Biochimica et Biophysica Acta, 307 (1973) 1–19

BBA 76305

THE EFFECT OF THE POLAR HEADGROUP ON THE LIPID–CHOLESTEROL INTERACTION: A MONOLAYER AND DIFFERENTIAL SCANNING CALORIMETRY STUDY

B. DE KRUYFF, R. A. DEMEL, A. J. SLOTBOOM, L. L. M. VAN DEENEN and A. F. ROSENTHAL*

Laboratory of Biochemistry, State University of Utrecht, Vondellaan 26, Utrecht (The Netherlands)

(Received December 11th, 1972)

SUMMARY

Since the sterol 3β-OH group is essential for the lipid–sterol interaction, the interaction of cholesterol with natural phospholipids and glycolipids and synthetic phospholipids and analogs, differing in the polar moiety was studied in monolayers and liposomes. In monolayers, the interaction was measured as a reduction in the mean molecular area (condensing effect). In liposomes by differential scanning calorimetry as a reduction in the energy content of the crystalline → liquid–crystalline phase transition (liquefying effect).

The presence of the oxygen atoms of the acyl ester linkages and of the oxygen atoms connecting phosphorus and carbon are not essential for the lipid–sterol interaction as determined by the above methods. Measurements with monoglucosyldiglyceride and diglucosyldiglyceride even reveal that the phosphorus and choline moiety are not required for the interaction. This indicates that it is unlikely that a specific binding of the sterol-OH group with any polar part of the lipid molecule is essential for the condensing or liquefying effect. The need for a 3β-OH group can neither be explained by a cooperative effect of the C_{19} methyl group and the 3β-OH group in the lipid–sterol interaction since 19-norcholesterol shows the same effect as cholesterol.

The effect of cholesterol upon the crystalline → liquid–crystalline phase transitions in a codispersion of (1,2-dioleoyl)lecithin and (1,2-distearoyl)lecithin was studied by differential scanning calorimetry. At low concentrations (less than 25 mole %) cholesterol preferentially associates with (1,2-dioleoyl)lecithin. At higher concentrations cholesterol interacts with (1,2-distearoyl)lecithin as well. This indicates that when cholesterol is present in a membrane with lipids in both the crystalline and liquid–crystalline state, cholesterol preferentially interacts with these lipids which are in the liquid–crystalline state.

* Present address: Long Island Jewish Medical Center, New Hyde Park, N.Y. 11040, U.S.A.

INTRODUCTION

The exact function of cholesterol, a major constituent of many biological membranes, is as yet not clearly understood. A variety of physical techniques have demonstrated that cholesterol can affect the chain interaction of lipids in model membranes and biological membranes.

A reduction in mean molecular area was demonstrated by monolayer and X-ray studies[1–4]. Monolayer experiments revealed that phospholipid–cholesterol interactions could occur only with particular fatty acid constituents of the phospholipid. Saturated lecithin films are condensed by cholesterol only when the film is of a liquid–crystalline type and the fatty acid chain length is more than 10 carbon atoms[3]. Unsaturated lecithin films are condensed by cholesterol when one of the acyl chains contains up to four double bonds[5]. Little or no interaction is found when both chains are poly-unsaturated, although these lecithins form liquid–crystalline films[5]. A reduction in chain mobility of the fatty acid constituents due to increased hydrophobic interactions is shown by ESR and NMR studies[6,10].

That the above effects influence the simple diffusion of solutes through the lipid bilayer is demonstrated by studies on liposomes[11,12], black lipid membranes[13], erythrocytes[14] and *Acholeplasma laidlawii* cell membranes[15–17].

A striking correlation was found between the reduction in molecular area in monolayers and the reduction in permeability in liposomes[18,19].

On the other hand, the chain interaction of phospholipids in the crystalline state, is reduced by the presence of cholesterol. This increase in chain mobility, the so-termed liquefying effect, has been observed by NMR[8,10] and differential scanning calorimetry[16,20] in model membrane systems. Natural membranes of *A. laidlawii* showed the same phenomenon[16]. Growth experiments with this organism furthermore indicated that the liquefying effect can be very essential for proper membrane functioning[17].

With respect to the structural requirements of the sterol moiety it is shown in ESR[7], monolayer[18], and permeability studies using liposomes[19] and *A. laidlawii* cells[17] that: a planar ring system, an intact side chain at C_{17}, and a 3β-OH group are essential for a condensing effect of the sterol. However, also for the liquefying effect, as visualised by a reduction in energy content of the crystalline $\rightarrow$ liquid–crystalline phase transition, the same functional groups are a prerequisite[16].

It is clear that the phospholipid–sterol interaction is highly hydrophobic in nature. Since however, also a 3β-OH is essential, interactions in the polar region have to be considered as well. In the present study we investigate whether the absolute requirement for a 3β-OH group can be explained by; (a) an interaction between the 3β-OH group and any specific polar moiety of the lipid molecule (b) a cooperative effect of the 18 and 19 methyl groups and the 3β-OH group of the sterol molecule in the lipid–sterol interaction. For this purpose we studied the effect of cholesterol upon the force–area curves and the phase transition of various lipids which differ in the polar part of the molecule. Furthermore, we investigated the effect of the C_{19} methyl group of cholesterol on the interaction.

Although cholesterol can interact with phospholipids in the liquid–crystalline state and in the crystalline state, nothing is known about the lipid–sterol interaction in a membrane when both phases are present simultaneously. Therefore, we investi-

gated the interaction in a codispersion of (1,2-dioleoyl)lecithin and (1,2-distearoyl)-lecithin by differential scanning calorimetry at various cholesterol concentrations.

MATERIALS AND METHODS

Sterols

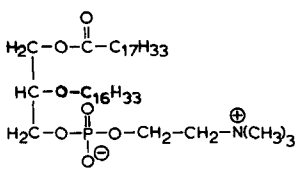
Cholest-5-en-3β-ol (cholesterol) was purchased from Fluka AG (Buchs, Switzerland). Cholest-5,22-dien-24-ethyl-3β-ol (stigmasterol) was obtained from Koch-Light laboratories, Colnbrook (Buchs, England). 19-Nor-Cholest-5-en-3β-ol (19-norcholesterol) was a generous gift of Dr P. P. Poirer from the Division Scientifique Roussel-Uclaf (Romainville, France). Cholesterol was recrystallised two times from ethanol. The purity of the sterols was checked by thin-layer chromatography as described before[18].

Synthetic phospholipids

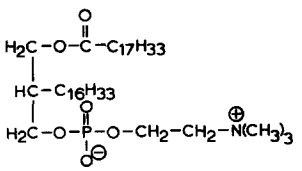
Phosphatidylcholines (*lecithins*). 1-Palmitoyl-2-oleoyl-*sn*-glycero-3-phosphorylcholine (16:0/18:1c-phosphorylcholine), 1,2-dipalmitoyl-*sn*-glycero-3-phosphorylcholine (16:0/16:0-phosphorylcholine), 1,2-distearoyl-*sn*-glycero-3-phosphorylcholine (18:0/18:0-phosphorylcholine), 1,2-dioleoyl-*sn*-glycero-3-phosphorylcholine (18:1c/18:1c-phosphorylcholine), 1-oleoyl-2-stearoyl-*sn*-glycero-3-phosphorylcholine (18:1c/18:0-phosphorylcholine), 1,2-dielaidoyl-*sn*-glycero-3-phosphorylcholine (18:1t/18:1t-phosphorylcholine) and 1-palmitoyl-2-stearoyl-*sn*-glycero-3-phosphorylcholine (16:0/18:0-phosphorylcholine) were synthesised as described before[21].

