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Cyclodextrin-catalyzed extraction of fluorescent sterols from monolayer membranes and small unilamellar vesicles

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Abstract

This study examined the kinetics of sterol desorption from monolayer and small unilamellar vesicle membranes to 2-hydroxypropyl-β-cyclodextrin. The sterols used include cholesterol, dehydroergosterol (ergosta-5,7,9,(11),22-tetraen-3β-ol) and cholestatrienol (cholesta-5,7,9,(11)-trien-3β-ol). Desorption rates of dehydroergosterol and cholestatrienol from pure sterol monolayers were faster (3.3–4.6-fold) than the rate measured for cholesterol. In mixed monolayers (sterol: 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine 30:70 mol%), both dehydroergosterol and cholestatrienol desorbed faster than cholesterol, clearly indicating a difference in interfacial behavior of these sterols. In vesicle membranes desorption of dehydroergosterol was slower than desorption of cholestatrienol, and both rates were markedly affected by the phospholipid composition. Desorption of sterols was slower from sphingomyelin as compared to phosphatidylcholine vesicles. Desorption of fluorescent sterols was also faster from vesicles prepared by ethanol-injection as compared to extruded vesicles. The results of this study suggest that dehydroergosterol and cholestatrienol differ from cholesterol in their membrane behavior, therefore care should be exercised when experimental data derived with these probes are interpreted.

Keywords: Desorption kinetics; Dehydroergosterol; Cholestatrienol; Phospholipid

1. Introduction

In recent years, cyclodextrins (CyDs) have been used as tools to manipulate the lipid composition of biological and model membranes (Asgharian et al., 1988; Kilsdonk et al., 1995; Klein et al., 1995; Ohvo and Slotte, 1996; Yancey et al., 1996; Ohvo et al., 1997). The CyD molecules have a hydrophilic outer surface and a hydrophobic inner core, thus they can form inclusion complexes by binding small amphiphilic molecules into their core. β-Cyclodextrins are known to be very effi-

Abbreviations: CTL, cholesta-5,7,9,(11)-trien-3β-ol (cholestatrienol); CyD, cyclodextrin; DHE, ergosta-5,7,9,(11),22-tetraen-3β-ol (dehydroergosterol); 2OHpβCyD, 2-hydroxypropyl-β-cyclodextrin; *N*-O-SM, *N*-oleoyl-sphingomyelin; POPC, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine.

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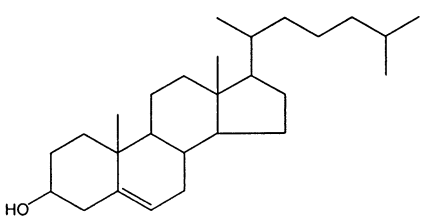
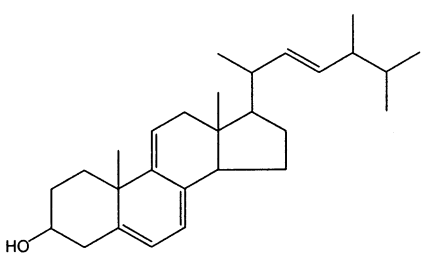
cient sterol acceptor molecules, apparently because their inner hydrophobic cavity matches the size of the sterol molecule (Pitha et al., 1988; Ohtani et al., 1989; Irie et al., 1992; Ohvo and Slotte, 1996). Cholesterol can be removed from cells by extraction into small unilamellar vesicles (Clejan and Bittman, 1984a,b; Bittman, 1988). However, the process is not very efficient and is also fairly slow ($t_{1/2}$ measured in hours). Using CyDs as cholesterol acceptors, the extraction of cholesterol from cells can be both efficient and rapid (Kilsdonk et al., 1995; Yancey et al., 1996; Ohvo et al., 1997).

