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THE EFFECT OF CHOLESTEROL AND EPICHOLESTEROL INCORPORATION ON THE PERMEABILITY AND ON THE PHASE TRANSITION OF INTACT *ACHOLEPLASMA LAIDLAWII* CELL MEMBRANES AND DERIVED LIPOSOMES

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SUMMARY

1. The effect of incorporated cholesterol and epicholesterol upon the glycerol and erythritol permeability through the membrane of *Acholeplasma laidlawii* (previously denoted as *Mycoplasma laidlawii*) is studied. Both sterols, when present in the growth medium, are incorporated to the same extent in the *A. laidlawii* membrane. Only the cholesterol-containing *A. laidlawii* membrane shows a reduced permeability towards glycerol and erythritol as compared to the sterol-free cells. The 3α-hydroxy isomer, epicholesterol, does not affect the membrane permeability.

2. Liposomes prepared from lipids isolated from cells grown on cholesterol-rich media also show a reduced glycerol and erythritol permeability as compared to liposomes prepared from lipids isolated from sterol-free control cells. This permeability-lowering effect can be correlated with a condensing effect of cholesterol upon a monolayer of total *A. laidlawii* lipids.

3. The phase transitions occurring in membranes and extracted lipids of *A. laidlawii* have been studied by differential scanning calorimetry. Incorporated cholesterol causes a considerable reduction of the energy content of this phase transition. This reduction in energy is the same for the intact *A. laidlawii* cell membrane as for the liposomal bilayer system of the extracted lipids dispersed in water.

4. Using the synthetic lecithin 1-oleoyl-2-stearoyl-*sn*-glycero-3-phosphorylcholine the same phenomenon is observed. 32 mole % cholesterol completely eliminated the phase transition of the lecithin. Epicholesterol 5α-androstan-3β-ol and cholest-4,6-dien-3-one are unable to show such an effect, also suggesting the importance of the 3β-OH group of the sterol molecule for the specific sterol–lecithin interaction.

INTRODUCTION

An interesting group of microorganisms with respect to their sterol growth requirements are the Mycoplasmas. Most Mycoplasmas including the T-strain Mycoplasmas depend for their growth upon the presence of sterols in the growth medium[1–6]. T-strain Mycoplasmas grow well on cholesterol and β-sitosterol and, to a lesser extent, on 7-dehydrocholesterol, cholestanol, stigmasterol, and ergosterol; but not on chole-

sterollaureate or cholestan-3-one[4]. Coprostanol, epicoprostanol and epicholestanol inhibited cell growth[4]. Growth of Mycoplasma strain 07 was supported only by cholesterol and cholestan-3β-ol[1]. Epicholesterol, cholestan-3α-ol, coprostan-3β-ol and coprostan-3α-ol did not show any significant growth response. These sterols even inhibited the cholesterol-supported growth of strain 07[1]. *Mycoplasma mycoides* required for growth a medium containing cholesterol or lathosterol[5]. Cholestenone, cholest-5-en-3-one, 7-dehydrocholesterol and progesterone inhibited cholesterol-promoted growth of *M. mycoides*[5].

Acholeplasma laidlawii strain B (previously denoted as *Mycoplasma laidlawii* strain B) and other Acholeplasmas differ from the Mycoplasmas and T-strain Mycoplasmas by the fact that these organisms have no sterol growth requirement[1-3,6,7]. Grown in the absence of exogenous sterols these Acholeplasmas do not contain sterol, however, when cholesterol is present in the growth medium, this cholesterol will be incorporated into the cell membrane[6,7]. Some fungi also display this property[8,9]. This makes these organisms very suitable for studying the role of sterol in a biological membrane.

Using model membrane systems it was found recently that the 3β-OH group is essential for the specific interaction of sterol and lecithin[10,11]. BRUCKDORFER *et al.*[12] have already shown that a hydroxyl group at the 3 position of the sterol molecule is essential to limit the permeability properties of the erythrocyte membrane. When part of the erythrocyte cholesterol was replaced by 3-ketosteroid by incubating erythrocytes with 3-ketosteroid-rich liposomes, these erythrocytes showed an increased glycerol permeability[12]. MCELHANEY *et al.*[19] showed that cholesterol incorporated in the membrane of *A. laidlawii* decreased the glycerol permeability of the cells.

In the present study, in order to investigate the role of cholesterol and particularly the function of the -OH group in more detail, we incorporated cholesterol and epicholesterol into the cell membrane of *A. laidlawii*. The permeability of intact *A. laidlawii* cells for glycerol and erythritol was studied and compared with the permeability properties of liposomes obtained from lipid extracts of *A. laidlawii* cells.

Lecithin shows a sharp phase-transition in systems containing excess water[14]. This phase transition is due to a conversion from the crystalline Lβ phase to the liquid crystalline Lα phase. Cholesterol reduces the energy of the phase transition of several synthetic lecithins[13,14]. We found it useful to establish whether the same phenomena can be observed for *A. laidlawii* membranes. Furthermore, differential scanning calorimetry spectra of 1-oleoyl-2-stearoyl-*sn*-3-phosphorylcholine in the absence and presence of cholesterol and epicholesterol respectively were studied to investigate the influence of the -OH group on the phase transition.

MATERIALS AND METHODS

Organisms and growth conditions

A. laidlawii strain B cells (a gift from Dr. R. N. McElhaney) were grown in 0.35–5 l quantities of lipid-poor tryptose medium[15] with addition of 1 ml penicillin (100 000 units/ml) per l of culture. Fatty acids (60–100 μM) were added to the growth medium as sterile ethanolic solutions. Cholesterol was added to the growth medium in two ways:

1. Together with the fatty acids in absolute ethanol so that total concentration

of fatty acids *plus* cholesterol was 10 mg/ml ethanol. By this method up to 25 mg cholesterol per l of culture medium could be dissolved at 37°. In some cases 15 μC of [1-^{14}C]cholesterol was added together with the fatty acids and non-radioactive cholesterol.

