsaturated resorcinols. A marked reduction of the resorcinol activity was found in the presence of fatty acid-free bovine serum albumin. The greatest reduction was found for pentadecylresorcinol. With a bovine serum albumin concentration of 120 μg/ml, the effects of pentadecyl- and the saturated resorcinols can be completely abolished (Fig. 5). At this concentration of bovine serum albumin, the effects of the unsaturated resorcinols can be reduced by 80%. Also, an amphiphatic membrane protein such as glycophorin can reduce the effects of resorcinols considerably. This protein shows the biggest effect on the unsaturated resorcinols (Table I). ^{31}P-NMR was used to determine whether the resorcinols can affect membrane permeability by inducing changes in the membrane structure. 18 mol% of saturated resorcinols showed no significant effect on the bilayer structure, showing the normal high-field peak and low-field shoulder (Fig. 6A). However, 33 mol% saturated resorcinols showed an additional isotropic peak and at 50 mol% only an isotropic peak was found (Fig. 6A). The unsaturated resorcinols showed also no significant change of the bilayer structure at a concentration of 18 mol% (Fig. 6B). However, at a concentration of 37 mol%, only the isotropic peak could be seen and at a concentration of 50 mol% a high-field shoulder occurs indicating a hexagonal phase.

Discussion

It has been shown in a previous paper that alkyl resorcinols can influence membrane properties dramatically. The membrane becomes highly permeable to small solutes such as K^+, glycerol and erythritol after the addition of pentadecylresorcinol and longer chain saturated analogs (15—31 carbon atoms) isolated from rye. Unsaturated resorcinols isolated from rye were found to be lytic as evidenced by the release of haemoglobin [29].

In the present paper, we have demonstrated the amphiphatic character of alk(en)yl resorcinols and their interfacial stability. These properties will enhance their ability to act on membranes. The molecular area of the saturated resorcinols is 0.28 nm^2/mol. This is 0.08 nm^2/mol more than for fatty acids and long-chain alcohols. The introduction of double bonds in the aliphatic chain leads to an increase of 0.09 nm^2/mol at 30 mN/m. In particular, the presence of these unsaturated resorcinol analogs in the membrane could disturb the structure by reducing the packing of the lipids. The saturated long chain analogs with an aliphatic chain of 15—31 carbon atoms could affect lipid packing by extending into the second half of the bilayer.

The DSC experiments, however, show that only the unsaturated analogs have a moderate effect on the heat content of the dipalmitoyl phosphatidylcholine. Also, lysophosphatidylcholines which are known lytic compounds show in mixtures with phosphatidylcholines at concentrations below 50 mol% only a small decrease in enthalpy [41,42]. The DSC experiments indicate a good miscibility of resorcinols and phosphatidylcholines in most mixtures. That the miscibility can be influenced by the fatty acid chain length has been shown also for mixtures of lysophosphatidylcholines with phosphatidylcholines [42].

The electron-microscopic pictures show that the lytic resorcinols can induce protein aggregation in membranes. This might be due to the interaction of resorcinols with membrane lipids or proteins. The possibility of a resorcinol-protein interaction, with proteins with a hydrophobic region, is demonstrated by the inhibition of the resorcinol-induced K^+ release by glycophorin and bovine serum albumin. Resorcinols can bind to bovine serum albumin like lysophosphatidylcholine and fatty acids [43,44] do. The binding of resorcinols to membrane proteins might also be involved in the induction of permeability changes in natural membranes. However, permeability changes in the presence of resorcinols have been noted in liposomes which do not contain protein [29]. The ^{31}P-NMR data indicate that structural rearrangements of the lipids might be primarily involved in the resorcinol permeability changes. Since resorcinols, except for 5-(*n*-pentyl)resorcinol, do not induce a selective proton permeability [29], a rapid translateral movement of resorcinols with an aliphatic chain of 15 or more carbon atoms seems to be unlikely. The occurrence of an isotropic signal in the presence of resorcinols and even a hexagonal signal in the case of unsaturated resorcinols indicate that highly mobile structures are formed. Recently, Cullis and de Kruijff [39] proposed that the formation of reversed micelles would account for the occurrence of an isotropic signal and a high membrane permeability. These non-lamellar structures were also induced in phosphatidylcholine by phosphatidylethanolamine, monoglucosyldiglyceride, cardiolipin in the presence of Ca^{2+} [29] and lysophosphatidylcholine (van Echteld, C.J.A., unpublished result). It is possible that resorcinols increase membrane permeability by the formation of reversed micelles. An altered osmotic equilibrium and an increasing percentage of non-bilayer structure can finally lead to disruption of the cell membrane.

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