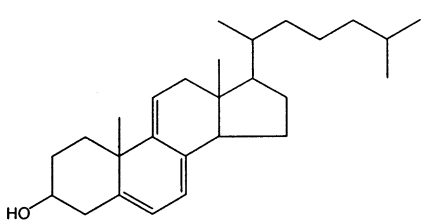
The kinetics for the formation of cholesterol/CyD inclusion complexes can be followed easily using a monolayer desorption assay, developed by us (Ohvo and Slotte, 1996). In this system, rates of cholesterol desorption to CyDs can be followed from the changes in monolayer area (at constant surface pressure) as a function of time. However, in order to measure cholesterol desorption kinetics from cell membranes or vesicles, one would need to either use labeled cholesterol or measure changes in cholesterol mass (chemical analysis) as a function of CyD-induced cholesterol extraction from the donor membrane surface. With a vesicle model system, fast kinetic processes cannot be studied however, since donor vesicles would need to be separated from the cholesterol/CyD complexes prior to analysis (Yancey et al., 1996).



Cholesterol



Dehydroergosterol



Cholestatrienol

Scheme 1. The structures of cholesterol, dehydroergosterol and cholestatrienol.

The aim of this study was to measure sterol desorption kinetics from monolayers and bilayers with CyDs as acceptors, using fluorescent sterols as reporter molecules. The sterols used were ergosta-5,7,9,(11),22-tetraen-3β-ol (dehydroergosterol) and cholesta-5,7,9,(11)-trien-3β-ol (cholestatrienol). The structures of the used sterols are shown in Scheme 1. These sterols have previously been used as cholesterol analogues in model membrane studies, and their transfer or exchange properties have been studied in vesicle systems (Yeagle et al., 1982; Schroeder et al., 1987, 1988a,b; Nemecz et al., 1988; Schroeder and Nemecz, 1989; Hyslop et al., 1990). Dehydroergosterol and cholestatrienol have been shown to act like cholesterol in altering water permeability of model membranes (Rogers et al., 1979), in lipid packing (Yeagle et al., 1982), and in condensing phosphatidylcholine monolayers (Hyslop et al., 1990). So far, there exists little data comparing the desorption kinetics of cholesterol with that of either dehydroergosterol or cholestatrienol (Nemecz et al., 1988; Nemecz and Schroeder, 1988). Nemecz and co-workers showed in their studies that dehydroergosterol desorbs with a similar rate as cholesterol, but they used different parameters to measure the amount of desorbed cholesterol and dehydroergosterol. We have in this study determined sterol desorption kinetics from monolayers and small unilamellar vesicles using 2-hydroxypropyl-β-CyD (2OHpβCyD) as a sterol acceptor.

2. Materials and methods

2.1. Materials

Cholesterol, 7-dehydrocholesterol, dehydroergosterol, 2-hydroxypropyl-β-CyD and the phospholipids were purchased from Sigma Chemicals (St. Louis, USA). Stock solutions of the lipids were prepared in hexane/2-propanol (3/2, v/v), stored in the dark at −20°C, and warmed up to ambient temperature before use. 2OHpβCyD stock solutions were prepared in pure water to a concentration of 40 mM (or 150 mM). Mercuric acetate was from J.T. Baker (the Netherlands) and acetic anhydride from Riedel-de Haën (Germany). The water used in all experiments was purified by a Millipore UF Plus water purification system providing a product with a resistivity of 18.2 MΩcm.