Phosphatidylcholine analogs. 3-Palmitoyl-2-oleoyl-*sn*-glycerol-1-phosphorycholine (D-16:0/18:1c-phosphorylcholine), *rac*-1-oleoyl-2-hexadecylglycero-3-phosphorylcholine (Formula I) and *rac*-1-oleoyl-2-C-hexadecylpropanediol-3-phosphorylcholine (Formula II) were synthesised as described before[22].

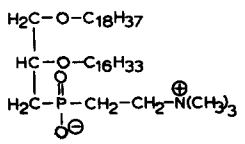
Phosphinate lipids. 2-Hexadecoxy-3-octadecoxypropyl [2′-(trimethylammonium) ethyl]-phosphinate (Formula III) and octadecyl [2′-trimethylammonium) ethyl]-phosphinate (Formula IV) were synthesised as described before[23]. The purity of all synthetic lipids was checked prior to use by thin-layer chromatography on silicagel G



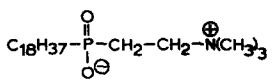
I



II



III

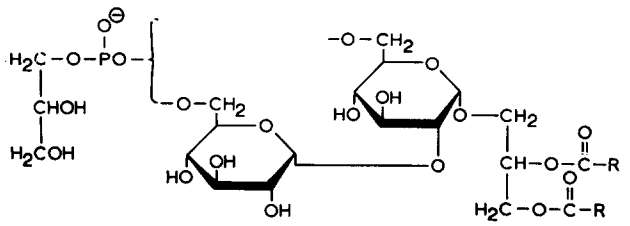


IV

in the solvent system chloroform–methanol–water, 65:35:4 (v/v/v). Because only small quantities of both phosphinate lipids were available no further purity checks were performed on these compounds.

Lipids isolated from A. laidlawii.

A. laidlawii strain B cells were grown in 5–22 l quantities of lipid-poor tryptose medium as described before[16]. For the isolation of diglucosyldiglyceride from *A. laidlawii* cells no fatty acids were added to the growth medium. For the isolation of other *A. laidlawii* lipids the growth medium was supplemented with 0.12 mM elaidic acid (18:1t). The total polar lipids were isolated as described previously[16]. For the isolation of 3-[*O*-α-D-glucopyranosyl-(1→2)-*O*-α-D-glucopyranosyl]-*sn*-1,2-diglyceride (diglucosyldiglyceride) the total polar lipids were fractionated first by thin-layer chromatography on silicagel G in the solvent system chloroform– methanol–water, 65:35:4 (v/v/v) and purified further by thin-layer chromatography on silicagel G in the solvent system chloroform–methanol–water, 65/15/1 (v/v/v). The final purification was performed by column chromatography over silicagel (Mallinckrodt, mesh 80–120). The column was eluted with chloroform with increasing amounts of methanol. Chromatographically pure diglycosyldiglyceride was obtained in the fractions containing 8–13% (v/v) of methanol. The compound had a molar ratio of glucose to fatty acid of 1.01, contained no phosphorus, and had an R_F value of 0.50 on silicagel G in the solvent system chloroform–methanol–water, 65:15:1 (v/v/v). For the isolation of the other major polar membrane lipids of *A. laidlawii* the total polar lipid extract was applied directly on a silicagel (Mallinckrodt, mesh 80–120) column. The column was eluted with chloroform containing increasing amounts of acetone. The 30–50% (v/v) acetone fraction contained chromatographically pure 3-(*O*-α-D-glucopyranosyl)-1,2-diglyceride (monoglucosyl-diglyceride). The compound contained no phosphorus, and had an R_F value of 0.65 on silicagel G in the solvent system chloroform–methanol–water, 65:15:1 (v/v/v). The molar ratio of glucose to fatty acid was 0.5. Phosphatidylglycerol and the phosphoglycolipid, 3-(*sn*-glycerol-3-phosphoryl-6′-[*O*-α-D-glycopyranosyl-(1→2)-*O*-α-D-glucopyranosyl])-*sn*-1,2-diglyceride (Formula V)[24] were isolated from the column by eluting succesively with 100% acetone (which removed the diglucosyldiglyceride), and chloroform with increasing amounts of methanol. Phosphatidylglycerol was eluted with 10–12% (v/v) methanol and the phosphoglycolipid (Formula V) with 25% (v/v) methanol. Both compound were chromatographically pure on silicagel G solvent system chloroform–methanol–water, 65:35:5 (v/v/v). Both compounds contained phosphorus and developed with Schiff



V

reagent the characteristic purple color. The phosphoglycolipid cochromatographed with the phosphoglycolipid (Formula V) isolated from *A. laidlawii* by Shaw *et al.*[24]. Phosphatidylglycerol isolated from *A. laidlawii* cochromatographed with synthetic phosphatidylglycerol.

Monolayer studies

Force–area measurements were performed at the air–water interface in a teflon trough 35.5 cm long and 17.3 cm wide with a total capacity of 800 ml. The trough was filled with unbuffered double-distilled water (pH 5.4). Known amounts of lipids dissolved in chloroform were released onto the interface using a aglamicrometer syringe. Mixtures of lipid–sterols were spread from premixed solutions. The lipid film was compressed by a barrier which was driven by an electromotor with a constant speed of 1.03 mm/s. The surface tension was automatically recorded using the Wilhelmy plate method and a R.I.I.C. L.M.500 electrobalance. Each force–area curve was recorded at least 3 times. The maximal variation between the various runs was not more than 2 $Å^2$/molecule.

Differential scanning calorimetry

The thermal transitions in the fatty acid chains of the different lipids were measured as described in detail previously[16]. Lipid dispersions were prepared by adding 50 μl of water–glycol, 1;1 (v/v) to about 6 μmole of dry lipid. Lipids were dispersed by agitating the tube for 5 min on a Vortex mixer above the temperature of the phase transition of the lipids. 40 μl of the lipid dispersions were sealed in specially constructed aluminum sample pans. The commercially available sample pans have a 17 μl capacity. A disadvantage of the 40 μl sample pans was that, because of slower temperature equilibrium, transitions became relatively broad. After the calorimetric measurements the amount of lipid present in the sample pan was determined by a phosphorus or a glucose determination. The maximal variation between the various determinations of the heat of transition of a lipid was about 10%.

Measurements of glucose permeability of liposomes

The release of glucose from liposomes at 40 °C was measured as described in detail before[12].

Analytical methods

Phosphorus was determined according to the method of Fiske and SubbaRow[25]. Glucose, liberated from glycolipid after acid hydrolysis[26], was measured quantitatively using the anthrone reagent[27]. The fatty acid pattern of the different lipids was determined as described before[16]. The amount of fatty acid was determined by reference to a C_{20} standard.

RESULTS

The effect of cholesterol upon the force–area curves of various pure lipids at the air–water interface

The pressure–area curves of 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoryl-

choline, *rac*-1-oleoyl-2-hexadecylglycero-3-phosphorylcholine (Formula I) and *rac*-1-oleoyl-2-C-hexadecylpropanediol-3-phosphorylcholine (Formula II) in the presence and absence of 50 mole % cholesterol are shown in Fig. 1. The pure lipids having at the 2 position an ester, ether or alkane linkage show similar force–areas curves. This indicates that the two oxygen atoms of the ester linkage at the 2-position of the phosphatidylcholine molecule do not significantly influence the molecular area. This finding is in agreement with the studies of Paltauf *et al.*[28].