2. It was found that especially with cultures larger than 1 l, cholesterol crystals were formed during the addition of the ethanolic solution of cholesterol. These crystals co-precipitated with the cells during the isolation procedure. For this reason and to attempt to increase the cholesterol concentration in the medium, in addition to 15 mg cholesterol/l culture medium added as described above (at this concentration cholesterol always stayed in solution), cholesterol was first incorporated into liposomes prepared from total polar *A. laidlawii* lipids and then also added to the growth medium.

Liposomes were formed in the following way: 90 mg of total *A. laidwaii* lipid (isolated from cells grown on 0.03 mM palmitic and 0.03 mM oleic acid) and 45 mg cholesterol were mixed in chloroform and evaporated to dryness, 120 ml of complete, fatty acid-free growth medium was added. This solution was sonicated at room temperature for 15 min at maximum power with a Branson sonic power instrument. The liposomes were centrifuged at 0° for 15 min at 17 500 rev./min in the SS 34 rotor of a Sörvall centrifuge to prevent contamination of the cells with liposomes during the isolation procedure. The supernatant, containing approximately 30 mg of cholesterol was added to 880 ml of growth medium. The final cholesterol concentration was about 45 mg cholesterol/l culture medium. Epicholesterol was added to the growth medium in the same way as described above for cholesterol except that, due to the lower solubility of epicholesterol in liposomes of Acholeplasma lipids, 90 instead of 45 mg epicholesterol was evaporated together with the polar lipids. The final epicholesterol concentration was about 22 mg per l of culture medium. The 15 mg epicholesterol per l medium added to the growth medium together with the fatty acids did not precipitate during the isolation of the cells.

Cells were harvested in late log phase by centrifugation for 15 min at 10000 rev./min in the GSA rotor of a Sörvall centrifuge. Cells were washed once with 200 mM sucrose. The cell pellet was finally suspended in 200 mM sucrose for the permeability experiments.

Membrane preparations

Membranes were prepared as described by VAN GOLDE *et al.*[16]. The membranes were concentrated for the differential scanning calorimeter by centrifugation for 3 h at 50 000 rev./min in the 50 rotor of the Spinco ultracentrifuge.

Lipid extraction

Lipids were extracted from the wet cells according to the BLIGH AND DYER procedure[17]. Residual protein was removed by column chromatography over silica gel mesh 80–100 (Mallinckrodt), neutral lipids were eluted with chloroform, polar lipids with chloroform–methanol (1:9, v/v).

Monolayer studies

Force area measurements were performed at the air–water interface in a paraffin-coated silica trough, 60 cm long × 14 cm wide with a total capacity of

1500 ml as described before[10]. The trough was filled with unbuffered (pH 5.4) water that had been distilled from alkaline permanganate and then redistilled. Known amounts of lipids dissolved in chloroform were released onto the interface using a Agla micrometer syringe.

Preparation of liposomes for permeability experiments

Liposomes of total *A. laidlawii* lipids were prepared in a solution containing 50 mM KCl and 50 mM $MgSO_4$ per l as described before[18]. The zeta potential of the liposomes prepared from different lipid samples ranged from 19–22 mV.

Permeability assay

Permeabilities of whole *A. laidlawii* cells and liposomes prepared from *A. laidlawii* lipids were determined by measuring the initial swelling rates in 200 mM glycerol and erythritol as described before[18,19]. The initial swelling rate is defined as the rate with which the reciprocal of the absorbance (A) changes after the addition of the cell/liposome sample to the isotonic solution of the permeant molecule[18,19] (see also Fig. 9B). Turbidity of the cell and liposome solutions were chosen such that in all cases when the 50 μl of cell/liposome sample was injected in 5 ml of isotonic glycerol or erythritol, the initial transmittance of the solution was 50 %. The permeability behavior of cells grown on cholesterol-rich medium (according to Method 2) was always compared with the permeabilities of cells grown in a medium containing an equivalent amount of *A. laidlawii* polar lipid liposomes without sterol.

Differential scanning calorimetry

A Perkin Elmer DSC-1B differential calorimeter operating at a scan rate of 4 or 8°/min was used for all calorimetric experiments. Lipid dispersions were scanned with identical results in either the commercially available aluminum 20 μl sample pans or in specially constructed brass 80 μl sample pans. 20 mg of lipid dissolved in chloroform was evaporated under vacuum in a test-tube to complete dryness. 90 μl of glycol–water was added to the dry lipid film, 1:1(v/v) and 1:2(v/v) for 1-oleoyl-2-stearoyl-*sn*-glycero-3-phosphorylcholine (18:1/18:0 phosphatidylcholine) sterol mixtures and total *A. laidlawii* lipids, respectively. The lipids were dispersed by vortexing the stoppered tube at 37° for 15 min. In control experiments it was shown that the glycol, added to reduce the freezing point of the solvent, had no significant effect upon the temperature and heat content of the phase transitions of the Acholeplasma lipids. An aliquot of this dispersion was sealed in the sample pan and scanned at least twice to show that the transitions were completely reversible. After the scans the amount of lipid in the sample pan was determined by a phosphorus determination. In case of the Acholeplasma lipids, the content of the sample pan was extracted according to the BLIGH AND DYER procedure[17]. After separation of the two phases, the chloroform phase was washed twice with fresh upper phase. The chloroform was removed under vacuum and the dry weight of the residue was taken as amount of lipid present in the sample pan. The reference pan contained an identical amount of the same water–glycol mixture as present in the sample pan containing lipids. Because of the relatively low lipid content of an aqueous membrane dispersion, scanning of the *A. laidlawii* membranes was always performed using the 80 μl brass sample pans. Each membrane preparation was scanned at least

3 times, first up to 45° (below the temperature of the protein denaturation), second and third scan up to about 70°. The reference pan in this case contained the same 0.001 M Tris, pH 7.4, buffer as used for the preparation of the membranes. After the scans the protein content of the sample pan was measured. The amount of lipid present in the sample pan could be calculated by determining the lipid:protein ratio in an aliquot of the same membrane preparation. The sample head of the calorimeter was cooled with liquid nitrogen or acetone–solid CO_2, sample pans were flushed with dried nitrogen gas. The calorimeter was calibrated using benzoic acid as a standard.