2.2. Synthesis and purification of cholestatrienol

Cholestatrienol was synthesized from 7-dehydrocholesterol by a slight modification of the method by Fischer et al. (1984). In all steps contact with light was avoided as much as possible to avoid photo-oxidation of the labile sterols. A total of 200 mg 7-dehydrocholesterol was dissolved in 5 ml pre-warmed acetic anhydride, and the esterification was then allowed to proceed for 4 h at 90°C. The mixture was then cooled to room temperature and 8 ml methanol was added. 7-Dehydrocholesterol-acetate was precipitated by cooling the mixture to 4°C. Then the mixture was centrifuged a few minutes at 3000 rpm and the supernatant was discharged. The precipitation procedure was repeated with 10 ml ice cold methanol. The precipitate was dissolved in 5 ml chloroform and added to 10 ml glacial acetic acid containing 0.62 g mercuric acetate. The reaction was allowed to proceed for 18 h at room temperature with continuous stirring. Mercuric and mercurous acetates were filtered from the reaction mixture and washed with chilled diethyl ether. The combined solvents were evaporated to near dryness in vacuo. The product was dissolved in 3 ml chilled diethyl ether and to precipitate remaining mercurous acetates it was left standing on ice for 15 min. The mixture was again filtered and the filtrate evaporated in vacuo. The dry lipids were saponified for 1.5 h at 75°C with 0.75 M NaOH in ethanol. Precipitates of mercury salts were then removed by filtration. The filtrate was extracted two times with 4 ml hexane and 2 ml water. The hexane-phases were dried with $CaCl_2$ and evaporated to dryness with argon. The product was purified by reversed phase high performance liquid chromatography on a LiChrospher 100 RP-18 column (5-μm particle size, 125 × 4 mm column dimensions, Merck, Germany). 100% methanol was used to elute the sterols at room temperature (flow rate 1 ml/min). After this purification step the product was identified as cholestatrienol by gas chromatography-mass spectrometry analysis and this analysis also confirmed the purity of the product. Cholestatrienol was stored dried in aliquots at −70°C and used within 5 months of synthesis. When needed cholestatrienol was dissolved in hexane/2-propanol and used within 2 days.

2.3. Force–area isotherms of sterol monolayers

Pure monolayers of each sterol were compressed on water at ambient temperature under an argon atmosphere with a KSV surface barostat (KSV Instruments Ltd., Helsinki, Finland). The barrier speed did not exceed 3.4 $Å^2$/molecule per minute during compression. Data were recorded using proprietary KSV software. The monolayer was protected from ambient light during compression.

2.4. Cyclodextrin-mediated desorption of sterols from monolayers

Monolayers containing pure sterols were prepared at the argon/water interface at 22°C and compressed to 20 mN/m with a KSV surface barostat. The trough used was of zero-order type, with a reaction chamber (5.1 ml volume) separated by a glass bridge from the lipid reservoir. After a stable baseline had been obtained 2OHpβCyD was injected into the stirred reaction chamber without penetrating the monolayer. The final concentration of 2OHpβCyD in the reaction

chamber was 1.6 mM. The removal of monolayer sterols to the subphase was determined from the monolayer area decrease at constant surface pressure. Knowing the mean molecular area at a given surface pressure and temperature, we could calculate the amount of sterol removed as a time function (Ohvo and Slotte, 1996). Experiments on mixed monolayers containing phospholipid and sterol (70:30 mol%) were done like the experiments with pure monolayers, except that 16.0 mM 2OHpβCyD was used to mediate desorption. All experiments with the fluorescent sterols were done in darkness to avoid photo-oxidation.

2.5. Preparation of ethanol-injected small unilamellar vesicles

To prepare vesicles lipid stock solutions were mixed in the desired proportions (phospholipid:sterol 70:30 mol%) and dried under a stream of argon leaving a thin film of lipids on the walls of the tube. The dried lipids were dissolved in ethanol to obtain a lipid concentration of 0.2 mM. Of this solution, 20 μl was then injected into water using a spring-loaded Hamilton syringe (Hamilton CO, Reno, NE) to generate small unilamellar vesicles (final lipid concentration 1.3 μM). To avoid photo-oxidation of the sterols the solutions were kept in dark during and after preparation.

2.6. Preparation of unilamellar vesicles by extrusion

Vesicles of various sizes were prepared by the extrusion technique described by Hope et al. (1985). Lipid solutions were mixed in the desired proportions (phospholipid:sterol 70:30 mol%) and dried under a stream of argon leaving a thin film of lipids on the walls of the tube. Water was added to the dry lipids and the solution was sonicated briefly to remove the lipids from the side of the tubes and to generate multilamellar vesicles. Extrusion of the dispersions was carried out at 55°C using a Lipextruder (Lipex Biomembranes Inc., Vancouver, CA) equipped with a water jacketed thermobarrel. The dispersions were extruded through a stacked pair of 25 mm polycarbonate filters of the desired pore size (50, 100 or 200 nm). After ten cycles of extrusion the dispersions were diluted with water to obtain a final lipid concentration of 1.3 μM. The nitrogen pressure employed to the Lipextruder never exceeded 1500 kPa. During preparation of the vesicles contact with light was avoided as much as possible to avoid oxidation of the sterols, and the vesicles were used on the same day that they had been prepared. The lipid composition of the extruded vesicles was determined by chemical analysis of each lipid component, and was found to be within $\pm$ 5% of the intended composition (i.e. 70:30).