Neither of the oxygen atoms appeared to be required for the interaction with cholesterol. Fig. 1 shows that the monolayers of the three different phosphatidylcholines are significantly condensed by cholesterol. The strongest effect is observed with the natural lecithin (16:0/18:1c-phosphorylcholine). Figs 2A and 2B reveal the pressure–area curves of mixtures of cholesterol with the phosphinate lipids (Formulas III and IV). The phosphinate lipid used (Formula III) differs from 18:0/16:0-phosphorylcholine by a direct binding of phosphorus to carbon and ether instead of ester bonds between the paraffinic residues and the glycerol backbone. The phosphinate lipid (Formula III) and 16:0/18:0-phosphorylcholine showed a similar pressure–area curve (Fig. 2A). The phosphinate lipid represented in Formula IV gives small area per molecule due to the presence of only one hydrocarbon chain (Fig. 2B). The presence of cholesterol affected the mean molecular area of both phosphinate lipids (Figs 2A and 2B). It has to be noted that 52.5 mole% cholesterol

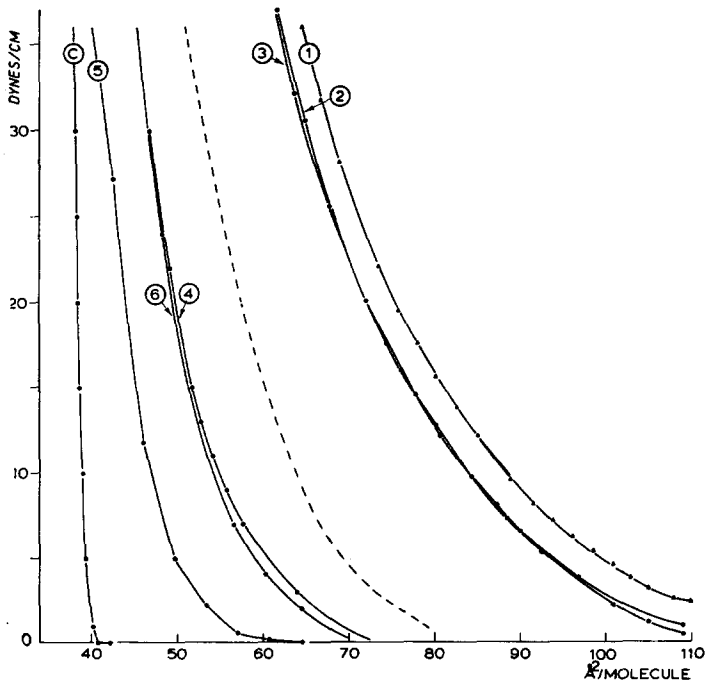


Fig. 1. Force–area curves at 22 °C at the air–water interface. (1) *rac*-1-Oleoyl-2-hexadecylglycero-3-phosphorylcholine; (2) 16:0/18:1c-phosphorylcholine; (3) *rac*-1-oleoyl-2-C-hexadecylpropanediol-3-phosphorylcholine; (4), (5), and (6) 1:1 mixtures of respectively (1), (2) and (3) with cholesterol. (C), cholesterol. The dotted line represents the calculated force–area curve of (4) assuming that no interaction occurs.

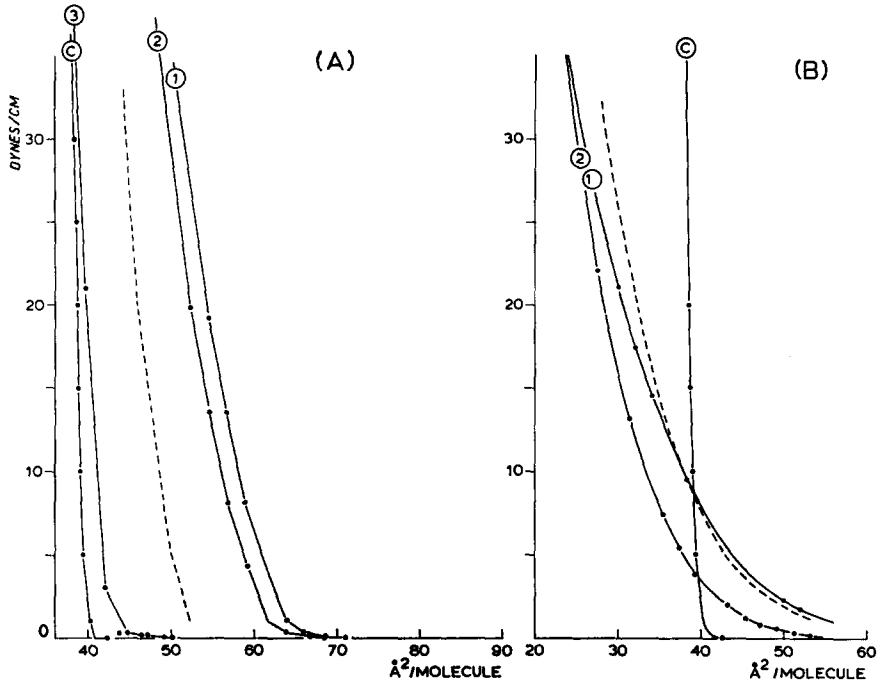


Fig. 2. Force–area curves at 22 °C at the air–water interface. (A) (1) Phosphinate lipid (Formula III), (2) 16:0/18:0-phosphorylcholine, (3) phosphinate lipid (Formula III) and 53.5 mole% cholesterol. (C),cholesterol. (B) (1) Phosphinate lipid (Formula IV), (2) phosphinate lipid (Formula IV) and 21.1 mole % cholesterol. (C), cholesterol. The dotted lines represent the calculated force–area curves of (A) (3) and (B) (2) assuming that no interaction occurs.

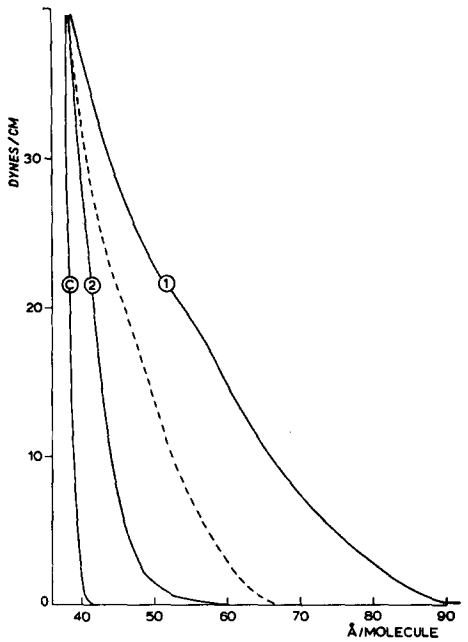


Fig. 3. Force–area curves at 22 °C at the air–water interface. (1) Monoglucosyldiglyceride, (2) monoglucosyldiglyceride and 52 mole % of cholesterol. (C), cholesterol. The dotted line represents the calculated force–area curve of (2) assuming that no interaction occurs. Monoglucosyldiglyceride was isolated from elaidic acid grown *A. laidlawii* cells and had the following fatty acid composition:

12:0	14:0	16:0	18:0	18:1t	18:2	short chain unknown
4.3	7.0	19.0	1.2	64.3	1.1	3.1

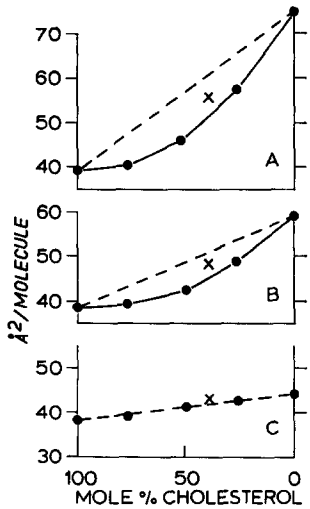


Fig. 4. Variation of the mean molecular area as a function of the composition at different pressures at 22 °C from mixed monolayers of cholesterol and monoglucosyldiglyceride. (A) 5 dynes/cm, (B) 15 dynes/cm and (C) 30 dynes/cm. ×, The mean molecular area of a mixture of monoglucosyldiglyceride and stigmasterol.