Analytical methods

Phosphorus was determined according to FISKE-SUBBAROW[20], protein using bovine serum albumine as standard was measured according to the method of LOWRY *et al.*[21], cholesterol and epicholesterol were quantitated with the modified Liebermann–Burchard method[22]. It was found that the molar extinction coefficient for epicholesterol was about 2.2 times larger than for cholesterol. Glucose liberated from glycolipid after acid hydrolysis[23] was determined using the anthrone reagent[24]. Methyl esters of fatty acids were prepared by heating about 5 mg of lipid in 10 ml of 5 % (w/w) H_2SO_4 in methanol at 70° under a nitrogen atmosphere. The methyl esters were extracted with pentane. The pentane was dried over anhydrous Na_2SO_4. The fatty acid pattern was determined by means of a F and M gas chromatograph equipped with a 3 % EGSS-X column (Applied Science) and a flame ionisation detector. The amount of fatty acid was determined by reference to a C_{20} standard. In the case that the *A. laidlawii* cells were grown on [1-^{14}C]cholesterol, the neutral lipid fraction, containing 99 % of the radioactivity present in the total lipid extract, was chromatographed on a 0.5 mm silica gel G plate, developed in chloroform–acetone (98:2, v/v) or benzene–ethylacetate (5:1, v/v). Radioactivity was localized using a Panax thin-layer scanner. The radioactive spots were scrapped into scintillation vials containing 16 ml of a scintillation solution with composition: 10 g naphthalene, 5 g PPO and 0.3 g dimethyl POPOP per l dioxan–water (5:1, v/v). The amount of ^{14}C was determined using a Packard-Tricarb scintillation instrument.

Materials

1-Oleoyl-2-stearoyl-*sn*-glycero-3-phosphorylcholine (18:1/18:0 phosphatidylcholine) was synthesized as described before[25]. Cholest-5-en-3β-ol (cholesterol) was obtained from Fluka AG (Buchs, Switzerland), cholest-5-en-3α-ol (epicholesterol) from Mann Research Lab (New York, N.Y., U.S.A.), androstan-3β-ol from Ikapharm (Ramat-Gan, Israel) and cholest-4,6-dien-3-one from British Drug Houses (Poole, England). Purity of sterols and lecithin was checked by thin-layer chromatography as described elsewhere[10].

RESULTS

A. laidwaii strain B incorporates cholesterol up to 8 % (by wt.) of the lipids of the cytoplasmic membrane, when the sterol is added together with fatty acids to the lipid-poor growth medium (Table I). The permeability behaviour of cells grown in the presence and in the absence of 25 mg cholesterol/l was determined by measuring the initial swelling rates of the cells in isotonic glycerol and erythritol

TABLE I

EFFECT OF CHOLESTEROL UPON FATTY ACID COMPOSITION AND CONTENT OF TOTAL LIPIDS FROM *A. laidlawii*

mg cholesterol per l growth medium	*Fatty acid composition of total lipid**								*Amount of cholesterol incorporated (weight % of total lipid)*	*Fatty acid content of the total lipids****
	12:0	*13:0*	*14:0*	*15:0*	*16:0*	*18:0*	*18:1c*	*18:2*		
—	3.4 ± 0.5	0.4 ± 0.1	9.9 ± 0.8	0.9 ± 0.1	48.4 ± 1.9	2.7 ± 1.8	35.8 ± 2.0	2.2 ± 0.6	0.0	51.4 ± 4.2
25	3.6 ± 0.3	0.5 ± 0.1	8.2 ± 0.7	1.1 ± 0.4	46.1 ± 1.5	2.3 ± 0.4	37.5 ± 1.9	2.3 ± 0.6	8.0 ± 0.9**	44.2 ± 3.7

* Cells grown on 0.03 mM 16:0 and 0.03 mM 18:1c (oleic acid), mean of 11 experiments given in mole % (± S.D.).
** Mean of eight experiments.
*** Mean of three experiments, given as (mg fatty acid)/(mg total lipid − mg cholesterol) × 100%.

(Fig. 1). It is obvious that the cells grown on the cholesterol-rich medium show lower $d(1/A)/dt$ % values as compared to the control cells. The osmotic behaviour of both cell types was tested by determining the swelling as measured by $1/A$, in different sucrose concentrations (Fig. 2). The cells act as ideal and comparable osmometers so it is apparent from Figs. 1 and 2 that the cholesterol-containing cells show lower

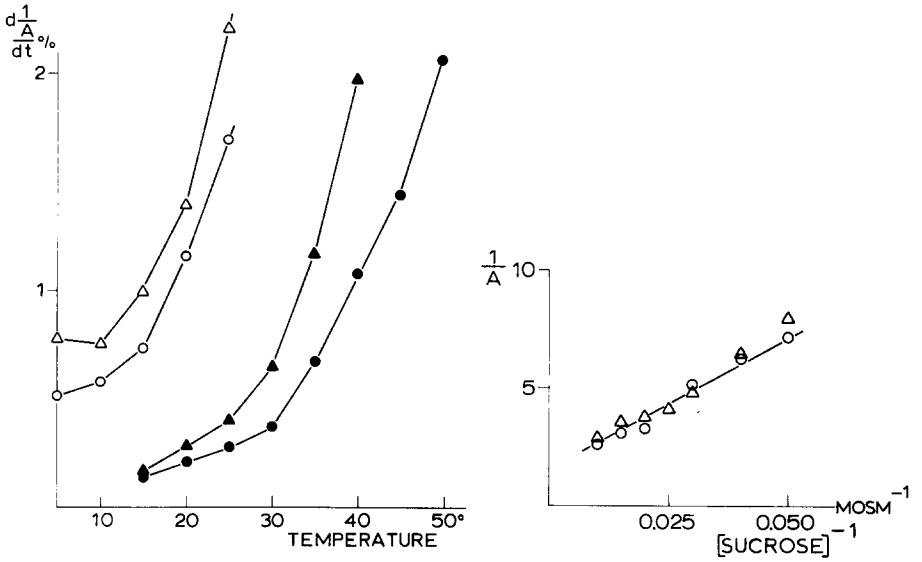

Fig. 1. Initial swelling rates of *A. laidlawii* cells in isotonic glycerol and erythritol. Cells were grown on 0.03 mM 16:0 and 0.03 mM 18:1c (oleic acid) with or without 25 mg cholesterol/l culture. Initial swelling rates in glycerol (△) and erythritol (▲) of cells grown without cholesterol, and initial swelling rates in isotonic glycerol (○) and erythritol (●) of cholesterol-containing cells are shown.