2.7. Fluorescence measurements of sterol desorption from vesicles

Fluorescence intensity was measured with a QuantaMaster-1 spectrofluorometer (Photon Technology International, Inc.) equipped with two 810 photomultiplier detection systems. Data were recorded using proprietary Felix software. Dehydroergosterol fluorescence emission was recorded at 390 nm (λ_{ex} 328 nm). The corresponding emission wavelength for cholestatrienol was 374 nm (λ_{ex} 324 nm). The vesicle preparation in the cuvette was continuously stirred and the temperature was kept constant at 37°C by a circulating water bath. The vesicles contained 30 mol% fluorescent sterol, which is a high enough concentration to cause self-quenching of sterol fluorescence in the vesicles (Schroeder et al., 1987, 1988b). After obtaining a stable baseline, 2OHpβCyD was injected into the vesicle preparation using a Hamilton syringe. The addition of 2OHpβCyD to the cuvette initiated sterol desorption and the formation of sterol/2OHpβCyD complexes, and lead to a rise in the relative fluorescence. The total concentration of lipids in the cuvette was always 1.3 μM and the final concentration of 2OHpβCyD in the cuvette was 2.5 mM (unless otherwise stated). Therefore the molar ratio sterol:2OHpβCyD was about 1:6300.

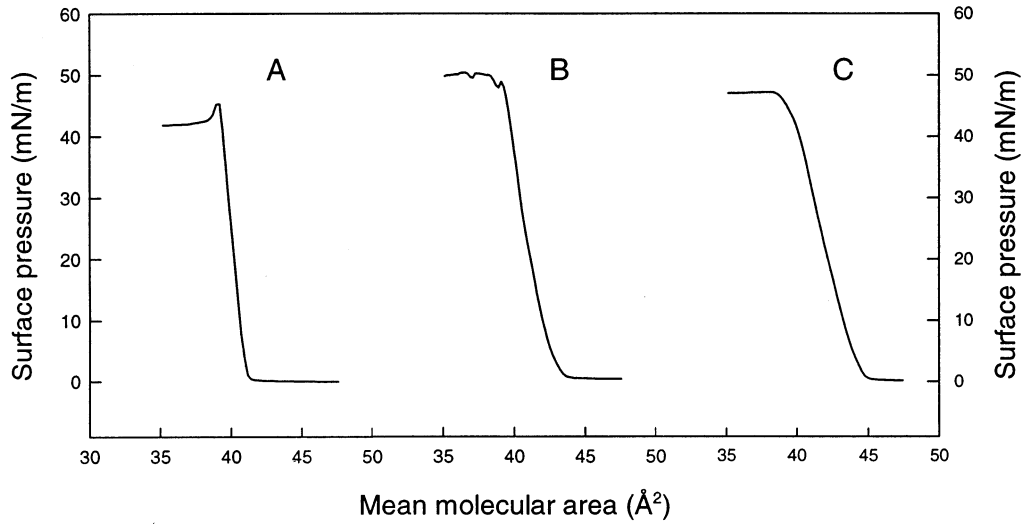


Fig. 1. Force–area isotherms of cholesterol, dehydroergosterol, and cholestatrienol. The isotherms were run at the argon/water interface at 22°C and in darkness to avoid oxidation of the fluorescent sterols. Panel A is cholesterol, B is dehydroergosterol, and C is cholestatrienol. The panels represent one isotherm out of three representative experiments.