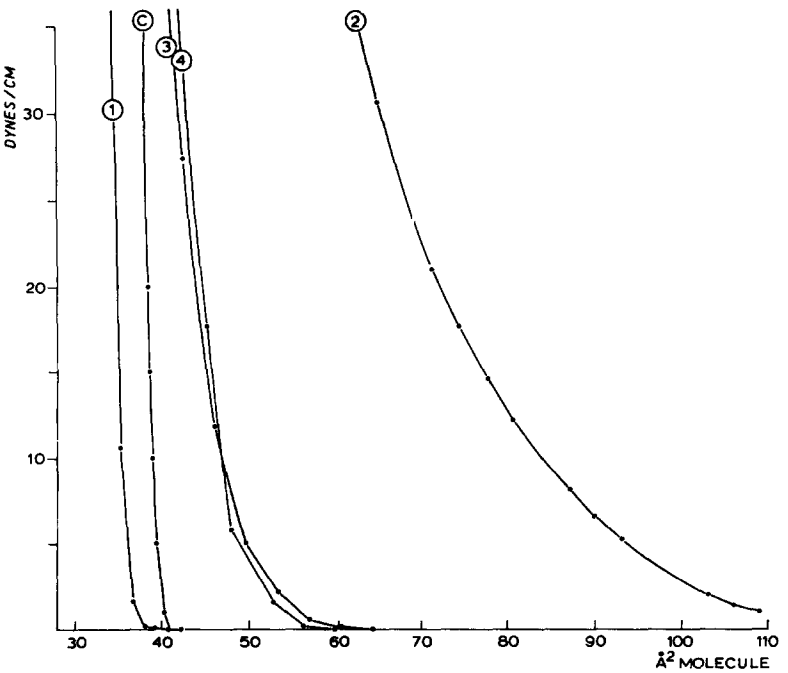


Fig. 5. Force–area curves at 22 °C at the air–water interface. (C), Cholesterol, (1) 19-norcholesterol, (2) 16:0/18:1c-phosphorylcholine (3) 16:0/18:1c-phosphorylcholine and 53.9 mole % cholesterol, (4) 16:0/18:1c-phosphorylcholine and 53.9 mole % 19-norcholesterol.

was present in the monolayer of phosphinate lipid (Formula III) and 21.1 mole% cholesterol in the monolayer of phosphinate lipid (Formula IV). This might explain the relative smaller reduction in mean molecular area of phosphinate lipid (Formula IV, Fig. 2B) as compared to the strong reduction as observed with phosphinate lipid (Formula III). From this it can be concluded that the oxygen atoms connecting the phosphorus with the glycerol and the choline moiety of the molecule do not play an important role in the interaction of cholesterol and phospholipids at the air–water interface.

In order to investigate whether the phosphate group is essential for the interaction between cholesterol and phospholipids we studied the interfacial behaviour of mixtures of cholesterol and monoglucosyldiglyceride. The force–area curve of monoglucosyldiglyceride isolated from elaidic acid grown *A. laidlawii* cells (Fig. 3) is at low surface pressures of a liquid-expanded type and shows a transition to a more condensed state at a surface pressure of about 20 dynes/cm. The force–area curve of a mixture of monoglucosyldiglyceride and cholesterol demonstrates (Figs 3 and 4) that a marked reduction in the mean area per molecule is observed especially at pressures below 20 dynes/cm indicating that an interaction between cholesterol and this glycolipid occurs. Fig. 4 demonstrates that a reduction in the mean area per molecule is also observed in a mixed monolayer of the plant sterol stigmasterol and monoglucosyldiglyceride, a typical plant lipid. This reduction in mean area per molecule is less than the reduction observed with cholesterol which is in agreement with the observations of Demel *et al.* [18] who demonstrated that at the air–water interface cholesterol gives a stronger interaction with phosphatidylcholine than stigmasterol.

To investigate the importance of the C_{19} methyl group of the cholesterol molecule for the interaction between cholesterol and phosphatidylcholine we compared the surface properties of pure 19-norcholesterol and cholesterol and of a mixture of these sterols with 16:0/18:1c-phosphorylcholine (Fig. 5). The pressure–area curve of 19-norcholesterol shows that the molecular area of this sterol is 3 $Å^2$/molecule smaller when compared with cholesterol. Both sterols show comparable interactions with 16:0/18:1c-phosphorylcholine (Fig. 5) at the air–water interface. In the liposomal bilayer system we determined that both sterols were equally effective in reducing the glucose permeability of the membranes of egg phosphatidylcholine liposomes (data not shown). Apparently the C_{19} methyl group of cholesterol plays no critical role in the interaction between sterols and phosphatidylcholines.

The effect of cholesterol upon the phase transition of various lipids dispersed in water

Different lipids varying in the polar and apolar part of the molecule were dispersed in water–glycol, 1:1 (v/v) in the presence or absence of 25, 33.3 or 50 mole % cholesterol. The energy contents of the crystalline→ liquid–crystalline phase transition of various synthetic phosphatidylcholines are summarised in Table I. The chain length as well as the presence of a double bond in the fatty acid chains influences the energy of the transition which is in agreement with the data of Ladbrooke *et al.*[29,30]. 16:0/18:1c-Phosphorylcholine shows a much lower kcal/mole value of the transition than the average value of 16:0/16:0-phosphorylcholine and 18:1c/18:1c-phosphorylcholine (Table I), this confirms the data of Phillips

TABLE I

REDUCTION OF THE ENERGY CONTENT OF THE PHASE TRANSITION OF VARIOUS LIPIDS BY CHOLESTEROL

*Sample**	*Mole % cholesterol*						
	0	*25*		*33.3*		*50*	
	kcal per mole	*kcal per mole*	*% reduction*	*kcal per mole*	*% reduction*	*kcal per mole*	*% reduction*
16:0/18:1c-phosphorylcholine	8.0	—	—	0.0	100	—	—
16:0/16:0-phosphorylcholine	8.6	3.7	57	—	—	—	—
18:0/18:0-phosphorylcholine	11.9	6.9	42	—	—	—	—
18:1c/18:1c-phosphorylcholine	11.2	6.2	45	—	—	—	—
18:1c/18:0-phosphorylcholine	6.7	2.0	70	0.1	98	0.0	100
18:1t/18:1t-phosphorylcholine	10.0	—	—	2.2	78	—	—
Phospholipid (Formula I)	5.0	—	—	0.7	86	—	—
Phospholipid (Formula II)	5.0	—	—	0.8	84	—	—
Phosphinate lipid (Formula III)	13.2	—	—	0.7	95	—	—
Phosphinate lipid (Formula IV)	8.0***	—	—	1.6	80	—	—
Phosphatidylglycerol	4.2	—	—	—	—	0.0	100
Phosphoglycolipid (Formula V)	9.9	—	—	—	—	0.0	100
Diglycosyldiglyceride	17.5	—	—	—	—	0.0	100
Total lipid extract of *A. laidlawii***	13.0	3.6	72	—	—	—	—

* The actual scans of most samples are presented in Figs 6, 7 and 8
** Cells were grown on elaidic acid (ref. 16)
*** Sum of the energy content of both transitions.

et al.[31], who showed that intermolecular mixing of hydrocarbon chains produces a decrease in the energy content of the phase transition. Cholesterol decreases the energy content of the transition of all the phosphatidylcholines tested (Table I). The phase transition in D-16:0/18:1c-phosphorylcholine, the stereo isomer of 16:0/18:1c-phosphorylcholine was completely eliminated by the presence of 33.3 mole % of cholesterol (data not shown).