Fig. 2. Osmotic swelling of *A. laidlawii* cells in different sucrose solutions. $1/A$ at 450 nm was measured at room temperature 1 h after the addition of 100 μl cells suspended in 200 mM sucrose to 5 ml of sucrose solutions of different concentrations. △, Cells grown without cholesterol; ○, cells grown with 25 mg cholesterol/l culture. Fatty acids supplemented in the growth medium: 0.03 mM 16:0 and 0.03 mM 18:1c.

glycerol and erythritol permeabilities. This is in agreement with the results obtained by McElhaney *et al.*[19]. Before one can conclude that cholesterol itself produces this reduction in permeability, it has to be proven (1) that cholesterol causes no changes in the membrane composition and (2) that the cholesterol is incorporated into the membrane as such. Fatty acid analysis revealed that cholesterol caused no changes in the fatty acid composition and content of the membrane lipids (Table I). The membrane lipid:protein ratio was 1.07 (mg total lipid/mg protein) in cells grown without cholesterol, and 0.91 in the cells grown on 25 mg cholesterol/l. The phospholipid and glycolipid content was measured by lipid phosphorus and glucose. When cells were grown in the absence of cholesterol 5.30 μmoles phosphorus and 9.95 μmoles glucose per 10 mg polar lipid were present; when the cells were grown in the presence of the 25 mg cholesterol per l culture medium, 5.69 μmoles phosphorus and 10.75 μmoles glucose per 10 mg polar lipid was found. Growth rate and morphological appearance under the phase contrast microscope of both cell types was com-

parable. [^{14}C]Cholesterol was incorporated into the membrane as such, more than 98 % of the radioactivity found in the cells was present in a single compound, which co-chromatographed with cholesterol in the chloroform–acetone (98:2, v/v) and the benzene–ethylacetate (5:1, v/v) systems. This was tested for cells harvested in early mid and late log phase of growth. No cholesterylglycoside or ester could be detected. When cells grown on cholesterol-rich medium were isolated and transferred to fresh growth medium without cholesterol, the amount of cholesterol in the membrane was only slightly reduced during incubation of the cells at 37° for 30 min. The glycerol and erythritol permeabilities of the cells did not change significantly, so it can be concluded that the cholesterol is not loosely bound in the *A. laidlawii* cell membrane.

Experiments using ultramembrane filter techniques for separating cells from medium showed that the rate of efflux of [^{14}C]erythritol from cells was decreased when cholesterol was incorporated into the membrane (to be published).

Liposomes are formed when the total lipid extract of *A. laidlawii* is hydrated with excess of a solution containing 50 mM KCl and 50 mM $MgSO_4$ per l. These liposomes behave like ideal osmometers (Fig. 3A). The glycerol permeability of these liposomes was measured in the same way as has been done with the whole cells. Fig. 3B shows that liposomes prepared from lipids isolated from cells grown on cholesterol show a lower glycerol permeability than liposomes prepared from the

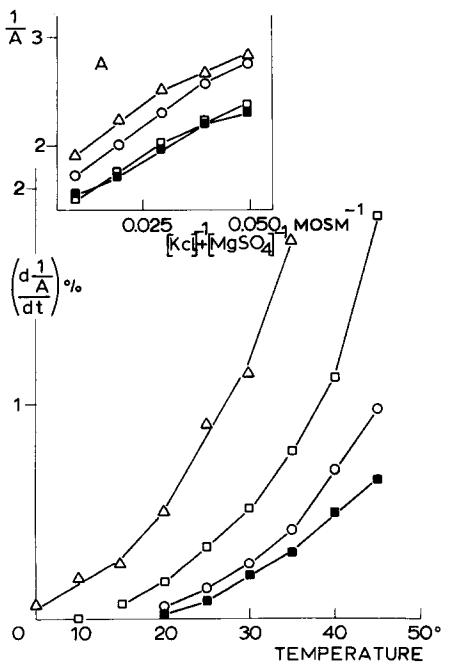

Fig. 3. Osmotic behavior of liposomes derived from total *A. laidlawii* lipids. A. Osmotic swelling as measured by $1/A$ at 450 nm 1 h after the addition of 100 μl liposome suspensions to 5 ml solutions of different KCl–$MgSO_4$ (1:1, mM/mM) concentrations. B. Initial swelling rates of liposomes in isotonic glycerol. Liposomes were prepared from lipids isolated from cells grown on 0.03 mM 16:0 and 0.03 mM 18:1c without cholesterol (△) and of the same lipids to which, before preparing the liposomes, 10 weight % (□) or 20 weight % (■) cholesterol was added. Liposomes prepared from lipids isolated from cells grown on 0.03 mM 16:0 and 0.03 mM 18:1c *plus* 25 mg cholesterol/l culture (○) contained 14.2 weight % cholesterol.

lipids extracted from the control cells in agreement with previous observations[19]. That this lowered permeability is indeed caused by cholesterol is demonstrated by the permeability behavior of liposomes prepared from the control lipids to which, before preparing the liposomes, a known amount of cholesterol was added. The rather high value of cholesterol incorporation (14.2 weight %) is probably caused by some co-precipitation of cholesterol during the isolation of the cells. The permeation of erythritol and glycerol through the *A. laidlawii* membrane is thought to proceed *via* a normal diffusion process. This suggestion is based on the following considerations: (1) the same effect of cholesterol upon the glycerol and erythritol permeability exists for both intact *A. laidlawii* membranes and derived liposomes, (2) the same activation energy of glycerol and erythritol permeation is found for both intact *A. laidlawii* membranes and derived liposomes,[41] and (3) the halftime of [^{14}C]erythritol efflux of *A. laidlawii* cells is independent of erythritol concentration up to 100 mM, no saturation of a hypothetical carrier is observed (to be published).