3. Results

3.1. Interfacial properties of the sterols

To determine how closely dehydroergosterol and cholestatrienol resemble cholesterol, we first set out to characterize their interfacial behavior in monolayer membranes. The force–area compression isotherm of cholesterol in Fig. 1 (obtained under an argon atmosphere) shows that cholesterol forms a condensed monolayer at ambient temperature that collapses at a surface pressure of about 45 mN/m. This isotherm is similar to numerous other previously published isotherms of cholesterol (Chapman et al., 1969; Slotte, 1992a). The lift-off area for cholesterol was about 41 Å^2 and the mean molecular area at 30 mN/m was 40 Å^2. Both dehydroergosterol and cholestatrienol had more expanded force–area isotherms than that seen for cholesterol (Fig. 1). This was evidenced by the larger lift-off areas (43.5 and 45 Å^2 for dehydroergosterol and cholestatrienol, respectively) and the larger mean molecular area at 30 mN/m (40.4 Å^2 for dehydroergosterol and 41.1 Å^2 for cholestatrienol). There was no large difference in monolayer stability to compression among the three sterols tested. Based on these interfacial properties, one can see that both dehydroergosterol and cholestatrienol formed stable monolayers and had mean molecular areas that were somewhat larger than those seen for cholesterol.

3.2. Sterol desorption from pure and mixed monolayers to cyclodextrin

Desorption rates of sterols from model membranes are markedly affected by the degree of hydrophobicity of the amphiphile, and by possible interactions with co-amphiphiles in the donor membrane (Phillips et al., 1987; Bittman, 1993). In a previous study we have developed a monolayer desorption assay that allows us to measure sterol desorption kinetics using CyDs as sterol acceptor molecules (Ohvo and Slotte, 1996). Using this technique, we could compare desorption kinetics of the three sterols at constant lateral compression (i.e. surface pressure). As shown in Fig. 2, the desorption of cholestatrienol from a pure sterol monolayer using 2OHpβCyD as acceptor was faster than the desorption of dehydroergosterol, while cholesterol desorption was the slowest. If the amount of sterol desorbed was calculated during the first 10 min of reaction, it was found that about 100 pmol of cholesterol had

desorbed per cm^2 of the monolayer, whereas 320 and 450 pmol of dehydroergosterol and cholestatrienol had desorbed, respectively (Table 1).

The rate of sterol desorption from monolayers containing phospholipids is known to be slower than rates measured in pure sterol monolayers, because molecular interactions between sterols and phospholipids retard the desorption process (Ohvo and Slotte, 1996). Cholesterol desorption from a mixed monolayer containing 70 mol% 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) was markedly slower than the desorption from a pure cholesterol monolayer, even though a 10-fold higher CyD concentration was used with the mixed monolayers (Table 1). The desorption rates of both dehydroergosterol and cholestatrienol, when calculated relative to cholesterol's, were even faster in the mixed monolayers as compared to pure sterol monolayers. This suggests that the fluorescent sterols did not interact as well with POPC as cholesterol did (Table 1).

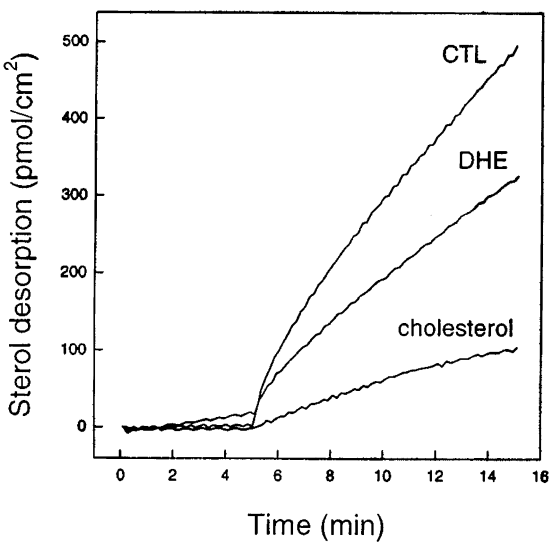


Fig. 2. 2OHpβCyD-mediated removal of cholesterol, dehydroergosterol, and cholestatrienol from pure sterol monolayers at constant lateral surface pressure. Monolayers were prepared at the argon/water interface and compressed to 20 mN/m. After a stable monolayer was obtained, 2OHpβCyD was injected into the stirred monolayer subphase (final concentration 1.6 mM), leading to a facilitated desorption of sterols from the monolayer. The curves represent values from one representative experiment out of three similar experiments.