The calorimetric scans of *rac*-1-oleoyl-2-hexadecylglycero-3-phosphorylcholine (Formula I), *rac*-1-oleoyl-2-C-hexadecyl-propanediol-3-phosphorylcholine (Formula II) and 16:0/18:1c-phosphorylcholine reveal that these lipids undergo an endothermic phase transition at about −5 °C (Fig. 6A). The presence of 33.3 mole % of cholesterol in the bilayers of these phosphatidylcholines almost completely eliminates this phase transition (Fig. 6B, Table I). These results indicate that the two oxygen atoms of the ester bond at the 2-position of lecithin play no important role in the interaction between cholesterol and 16:0/18:1c-phosphorylcholine in the liposomal bilayer system. Phosphinate lipid (Formula III) could be easily dispersed in water–glycol, 1;1 (v/v). The resulting turbid suspension had the typical liposomal like appearance. This compound showed a sharp phase transition at 65 °C (Fig. 7A). Phosphinate lipid (Formula IV) which contains only one fatty acid probably forms micelles in water because we obtained a completely clear solution after dispersing this lipid in water–glycol, 1:1 (v/v). The calorimetric scan of this

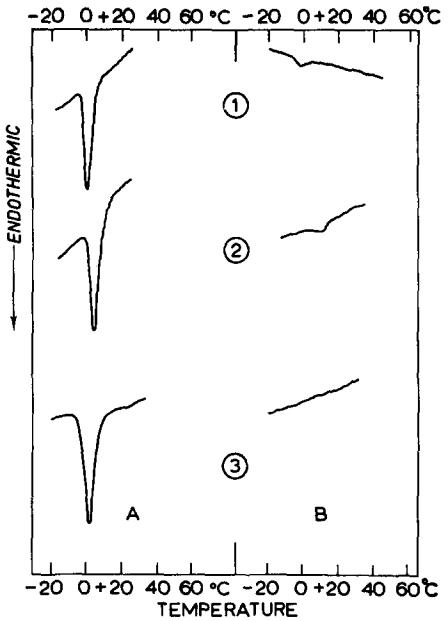


Fig. 6. Calorimetric scans of various lecithins. (1) *rac*-1-Oleoyl-2-hexadecylglycero-3-phosphorylcholine, (2) *rac*-1-oleoyl-2-C-hexadecylpropanediol-3-phosphorylcholine, (3) 16:0/18:1c-phosphorylcholine, in the absence (A) or presence (B) of 33.3 mole % cholesterol.

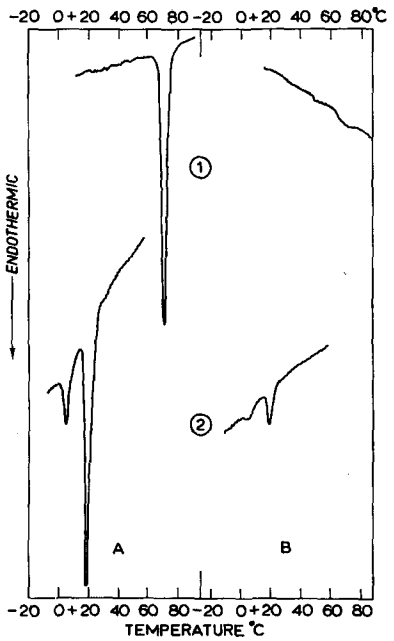


Fig. 7. Calorimetric scans of phosphinate lipids. (1) Phosphinate lipid (Formula III), (2) phosphinate lipid (Formula IV), in the absence (A) and presence (B) of 33.3 mole % cholesterol.

solution showed two endothermic transitions in the temperature range studied (Fig. 7A). A small transition was observed at 0 °C and a major one at about 15 °C. The presence of 33 mole % of cholesterol caused a more turbid dispersion of phosphinate lipid (Formula IV). The energy content of the phase transitions in both phosphinate lipids was reduced to 5 and 20% of the orginal value by 33.3 mole % cholesterol (Fig. 7A, Table I). These results confirm the observations described above that the oxygen atoms in the ester bond between the glycerol molecule and the fatty acid chains are not important for the interaction with cholesterol. Moreover, the same is true with regard to the oxygen atoms which connect the phosphorus atom with the glycerol and the choline part of the molecule. We were unable to prepare a proper dispersion of pure monoglucosyldiglyceride, isolated from *A. laidlawii*, in water–glycol, 1:1 (v/v). From the other pure polar lipids of *A. laidlawii e.g.* diglucosyldiglyceride, the α-glycerophosphate derivative of this compound (Formula V) and phosphatidylglycerol we could produce homogeneous liposomal-like dispersions. The calorimetric scans of these lipids (Fig. 8) show a rather wide endothermic transition as has been demonstrated for the membranes and the total lipid extract of *A. laidlawii*[16,32,33]. The phase transition in diglucosyldiglyceride

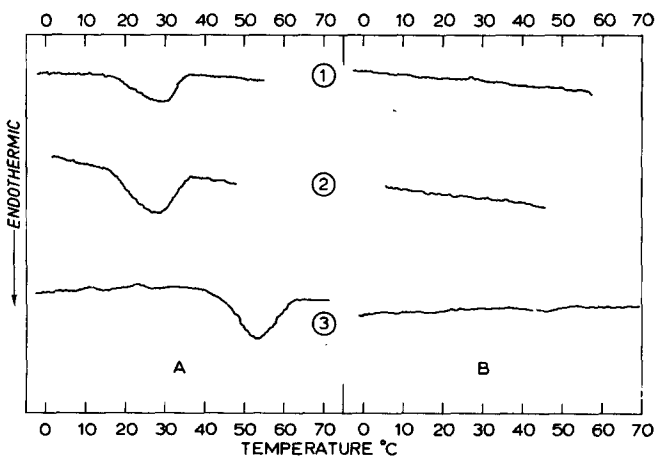


Fig. 8. Calorimetric scans of various pure lipids isolated from *A. laidlawaii* in the presence or absence of 50 mole % cholesterol. (1) Phosphatidylglycerol, (2) phosphoglycolipid (Formula V), (3) diglucosyldiglyceride, in the absence (A) or presence (B) of 50 mole % cholesterol.

Fatty acid pattern of the various lipids:

	12:0	*14:0*	*16:0*	*18:0*	*18:1c*	*18:1t*	*18:2*	*short chain unknown*
(1)	1.1	2.2	17.3	1.7	—	70.6	2.2	4.9
(2)	13.4	14.3	19.8	1.8	—	43.1	1.8	5.8
(3)	3.4	34.2	42.4	6.2	3.6	—	1.4	8.8

occurs at a higher temperature than the phase transitions in phosphoglycolipid (Formula V) and phosphatidylglycerol, this might in part be caused by differences in fatty acid composition. Diglucosyldiglyceride was isolated from cells grown without

fatty acid and contains palmitic acid as the most abundant fatty acid. Phosphoglycolipid (Formula V) and phosphatidylglycerol were isolated from elaidic acid grown cells and contained mainly 18:1t (legend Fig. 8). The energy contents of the phase transitions of these lipids differ considerably (Table I). The amounts of energy adsorbed in the transition of diglucosyldiglyceride and phosphoglycolipid (Formula V) are respectively 17.5 and 9.9 kcal/mole. This large difference cannot be explained solely by differences in fatty acid composition because the energy contents of the phase transition in 18:1t/18:1t-phosphorylcholine and 16:0/16:0-phosphorylcholine are rather similar (Table I). The differences in headgroup or charge of phosphoglycolipid (Formula V) could possibly explain the lower energy content of the phase transition of phosphoglycolipid (Formula V) as compared to diglucosyldiglyceride.

The phase transition in phosphatidylglycerol isolated from elaidic acid grown *A. laidlawii* cells occurs at the same temperature as phosphoglycolipid (Formula V) isolated from the same culture (Fig. 8). However, the energy content of the transition is relatively low as compared to the energy content of the transition in phosphoglycolipid (Formula V), diglucosyldiglyceride and the total lipid extract of *A. laidlawii* (Table I). A similar observation has been made by Chapman and Urbina[33] who demonstrated that the total glycolipids had a higher energy content of the transition than the total phospholipids. In their experiments however, no comparison was made between the energy contents of the transition of the pure lipids. Moreover, the difference in kcal/mole value of the transition of total glyco- and total phospholipids is difficult to interpret because their total phospholipid fraction contained about 40% of phosphoglycolipid (Formula V). It may be suggested that the lower energy content of the transition in phosphatidylglycerol must be caused by electrostatic repulsion forces between the negative charges at the phosphate groups of different molecules.