Because it is well established that the permeability-lowering effect of cholesterol in model membrane systems can be correlated with a condensing effect of

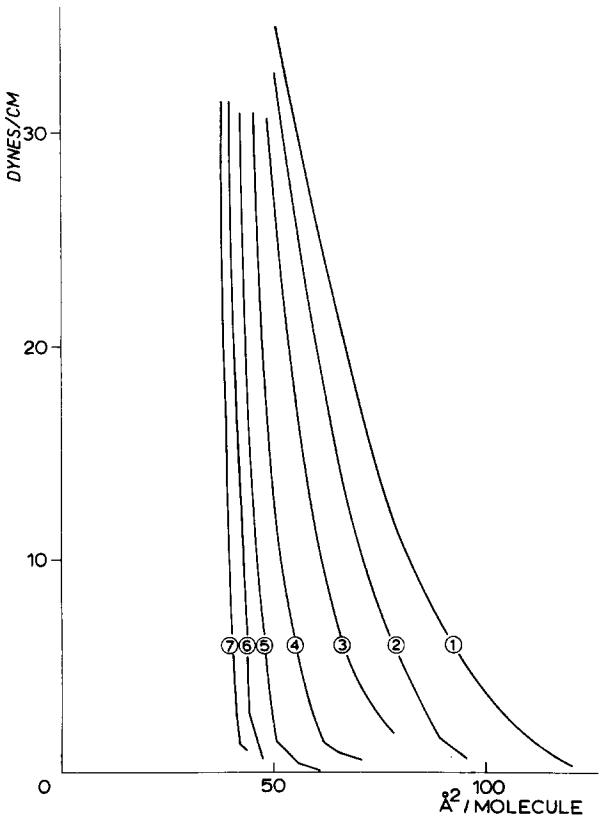

Fig. 4. Surface pressure–area curve at 22° at the air–water interface. (1), total *A. laidlawii* lipids isolated from cells grown on 0.03 mM 16:0 and 0.03 mM 18:1c without cholesterol. (2)–(7), Mixtures of these lipids with different amounts of cholesterol: (2), 19.4%; (3), 35.2%; (4), 60.1%; (5), 77.2%; (6), 95.1%; and (7), 100 mole % of cholesterol.

cholesterol in a mono- or bilayer of lipid[18,26–28], it was thought of interest to see whether this was also the case for the *A. laidlawii* lipids.

The pressure–area curve for a monolayer of total *A. laidlawii* lipids is shown in Fig. 4. The monolayer is of the liquid-expanded type as has been described for several naturally occurring lipids[29]. The effect of increasing amounts of cholesterol on the interfacial behavior was also studied. When the mean area per molecule is plotted *versus* the mole fraction of cholesterol according to the Goodrich method a reduction in the mean area per molecule by cholesterol is observed at all surface pressures. With 20 mole % cholesterol a reduction of the area per molecule of 3 and 5 Å^2 is observed at pressures of 5 and 20 dynes/cm, respectively. This amount of cholesterol can be incorporated into the *A. laidlawii* cell membrane (Table I), so it is obvious that the reduction of the glycerol and erythritol permeability of the *A. laidlawii* cell membrane and derived liposomes by cholesterol can be correlated with a condensing effect of cholesterol in a monolayer of *A. laidlawii* lipids. The area per molecule was calculated using a value of 870 for the mean molecular weight of the *A. laidlawii* lipids. This value was estimated in two independent ways, first by determining the amount of fatty acid per mg total lipid and second by calculating using literature values of the lipid composition of *A. laidlawii*[23,30].

To study the effect of the 3-hydroxyl group, *A. laidlawii* cells were grown in the absence of sterol or in the presence of cholesterol or epicholesterol, respectively (the first sterol being the 3β-hydroxy isomer and the latter the 3α-hydroxy isomer). The sterols were added as described under Method 2 in MATERIALS AND METHODS. The amount of epicholesterol solubilized by total *A. laidlawii* lipids was found to be 4.7 times smaller than the amount of cholesterol solubilized. Analysis showed that in this experiment 6.9 weight % of cholesterol and 7.4 weight % of epicholesterol was incorporated into the *A. laidlawii* membrane. The epicholesterol-containing cells showed the same osmotic behaviour and membrane fatty acid composition as the control cells. It is apparent that only cholesterol has a significant effect on the permeability of glycerol and erythritol through *A. laidlawii* membranes (Fig. 5). Incorporation of epicholesterol does not result in a reduced permeability for either solute. It can be concluded that also in biological membranes the 3β-hydroxy group of cholesterol is a prerequisite for a reduction in permeability of the membrane.

Although the amount of cholesterol in the growth medium was increased about 2-fold by adding the cholesterol in part in a liposomal form, no big difference in amount of cholesterol incorporated into the *A. laidlawii* cell membrane could be detected (9.3 $\pm$ 1.9 weight % cholesterol; *cf.* Table I). The amount of epicholesterol incorporated into the *A. laidlawii* membrane did not differ significantly from the amount of cholesterol incorporated.

LADBROOKE *et al.*[13] showed that cholesterol incorporated into the bilayers of dipalmitoyl lecithin in a 50 % water system reduced the amount of energy necessary to melt the hydrocarbon chains in the interior of the bilayer when these bilayers were brought from the crystalline Lβ to the liquid crystalline Lα form. This was confirmed for the unsaturated lecithin, 1-oleoyl-2-stearoyl-*sn*-glycero-3-phosphorylcholine (Fig. 6). In the temperature range from $-20°$ to $+90°$ this lecithin showed one main endothermic transition between $+4$ and $+22°$, the ΔH for this transition was 6.7 kcal/mole. For 18:0/18:0 phosphatidylcholine and 18:1/18:1 phosphatidylcholine ΔH values of 10.7 and 7.6 kcal/mole, respectively, are reported[14]. When