Table 1
Rates of cholesterol, dehydroergosterol and cholestatrienol desorption to 2OHpβCyD from pure and mixed monolayers[a]

Sterol species	Desorption rate (pmol/cm^2·per min)	Rate relative to cholesterol desorption
Pure sterol monolayers		
Cholestatrienol[b]	45.2 ± 2.8	4.6
Dehydroergosterol[b]	32.1 ± 0.1	3.3
Cholesterol[b]	9.8 ± 0.3	1.0
Mixed monolayers		
Cholestatrienol[c]	5.3 ± 0.3	13.3
Dehydroergosterol[c]	5.2 ± 0.3	13.0
Cholesterol[c]	0.4 ± 0.2	1.0

[a] Pure and mixed lipid monolayers were prepared at the argon/water interface. The monolayer was compressed at 22°C and held at a constant lateral surface pressure of 20 mN/m. The rate of desorption to 2OHpβCyD in the subphase was determined from the rate of monolayer area decrease. Values are means ± S.E.M. from three different monolayer experiments of each composition.

[b] 1.6 mM 2OHpβCyD was used for desorption.

[c] 16.0 mM 2OHpβCyD was used for desorption.

3.3. Dehydroergosterol desorption from POPC vesicles to 2OHpβCyD

Next, we wanted to develop a method with which we could follow the kinetics of the desorption of fluorescent sterols from vesicles to 2OHpβCyD in a spectrofluorometer. The fluorescence spectrum of dehydroergosterol in POPC vesicles gave a maximal excitation at 328 nm and a maximal emission at 390 nm (Schroeder et al., 1987). The interaction of dehydroergosterol with *N*-oleoyl-sphingomyelin (*N*-O-SM) vesicles did not affect its spectrum, neither did the addition of 2OHpβCyD (data not shown). The vesicles used in this study contained 30 mol% dehydroergosterol in a POPC matrix, which is a high enough concentration of dehydroergosterol to cause self-quenching in the vesicles (Schroeder et al., 1987, 1988b). Addition of 2OHpβCyD to the vesicles initiated dehydroergosterol desorption and the formation of dehydroergosterol/CyD complexes, a process which lead to a rise in the relative fluorescence (reduction of quenching; Fig. 3).

Desorption of the fluorescent sterol was fast and the process reached an equilibrium (maximum fluorescence) in a few minutes. To determine optimal vesicle/CyD ratio for our experiments we tested the concentration dependence of CyD-mediated dehydroergosterol desorption from POPC vesicles (Fig. 4). Maximal fluorescence was measured at a 2OHpβCyD concentration of about 2.5 mM, a concentration used in further experiments. Although not shown, the CyD concentration dependence of cholestatrienol desorption was qualitatively similar to that observed for dehydroergosterol.

3.4. Sterol desorption kinetics from POPC and N-O-SM vesicles

To study the effects of the phospholipid matrix, and the size of small unilamellar vesicles on sterol desorption kinetics to 2OHpβCyD, we prepared small unilamellar vesicles either by ethanol injection or by the extrusion technique (Hope et al., 1985). The vesicles contained either dehydroergosterol or cholestatrienol (at 30 mol%) and either POPC or *N*-O-SM (70 mol%). As shown in Fig. 5, the time needed for 50% desorption was much longer for *N*-O-SM vesicles as compared to POPC vesicles, irrespective of the sterol examined. It was also observed that cholestatrienol desorption was faster than dehydroergosterol desorption (Fig. 5), in good agreement with the results obtained from monolayer desorption studies (Fig. 2). For sterol desorption from POPC vesicles the rate did not change with donor vesicle size, whereas sterol desorption from *N*-O-SM vesicles was slower from larger vesicles than from smaller ones. Interestingly, sterol desorption rates were very fast from ethanol-injected vesicles, and there was no marked difference in sterol desorption kinetics when the vesicle matrix was changed from POPC to *N*-O-SM.