The presence of 50 mole % cholesterol completely abolishes the phase transition occurring in diglucosyldiglyceride, phosphoglycolipid (Formula V) and phosphatidylglycerol (Fig. 8). This indicates that in the liposomal bilayer system the presence of a phosphate or a choline group is not required for the interaction between cholesterol and other lipids.

The effect of cholesterol upon the transitions of a mixture of 18:1c/18:1c -phosphoryl choline and 18:0/18:0-phosphorylcholine

Pure 18:1c/18:1c-phosphorylcholine and 18:0/18:0-phosphorylcholine show phase transitions at respectively −20 °C and +50 °C (Figs 9a and 9b). The scan of a mixture of 57.5 mole % 18:1c/18:1c-phosphorylcholine and 42.5 mole % 18:0/18:0-phosphorylcholine reveals the transition of both lecithins (Fig. 9c). The temperature and energy content of the transition of 18:1c/18:1c-phosphorylcholine is not affected by the presence of 18:0/18:0-phosphorylcholine*. Due to the presence of 18:1c/18:1c-phosphorylcholine, however, the transition of 18:0/18:0-phosphorylcholine is shifted to a lower temperature, the energy content of this transition also is decreased (Fig. 9c, Table II). This experiment, first described by Phillips *et al.*[31] demonstrates that between −10 °C and +40 °C, phase separa-

* It has to be noted that transition temperatures in cooling curves often are shifted to lower temperatures caused by undercooling of the phase. Transition temperatures only can be determined accurately from heating curves.

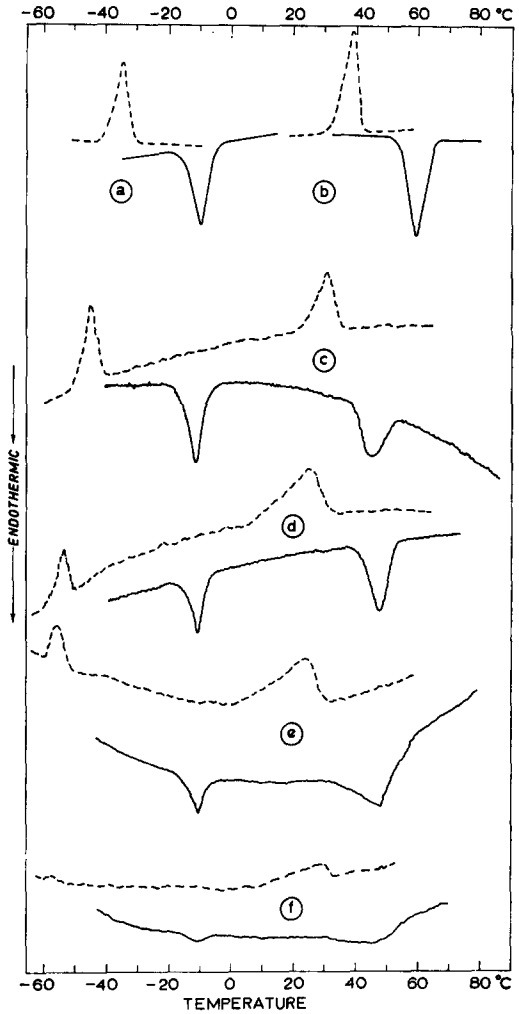


Fig. 9. Calorimetric scans of a mixture of 18:1c/18:1c-phosphorylcholine and 18:0/18:0-phosphorylcholine in the presence of various amounts of cholesterol. (a) 18:1c/18:1c-phosphorylcholine, (b) 18:0/18:0-phosphorylcholine, (c) 57.5 mole % 18:1c/18:1c-phosphorylcholine and 42.5 mole % 18:0/18:0-phosphorylcholine, (d) mixture (c) and 11.9 mole % cholesterol, (e) mixture (c) and 23.3 mole % cholesterol and (f) mixture (c) and 40.0 mole % cholesterol. The percentage of cholesterol is based on the total amount of phosphatidylcholine present. ——, heating curves; . . . ., cooling curves.

tion occurs. In this temperature range both liquid parts of 18:1c/18:1c-phosphorylcholine and liquid–crystalline parts of 18:0/18:0-phosphorylcholine must be present in the bilayer. Cholesterol lowers the energy content of the phase transition of pure 18:1c/18:1c-phosphorylcholine and 18:0/18:0-phosphorylcholine to the same extent (Table I). In the mixture of both lecithins it is clear that this is not the case. The presence of 11.9 mole % cholesterol causes a decrease in the energy content of the phase transition of 18:1c/18:1c-phosphorylcholine and a small increase in the

TABLE II

EFFECT OF CHOLESTEROL UPON THE ENERGY CONTENT OF THE PHASE TRANSITIONS OCCURRING IN A MIXTURE OF 18:1c/18:1c-PHOSPHORYLCHOLINE AND 18:0/18:0-PHOSPHORYLCHOLINE

PC, Phosphorylcholine
The calorimetric scan of the various samples are presented in Fig. 9.

Sample	*Energy content of the phase transition (kcal/mole)*	
	18:1c/18:1c-PC	*18:0/18:0-PC*
(a) 18:1c/18:1c-PC	11.2	—
(b) 18:0/18:0-PC	—	11.9
(c) 57.5 mole % 18:1c/18:1c-PC + 42.5 mole % 18:0/18:0-PC	10.9	8.4
(d) 57.5 mole % 18:1c/18:1c-PC + 42.5 mole % 18.0/18:0-PC + 11.9 mole % cholesterol*	6.4	9.1
(e) 57.5 mole % 18:1c/18:1c-PC + 42.5 mole % 18.0/18:0-PC + 23.3 mole % cholesterol*	6.0	13.0
(f) 57.5 mole % 18:1c/18:1c-PC + 42.5 mole % 18.0/18:0-PC + 40.0 mole % cholesterol*	$\leqslant$ 0.8	3.3

* Percentage of cholesterol is based on the total amount of phosphatidylcholine present.

energy content of the transition of 18:0/18:0-phosphorylcholine (Fig. 9d, Table II). At a cholesterol concentration of 23.3 mole % this effect is even more pronounced (Fig. 9e, Table II). The kcal/mole value of the transition of 18:0/18:0-phosphorylcholine is now close to the kcal/mole value of the transition of pure 18:0/18:0-phosphorylcholine, whereas the kcal/mole value of the transition of 18:1c/18:1c-phosphorylcholine is now reduced almost to half the value observed in the absence of cholesterol. This strongly indicates that in this case cholesterol interacts preferentially with 18:1c/18:1c-phosphorylcholine causing a decrease in the energy content of the 18:1c/18:1c-phosphorylcholine transition. The 18:1c/18:1c-phosphorylcholine which interacts with cholesterol is no longer available for association with 18:0/18:0-phosphorylcholine, this explains the increase in the energy content of the phase transition of 18:0/18;0-phosphorylcholine up to the value obtained in the absence of 18:1c/18:1c-phosphorylcholine. At a still higher cholesterol concentration of 40.0 mole % we observed also a decrease in the energy content of the transition of 18:0/18:0-phosphorylcholine (Fig. 9f, Table II); apparently almost all the 18:1c/18:1c-phosphorylcholine has now interacted with cholesterol. The excess cholesterol can now interact also with 18:0/18:0-phosphorylcholine. Identical results were obtained from the energy contents of the phase transitions in the heating and cooling curves of the different samples. The experiment strongly suggests that if both lipids with crystalline and liquid–crystalline fatty acid chains are present in the membrane, cholesterol complexes specifically with these fatty acid chains which are in the liquid–crystalline state.