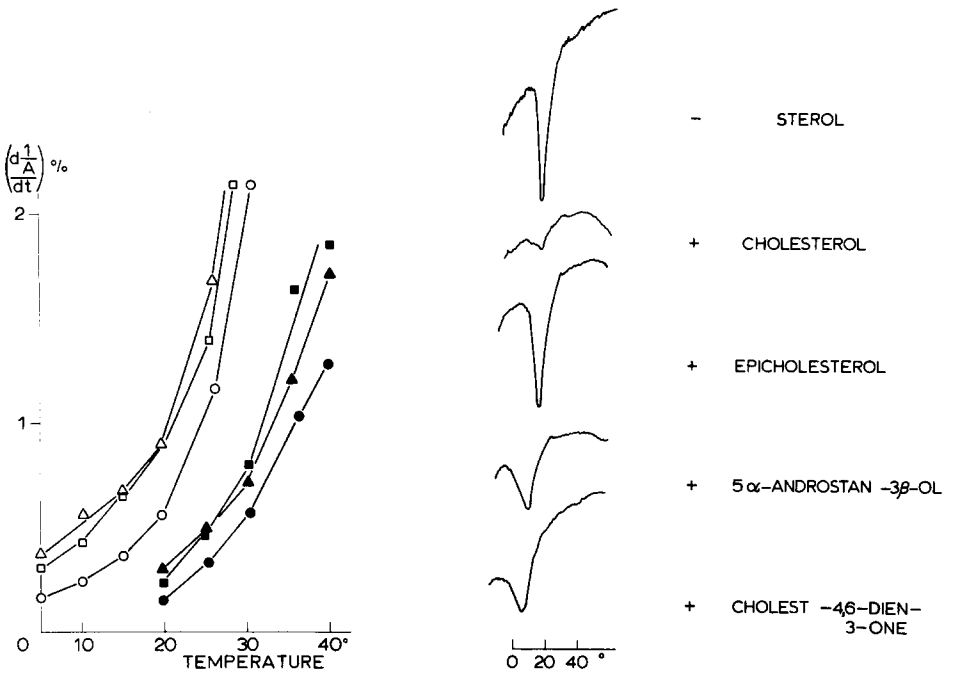

Fig. 5. Initial swelling rates of *A. laidlawii* cells in isotonic glycerol and erythritol. Cells were grown on 0.03 mM 16:0 and 0.03 mM 18:1c without sterol (△, ▲) with cholesterol (○, ●) or epicholesterol (□, ■) in the medium. Sterols were added as described under Method 2 in MATERIALS AND METHODS. The swelling rates in isotonic glycerol and erythritol are represented by open and closed symbols, respectively.

Fig. 6. Differential calorimeter scans of mixtures of 1-18:1/2-18:0 phosphatidylcholine with 20 mole % of different sterols. All sample pans were scanned by heating at a rate of 8°/min and contained identical amounts of lipids.

20 mole % of cholesterol was present the energy content of the phase transition of the lecithin was markedly reduced. Epicholesterol and a steroid having a keto group at the 3-position, cholest-4,6-diene-3-one, were unable to reduce the ΔH value of the lecithin phase transition to the same extent as cholesterol. 5α-Androstan-3β-ol, a sterol missing the side chain at C-17, was also unable to show the reduction in ΔH value of the lecithin phase transition. Fig. 7 shows the effect of different concentrations of sterol upon the phase transition of the lecithin. The phase transition was completely absent after the addition of about 32 mole % of cholesterol. However, after addition of 75 mole % of epicholesterol a definite phase transition of the lecithin is still present. The plateau of the energy value for the phase transition of the lecithin that is reached at higher epicholesterol concentrations might be explained by the only limited solubilities of epicholesterol in lecithin bilayers[34]. Preliminary X-ray work done by Dr. Gulik revealed that only about 25 mole % epicholesterol can be incorporated homogeneously in egg lecithin bilayers. The reduction in energy in the presence of cholesterol was also observed when the total *A. laidlawii* lipids were examined by differential scanning calorimetry (Figs. 8 a and b). The phase transition of the *A. laidlawii* lipids is broad when compared with the transition of the synthetic lecithin because of the more complex composition of the former. Incorporation of 11.9

weight % of cholesterol into the membrane lipids produced a 72 % reduction of the energy content of the phase transition. As found also with the synthetic lecithin, not only the amount of energy of the phase transition is reduced, but also a small shift of the transition to lower temperatures was observed when cholesterol was present in the *A. laidlawii* lipids. When cholesterol was added to a total lipid extract of the control cholesterol-free cells similar effects were observed.

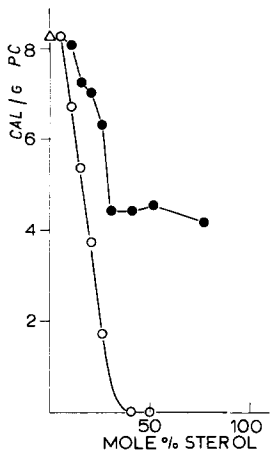

Fig. 7. Effect of different sterol concentrations on the phase transition of 18:1/18:0 phosphatidylcholine (PC). Different mixtures of 18:1/18:0 phosphatidylcholine (△) with cholesterol (○) or epicholesterol (●) were scanned by heating at a rate of 8°/min.

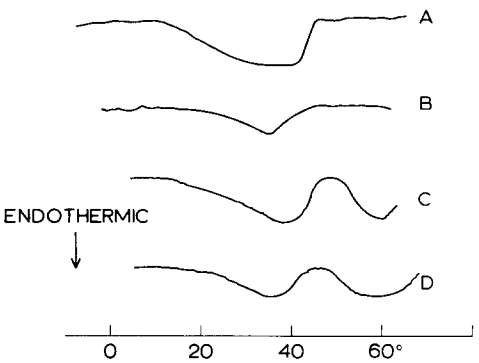

Fig. 8. A. Differential calorimeter scan of *A. laidlawii* lipids isolated from cells grown on 0.03 mM 16:0 and 0.03 mM 18:1t (elaidic acid). B. Scan of *A. laidlawii* lipids isolated from cells grown on 0.03 mM 16:0 and 0.03 mM 18:1t *plus* 25 mg cholesterol per l culture. C. Scan of *A. laidlawii* membranes derived from cells grown on 0.06 mM 18:1t without cholesterol. D. Scan of *A. laidlawii* membranes derived from cells grown on 0.06 mM 18:1t and 25 mg cholesterol per l of culture. Fatty acid composition of all samples was comparable; 18:1t (52%) and 16:0 (33%) were the predominant fatty acids. The samples were scanned by heating at a rate of 8°/min.