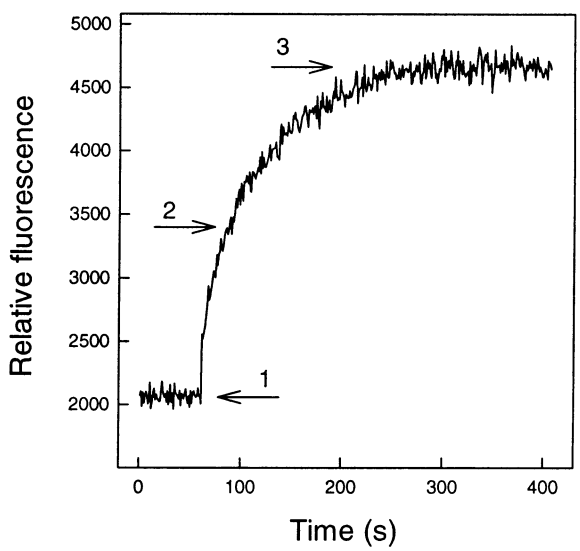


Fig. 3. Time course of dehydroergosterol desorption from POPC vesicles to 2OHpβCyD. The desorption of dehydroergosterol to 2OHpβCyD (final concentration 2.5 mM) resulted in an increased relative fluorescence intensity, which was detected as a time function at 37°C. Arrow 1 indicates the basal fluorescence intensity before addition of CyD. Arrow 2 indicate half-maximal reaction, and arrow 3 the final equilibrium state. The vesicles were extruded through 100 nm pores (final concentration in the cuvette 1.3 μM lipid) and contained 70 mol% POPC and 30 mol% dehydroergosterol.

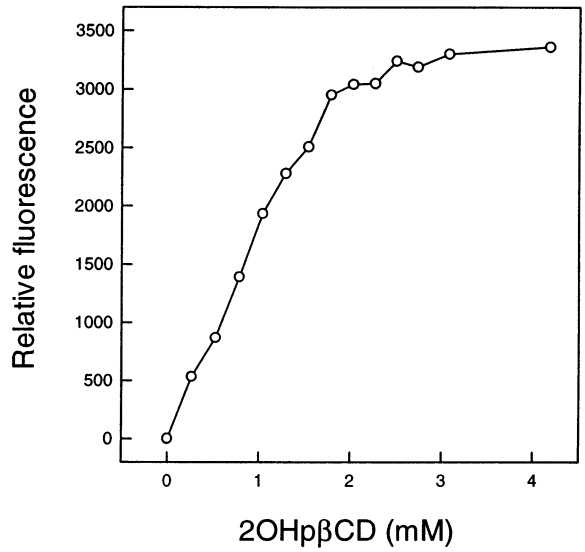


Fig. 4. Concentration dependence of 2OHpβCyD-mediated dehydroergosterol-efflux from POPC vesicles. The vesicles were extruded through 100 nm pores and contained 70 mol% POPC and 30 mol% dehydroergosterol. The maximal increase in fluorescence (relative fluorescence increase at equilibrium) was measured at different concentrations of 2OHpβCyD at 37°C.

4. Discussion

Earlier studies have suggested that dehydroergosterol and cholestatrienol behave like cholesterol in model membranes. The similarity in physical properties of the three sterols has been inferred from findings which show that all three sterols have a similar capacity to alter the water permeability of model membranes (Rogers et al., 1979), affect lipid packing (Yeagle et al., 1982), and to abolish the phase transition of dipalmitoyl phosphatidylcholine (Hale and Schroeder, 1982). In this study we found, however, that the interfacial properties of dehydroergosterol and cholestatrienol differed to some extent from the properties of cholesterol. The fact that dehydroergosterol and cholestatrienol showed more expanded isotherms than cholesterol implies that the van der Waals forces acting between the sterol molecules were weaker than in a cholesterol monolayer (Slotte, 1995). This finding is not really surprising since the three double bonds in the B- and C-rings of dehydroergosterol and cholestatrienol will shift the ring geometry in this part of the molecule, compared to cholesterol which only has a single double bond in the B-ring (Child and Kuksis, 1983; Slotte, 1992b). Our monolayer desorption method clearly showed that the fluorescent sterols displayed desorption rates that were markedly faster than the rate measured for cholesterol. This was true both for pure sterol monolayers and for mixed monolayers containing POPC as the matrix phospholipid. The rate of desorption of amphiphiles from a monolayer depends to varying degrees on the hydrophobicity of the molecule (Kan et al., 1992), on intermolecular interactions in the membrane (Clejan and Bittman, 1984a; Fugler et al., 1985; Bhuvaneswaran and Mitropoulos, 1986; Thomas and Poznansky, 1988a; Kan et al., 1991), on the lateral surface pressure of the monolayer membrane (Ohvo and Slotte, 1996; Slotte and Illman, 1996), and on the type of acceptors used (Phillips et al., 1987). With our model system, desorption of sterols from the monolayer is likely to depend on the hydrophobicity of the sterols, on intermolecular interactions (sterol–sterol and sterol–phospholipid), and possibly to some extent on the relative affinity of the sterols to 2OHpβCyD. Since double bonds are polar in their nature, the