DISCUSSION

The chain interaction in lipids can be affected by cholesterol in both the liquid–

crystalline and the crystalline state. When the fatty acid chains of phosphatidylcholine are in the liquid–crystalline state (this is above the transition temperature) cholesterol shows a condensing effect which results in a decreased mobility of the fatty acid chains[1–10]. When the fatty acid chains are in the crystalline or gel state (this is below the transition temperature) cholesterol shows a liquefying effect which results in an increased mobility of the fatty acid chains[8,10]. Both the condensing and liquefying effect of cholesterol results in an intermediate fluidity of the fatty acid chains which is between the crystalline and the liquid crystalline state[20].

A planar ring-system and a hydrophobic side chain at C_{17} have been shown to be essential structural requirements of the sterol molecule for its interaction with other lipids[7,17–19]. These two requirements indicate the hydrophobic nature of the interaction. However, there is now also manifold evidence that the 3β-OH group is essential for the lipid–sterol interaction. Model systems have demonstrated that (a) epicholesterol, the 3α-OH isomer of cholesterol brings about very little condensation of a 18:1c/18:0-phosphorylcholine monolayer[18] as compared to the strong reduction in mean area observed with cholesterol[18]. (b) The mobility of a spin probe incorporated in bilayers of egg lecithin was decreased only when cholesterol was present, but not when epicholesterol was incorporated[7]. (c) The energy content of the crystalline→ liquid–crystalline transition in 18:1/18:0-phosphorylcholine was drastically reduced by cholesterol; epicholesterol had almost no effect upon the energy content of the phase transition[16]. (d) The glycerol, erythritol, glucose and Rb^+ permeability through the bilayers of egg-lecithin liposomes was almost not affected by epicholesterol as compared to the strong reduction in permeability observed with cholesterol[19]. Experiments with natural membranes showed; (e) Both sterols, cholesterol and epicholesterol, can be incorporated to the same extent in the *A. laidlawii* cell membrane ($\pm$9 wt % of total lipids[16]). The permeability of glycerol and erythritol through the membranes of *A. laidlawii* was reduced by the incorporation of cholesterol and not by epicholesterol[16]. (f) Mycoplasma strain 07 and the T-strain mycoplasmas have an absolute sterol requirement for growth[34,35]. The membranes of these organisms contain a substantial amount of cholesterol when they are grown in a cholesterol rich medium. When the growth medium contained epicholesterol as the only sterol growth was inhibited[34,35].

In order to explain the absolute requirement of a 3β-OH group for the lipid–sterol interaction the following possibilities can be considered; (1) Is the orientation of the sterol molecule in the membrane determined by the configuration of the OH group? In the monolayer studies of Demel *et al.*[18] it was demonstrated that cholesterol and epicholesterol have an identical orientation at the air–water interface. This indicates that the configuration of the OH-group is not of critical importance for the orientation of the sterol molecule. (2) Is the configuration of the OH group of the sterol molecule of importance in determining the solubility of the sterol in a membrane? Cholesterol can be incorporated in a liposomal membrane up to 50 mole % of total lipids[14,19,36]. Differential scanning calorimetry[16], X-ray (Gulik, T., personal communication) and solubilization studies[19] have demonstrated that up to 25 mole % epicholesterol can be incorporated homogeneously in the liposomal bilayer system. Above 25 mole %, a second phase of crystalline epicholesterol was detected by X-ray (Gulik, T., personal communication). The configuration of the sterol OH group apparently does affect in the solubility of the sterol in the bilayer,

but at lower (less than 25 mole %) sterol concentration both cholesterol and epicholesterol are homogeneously incorporated in the membrane. At this sterol concentration there is still a considerable difference between the lipid–cholesterol and lipid–epicholesterol interaction. The exclusion of possibilities 1 and 2 indicates that the sterol 3β-OH group is directly involved in the lipid–sterol interaction. In this paper we investigated the specificity of the polar part of the lipid molecule for the lipid–sterol interaction in monolayers and in liposomal bilayer systems. A reduction in the area per molecule was observed in mixed monolayers of cholesterol with either synthetic 16:0/18:1c-phosphorylcholine, 18:1/18:1-phosphatidylethanolamine[2] *rac*-1-oleoyl-2-hexadecylglycero-3-phosphorylcholine (Formula I), *rac*-1-oleoyl-2-C-hexadecylpropanediol-3-phosphorylcholine (Formula II), phosphinate lipids (Formulas III and IV) and monoglucosyldiglyceride isolated from *A. laidlawii* (Figs 1–4). Cholesterol showed a comparable reduction in the heat content of the crystalline→liquid–crystalline phase transition of various synthetic phosphatidylcholines, *rac*-1-oleoyl-2-hexadecylglycero-3-phosphorylcholine (Formula I), *rac*-1-oleoyl-2-C-hexadecylpropanediol-3-phosphorylcholine (Formula II) phosphinate lipids (Formulas III and IV) (Figs 7 and 8, Table I), and different lipids isolated from *A. laidlawii e.g.* phosphatidyl glycerol, phosphoglycolipid (Formula V), diglucosyldiglyceride (Fig. 6, Table I). This means that the presence of the oxygen atoms of the ester bonds of phosphatidylcholine, and the oxygen atoms connecting the phosphorus atom with the choline and the glycerol part of the molecule are *not* essential for the phosphatidylcholine–cholesterol interaction in mono- and liposomal bilayers, nor is the choline group essential because we observe a similar interaction with phosphatidylglycerol. Most significant are the strong interactions observed between cholesterol and glycolipids (Figs 3, 4, 8). The phosphorylcholine group is apparently not essential for the lipid–sterol interaction in monolayers and liposomal bilayers. The presence of a hydrogen bond between the phosphate group of phosphatidylcholine and the OH group of cholesterol has been suggested by various authors[37,38]. Our experiments, together with some recent findings of Oldfield and Chapman[8] demonstrate that such a hydrogen bond is unlikely to play an important role in the lipid–sterol interaction. Furthermore, we can conclude that the requirement for a 3β-OH group for the lipid–sterol interaction cannot be explained by a direct interaction between the 3β-OH group and any specific polar part of the lipid molecule. Another possible explanation of the difference between cholesterol and epicholesterol in their interactions with lipids is that a cooperative effect of the 3β-OH group and the C_{18} and C_{19} methyl groups may be required for a proper lipid–sterol interaction. In cholesterol the 3β-OH group and the methyl groups at C_{18} and C_{19} are located at the same site of the ring system. In epicholesterol the 3α-OH group is located at the opposite site of the C_{18} and C_{19} methyl groups. Cholesterol and 19-norcholesterol (missing the C_{19} methyl group) show comparable interactions with 16:0/18:1c-phosphorylcholine (Fig. 5). The C_{19} methyl group of cholesterol plays no important role in the interaction with phosphatidylcholine. No cooperative effect of the 3β-OH group and the C_{19} methyl group is required for the lipid–sterol interaction. As yet nothing is known about the importance of the C_{18} methyl group of cholesterol. Discussing the absolute requirement of a 3β-OH group for the lipid–sterol interaction it seems relevant to consider the role of water in the interaction. X-Ray

analyses on 16:0/16:0-phosphorylcholine–cholesterol–water systems demonstrated that the thickness of the waterlayer increases between 0 and 7.5 mole % cholesterol and is constant between 7.5 and 42 mole % cholesterol[20]. The amount of "unfreezable bound" water has been shown calorimetrically to be constant from 0–7.5 mole % cholesterol and then to increase between 7.5 and 50 mole % cholesterol[20]. These findings indicate that the lipid–sterol interaction is accompanied by an increase in the amount of bound water. Monolayers of cholesterol and epicholesterol show comparable force–area curves only the collapse pressure of epicholesterol is significantly reduced as compared to cholesterol[18]. This difference in collapse pressure might indicate a difference in the hydration of the 3β-OH and the 3α-OH group. We suggest from these results that the hydration of the 3β-OH group of cholesterol is very important for the lipid–sterol interaction.