Figs. 8c and d show the scanning of intact *A. laidlawii* membranes. Two transitions are visible; the lower temperature transition is the lipid phase transition and has the same temperature characteristics as the transition observed by scanning of the total lipids (Fig. 8a). The second transition represents protein denaturation[31-33]. The control membranes showed a ΔH value for the lipid phase transition of 9.8 ± 0.6 cal/g total lipid (mean of three experiments). The 7.6 % cholesterol-containing membranes show a reduction of this energy quantity to 6.8 ± 0.8 cal/g total cholesterol-free lipid (mean of three experiments). The energy content of the phase transition in the total cholesterol-free lipids isolated from cells grown in a medium supplemented with elaidic acid was 11.3 ± 2.4 cal/g lipid (three experiments). In the presence of 11.9 % cholesterol this value was 3.2 ± 0.5 cal/g cholesterol-free lipids (Figs. 8a and b). The ΔH values for the transition in the *A. laidlawii* membranes and lipids are rather high as compared to previously reported values. REINERT AND STEIM[32] and CHAPMAN AND URBINA[30] found ΔH values for the total *A. laidlawii* lipids of 3.9 ± 0.2 and 3.8 ± 0.1 cal/g lipid, respectively, when the cells were grown

in a medium supplemented with palmitic acid. This difference might be caused by differences in the fatty acid composition of the lipids.

A phase transition in the lipid moiety of the membrane is also observed in the swelling experiments (Fig. 9). It was noticed that when cells were grown on 0.03 mM palmitic and 0.03 mM elaidic acid below a certain temperature the swelling of these cells in isotonic glycerol or erythritol was apparently rapid. The very rapid change

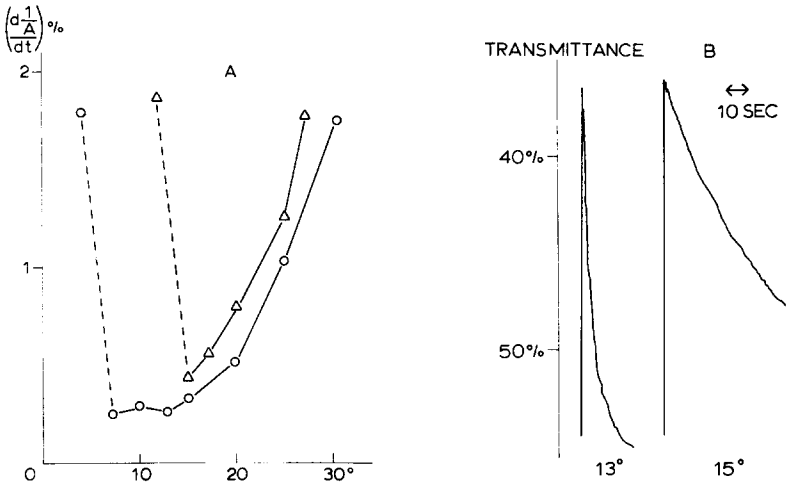

Fig. 9. Initial swelling rates of *A. laidlawii* cells in isotonic glycerol. Cells were grown on 0.03 mM 16:0 and 0.03 mM 18:1t with (○) or without (△) 25 mg cholesterol per l medium. A. Initial swelling rates in isotonic glycerol determined as a function of the temperature. B. Actual tracings of swelling curves of the cholesterol-free cells. The left hand figure represents the "swelling" of the cells at 13°; the right hand figure gives the swelling of the cells at 15°. In both cases 50 μl cell suspensions were injected in 5 ml of stirred 200 mM glycerol; the change in absorbance was recorded.

in the transmittance that was observed, a change in rate comparable to the change in transmittance when cells are brought in distilled water at this temperature, probably reflects the bursting of the cells. This phenomen is still under investigation. That this tempearture probably is correlated with a lipid phase transition is based on three observations:(a) The temperature at which the bursting happens is correlated with the fatty acid composition of the *A. laidlawii* membrane. When cells are grown on 0.03 mM palmitic and 0.03 mM oleic acid this temperature is 2–5°. When the cells are grown on 0.03 mM palmitic *plus* 0.03 mM elaidic acid this temperature is 12–15°. (b) Cholesterol incorporated into the membrane lowers these temperatures with 5–8°. This is in line with the finding that cholesterol can act as a liquifier in a solid lipid bilayer. (c) These temperatures were in reasonable agreement with the temperatures measured by differential scanning calorimetry (*cf.* Fig. 8a). The exact temperature of the beginning of the melting of the hydrocarbon chains in the *A. laidlawii* lipids is, however difficult to determine by differential scanning calorimetry. Fig. 9, furthermore, shows that cholesterol lowers the permeability of the elaidic acid-grown cells. The oleic acid-rich cells showed higher glycerol and erythritol permeabilities than the elaidic acid-containing cells (*cf.* Figs. 1 and 9), which can be explained by a more condensed character of the *trans* fatty acid.

DISCUSSION

It is demonstrated that cholesterol is incorporated into the plasma membrane of *A. laidlawii* cells. Epicholesterol is incorporated in the membrane of this organism* to the same extent as cholesterol. The sterol was not esterified by the *A. laidlawii* cells and incorporated in amounts up to 8 ± 1 weight %. The incorporation of cholesterol or epicholesterol did not influence the fatty acid composition of the membrane.

The membrane permeability of *A. laidlawii* cells is reduced for glycerol and erythritol when cholesterol is present in the plasma membrane. These data are in agreement with earlier observations[19]. However, the permeability for glycerol and erythritol is not affected by the incorporation of epicholesterol. It can be concluded that also in biological membranes the 3β-hydroxy group of cholesterol is a prerequisite for a reduction in permeability of the membrane. These results are in agreement with the work of DEMEL *et al.*[34]. These authors found that epicholesterol incorporated into egg lecithin liposomes did not affect the permeability of the liposomes for glycerol, erythritol and glucose. Cholesterol present in biological membranes and in lecithin liposomes lowered the permeability of the membranes for various substances[8-10,18,34]. Liposomes prepared from lipid extracts of *A. laidlawii* cells grown in the presence or absence of cholesterol showed a permeability behavior comparable to that of intact *A. laidlawii* cells. The presence of cholesterol caused a reduction in the glycerol and erythritol permeability of liposomes derived from *A. laidlawii* cells which incorporated the sterol in the cell membrane.