Biological membranes contain many molecular species of different lipids. Both molecular species with relatively high transition temperatures and molecular species with relatively low transition temperatures are found. Depending upon the temperature, lipids with both crystalline and liquid–crystalline fatty acid chains might be present in the membrane. This phase separation has been observed in *A. laidlawii*[16,32,39,40] and *E. coli*[41]. Cholesterol can interact with phosphatidylcholine when the fatty acid chains are in the liquid–crystalline state (condensing effect) or in the crystalline state (liquefying effect). In a codispersion of 18:1c/18:1c-phosphorylcholine and 18:0/18:0-phosphorylcholine both crystalline and liquid–crystalline fatty acid chains are present between -10 °C and $+40$ °C. When cholesterol is present in such a codispersion we observed that at low (less than 25 mole %) cholesterol concentrations only the heat content of the transition of 18:1c/18:1c-phosphorylcholine was drastically reduced. The heat content of the transition of 18:0/18:0-phosphorylcholine was not decreased by cholesterol at this concentration (Fig. 9, Table II). This means, cholesterol associates specifically with 18:1c/18:1c-phosphorylcholine at this concentration or 18:0/18:0-phosphorylcholine. At a higher concentration (40 mole%) cholesterol interacts with 18:0/18:0-phosphorylcholine as well (Fig. 9, Table II). In control experiments we demonstrated that cholesterol showed a comparable interaction with pure 18:1c/18:1c-phosphorylcholine. From these results we conclude that when cholesterol is present in a membrane with both crystalline and liquid–crystalline fatty acids, the sterol interacts preferentially with fatty acid chains which are in the liquid–crystalline state. The biological significance of this finding would be that in a natural membrane with a cholesterol content smaller than 25 mole%, *e.g.* the membranes of cholesterol grown *A. laidlawii*, cholesterol is not randomly distributed when both crystalline and liquid–crystalline phases are present in the bilayer.

ACKNOWLEDGEMENTS

We are indebted to Dr A. Schuyff of the Department of Crystal Chemistry of the State University of Utrecht, for the use of the calorimeter. The synthesis of some of the lecithins was excellently performed by W. S. M. Geurts van Kessel.

REFERENCES

1 de Bernard, L. (1958) *Bull. Soc. Chim. Biol.* 40, 161–170
2 Demel, R. A., van Deenen, L. L. M. and Pethica, B. A. (1967) *Biochim. Biophys. Acta* 135, 11–19
3 Joos, P. and Demel, R. A. (1969) *Biochim. Biophys. Acta* 183, 447–457
4 Rand, R. P. and Luzzati, V. (1968) *Biophys. J.* 8, 125–137
5 Demel, R. A., Geurts van Kessel, W.S.M. and van Deenen, L. L. M. (1972) *Biochim. Biophys. Acta* 266, 26–40
6 Hubbell, W. L. and McConnel, H. M. (1971) *J. Am. Chem. Soc.* 93, 314–326
7 Butler, K. W., Smith, I.C.P. and Schneider, H. (1970) *Biochim. Biophys. Acta* 219, 514–517
8 Oldfield, E. and Chapman, D. (1972) *FEBS Lett.* 21, 302–306
9 Darke, A., Finer, E. G., Flook, A. G. and Phillips, M.C. (1971) *FEBS Lett.* 18, 326–330
10 Oldfield, E., Chapman, D. and Derbyshire, W. (1971) *FEBS Lett.* 16, 102–106
11 de Gier, J., Mandersloot, J. G. and van Deenen, L. L. M. (1968) *Biochim. Biophys. Acta* 150, 666–675
12 Demel, R. A., Kinsky, S. C., Kinsky, C. B. and van Deenen, L. L. M. (1968) *Biochim. Biophys. Acta* 150, 655–665
13 Finkelstein, A. and Cass, A. (1967) *Nature* 216, 717–729
14 Bruckdorfer, K. R., Demel, R. A., de Gier, J. and van Deenen, L. L. M. (1969) *Biochim. Biophys. Acta* 183, 334–345
15 McElhaney, R. N., de Gier, J. and van Deenen, L. L. M. (1970) *Biochim. Biophys. Acta* 219, 245–247
16 de Kruyff, B., Demel, R. A. and van Deenen, L. L. M. (1972) *Biochim. Biophys. Acta* 255, 331–347
17 de Kruyff, B., de Greef, W. J., van Eyk, R. V. W., Demel, R. A. and van Deenen, L. L. M. (1973) *Biochim. Biophys. Acta* 298, 479–499
18 Demel, R. A., Bruckdorfer, K. R. and van Deenen, L. L. M. (1972) *Biochim. Biophys. Acta* 255, 311–320
19 Demel, R. A., Bruckdorfer, K. R. and van Deenen, L. L. M. (1972) *Biochim. Biophys. Acta* 255, 321–330
20 Ladbrooke, B. D., Williams, R. M. and Chapman, D. (1968) *Biochim. Biophys. Acta* 150, 333–340
21 van Deenen, L. L. M. and de Haas, G. H. (1964) *Adv. Lipid. Res.* 2, 168–229
22 Slotboom, A. J., de Haas, G. H., Bonsen, P. P. M., Burbach-Westerhuis, G. J. and van Deenen, L. L. M. (1970) *Chem. Phys. Lipids* 4, 15–19
23 Rosenthal, A. F. and Chodsky, S. V. (1971) *J. Lipid. Res.* 12, 277–285
24 Shaw, N., Smith, P. F. and Verheij, H. M. (1972) *Biochem. J.* 128, 167–173
25 Fiske, C. H. and SubbaRow, Y. (1925) *J. Biol. Chem.* 66, 375–379
26 Shaw, N., Smith, P. F. and Koostra, W. L. (1968) *Biochem. J.* 107, 329–333
27 Scott, T. A. and Melwin, E. H. (1953) *Anal. Chem.* 25, 650–657
28 Paltauf, F., Hauser, H. and Phillips, M. C. (1971) *Biochim. Biophys. Acta* 249, 539–547
29 Chapman, D., Williams, R. M. and Ladbrooke, B. D. (1967) *Chem. Phys. Lipids* 1, 445–455
30 Ladbrooke, B. D. and Chapman, D. (1969) *Chem. Phys. Lipids* 8, 127–133
31 Phillips, M. C., Hauser, H. and Paltauf, F. (1972) *Chem. Phys. Lipids* 8, 127–133
32 Steim, J. M., Tourtelotte, M. E., Reinert, J. C., McElhaney, R. N. and Rader, R. L. (1969) *Proc. Natl. Acad. Sci. U.S.* 63, 104–109
33 Chapman, D. and Urbina, J. (1971) *FEBS Lett.* 12, 169–172
34 Smith, P. F. (1964) *J. Lipid Res.* 5, 121–125
35 Rottem, S., Pfendt, E. A. and Hayflick, L. (1971) *J. Bacteriol.* 105, 323–330
36 Lecuyer, H. and Dervichan, D. G. (1969) *J. Mol. Biol.* 45, 39–57
37 Darke, A., Finer, E. G., Flook, A. G. and Philips, M. C. (1972) *J. Mol. Biol.* 63, 265–279
38 Zull, J. E., Greenoff, S. and Adam, H. K. (1968) *Biochemistry* 7, 4172–4176
39 Oldfield, E., Chapman, D. and Derbyshire, W. (1972) *Chem. Phys. Lipids* 9, 69–81
40 Engelman, D. M. (1971) *J. Mol. Biol.* 58, 153–165
41 Esfahani, M., Limbrick, A. R., Knutton, S., Oka, T. and Wakil, S. J. (1971) *Proc. Natl. Acad. Sci. US.* 68, 3180–3184