Monolayer studies showed that mixing of lecithin with cholesterol resulted in a decrease of the mean molecular area[10,36]. Van der Waals' forces are thought to be important in bringing about this condensation effect. This conclusion is supported by X-ray and electron spin resonance data which show a reduction in chain mobility when cholesterol is added[28,11,35]. Monolayers of total lipid extract of *A. laidlawii* cells also show a reduction in mean molecular area denoted as condensation effect. The total lipid extract of *A. laidlawii* consists of about 40 % glycolipid (mono- and di-glucosyl diglyceride), 45 % phospholipids (phosphatidylglycerol with its lysyl ester and an α-glycerophosphate derivative of diglucosyl diglyceride) and 15 % neutral lipids including the yellow colored carotenoids[23,29,37]. The pressure–area curve of a monolayer of this total lipid extract is of the liquid-expanded type similar to the curves obtained for the cholesterol-free erythrocyte and myelin lipids[29]. 50 mole % cholesterol causes a reduction in the mean molecular area of about 8 and 4 Å^2 per molecule at pressures of 5 and 10 dynes/cm, respectively. Mixed monolayers of 18:1/18:0 lecithin and epicholesterol showed especially at 37° only a very limited condensation effect when compared with cholesterol[10].

Differential scanning calorimetry, X-ray and ESR studies showed that phase

* That epicholesterol is located in the *A. laidlawii* membrane is supported by experiments with the polyene antibiotic filipin[40]. Sterols such as cholesterol and epicholesterol changed the ultraviolet absorption spectrum of filipin when the sterols were added in a microcrystalline state. However, when the sterols were present in egg lecithin liposomes, only 3β-hydroxysterols such as cholesterol produced a spectral shift. Epicholesterol incorporated into the liposome bilayer did not show an interaction with filipin. Cholesterol incorporated into the *A. laidlawii* membrane produced a change in the ultraviolet spectrum characteristic for the filipin–cholesterol complex. Epicholesterol incorporated into the *A. laidlawii* membrane could not interact with filipin suggesting that the orientation of epicholesterol in the *A. laidlawii* membrane is the same as in the liposomal bilayers system.

transitions occur in *A. laidlawii* lipids, membranes and cells[31–33,38,39]. LADBROOKE and co-workers[13,14] noticed that cholesterol could cause a decrease in ΔH value of the lipid transition of different synthetic and natural lecithins as measured by differential scanning calorimetry. This paper describes the same effects occurring in total *A. laidlawii* lipids. Lipids isolated from cells containing 11.9 weight % cholesterol showed a reduction of 72 % of the ΔH for the lipid transition as compared to the ΔH value for the transition of *A. laidlawii* lipids isolated from cells grown without cholesterol. This reduction is also found when 11.9 weight % cholesterol is present in 18:1/18:0 phosphatidylcholine liposomes (Fig. 7). Cholesterol can produce such an effect also in biological membranes. The energy of the lipid transition in the intact *A. laidlawii* membrane containing 7.6 weight % cholesterol was reduced by 31 % as compared to the lipid transition in the membranes obtained from cells grown without sterol (Fig. 8). This reduction is comparable to the 35 % reduction in energy observed in the 18:1/18:0 lecithin bilayers containing 7.6 weight % cholesterol, which might indicate that cholesterol in the *A. laidlawii* membrane is present in an environment comparable to a lipid bilayer. Comparison of ΔH values of the lipid transition in total lipids and membranes both isolated from organisms grown without sterol, revealed that 87 % of the cal/g lipid value for the total lipids was recovered in the membrane lipid transition. This is in good agreement with the data given by STEIM and co-workers[31,32]. These authors concluded from their data that almost all of the lipids in the *A. laidlawii* membrane are in a bilayer structure according to the Danielli–Davson membrane model. CHAPMAN AND URBINA[30] recently challenged this conclusion by pointing out that several assumptions made by these workers might not be valid.

A specific involvement of the 3β-OH group in the interaction of lecithin and cholesterol can also be demonstrated by differential scanning calorimetric measurements. The transition of the 18:1/18:0 lecithin dissappears completely with 32 mole % cholesterol. Epicholesterol at lower concentrations produced a decrease in ΔH value for the transition of the 18:1/18:0 lecithin. This decrease is small as compared to the reduction in energy produced by cholesterol. At higher epicholesterol concentrations no further decrease in energy of the 18:1/18:0 lecithin transition was observed. A definite phase transition of the 18:1/18:0 lecithin was still present with 75 mole % of epicholesterol. Cholestan-4,6-dien-3-one, a steroid having a keto group at the 3 position of the molecule could not produce the reduction in energy of the 18:1/18:0 lecithin phase transition as observed with cholesterol; this indicates the necessity of the hydroxyl group of cholesterol for the interaction with lecithin. The 3-ketosteroid is solubilized well by lecithin[12], which is probably also reflected by a broadening of the lecithin phase transition as found also with epicholesterol. The 5α-androstan-3β-ol present in the 18:1/18:0 lecithin liposomes does not influence the phase transition of the lecithin. This is in agreement with the work of LADBROOKE AND CHAPMAN[14]. A side chain at C-17 of the sterol molecule is a necessity for the specific cholesterol–lecithin interaction[10,34]. One has to be aware that the solubilization of the sterols in the lecithin bilayer can be different[12].

Spin label[11], monolayer[10] and permeability studies done with sterol–lecithin liposomes[34] revealed that the 3β-OH group is an essential part in the sterol molecule for a specific sterol–lecithin interaction. Also in the biological membrane a 3β-OH group seems to be a prerequisite for a specific interaction of the sterol in the membrane.

Considering the role of the 3β-OH group of a sterol as a necessity for a cholesterol like functioning in a membrane, it is relevant that the parasitic and T-strain Mycoplasmas, which have an absolute sterol growth requirement, cannot grow when epicholesterol or epicholestanol is present as the only sterol in the growth medium[1,4]. These Mycoplasmas contain rather high amounts of cholesterol, which plays an essential structural role in the membrane of these organisms. Apparently a 3α-OH sterol cannot take over this structural role.

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