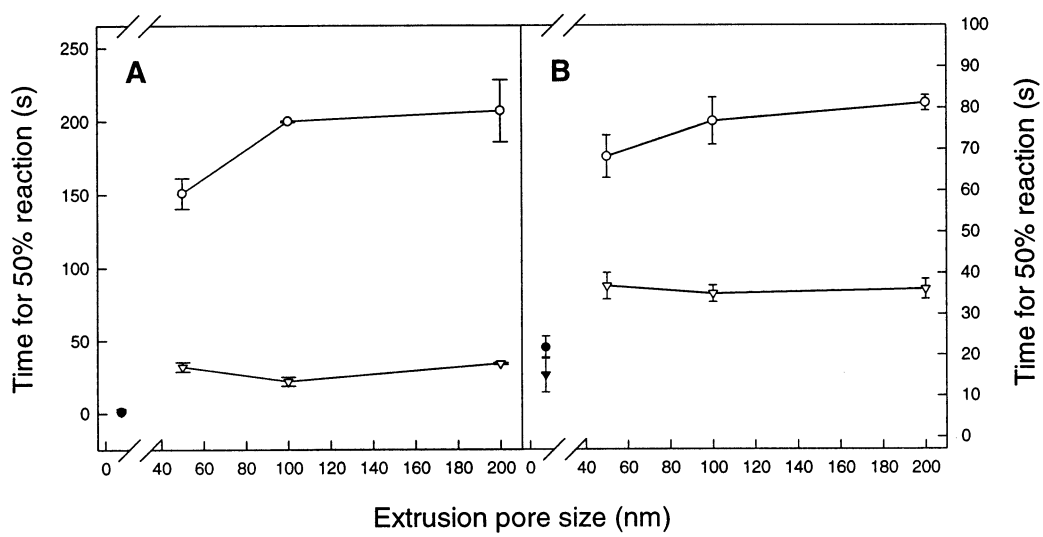


Fig. 5. The effect of vesicle size on dehydroergosterol and cholestatrienol desorption kinetics from POPC and *N*-O-SM vesicles. Vesicles of various sizes were prepared either by extrusion (hollow symbols) or by ethanol injection (filled symbols). 2.5 mM 2OHpβCyD was added and the time needed for 50% reaction was calculated. Panel A shows dehydroergosterol desorption from POPC (▽) and *N*-O-SM (○) vesicles. Panel B represents cholestatrienol desorption from POPC (▽) and *N*-O-SM (○) vesicles. Values are averages ± SEM from three experiments with each vesicle type.

fluorescent sterols are consequently more polar than cholesterol and are expected to desorb with a faster rate (Kan et al., 1992). Our results clearly showed that both dehydroergosterol and cholestatrienol desorbed faster from monolayer membranes than cholesterol. It is more difficult to assess the extent of intermolecular interactions in the monolayers. The fluorescent sterols gave more expanded isotherms than cholesterol, suggesting that their intermolecular interactions were weaker in pure sterol monolayers. This observation could therefore contribute to the faster desorption rates seen for fluorescent sterols in pure sterol monolayers. In mixed monolayers, the desorption rates of the fluorescent sterols differed even more from that of cholesterol, suggesting that neither dehydroergosterol nor cholestatrienol interacted favorably with POPC. This finding may be explained by the location of the polar double bonds, distal from the interface (in the B- and C-rings of the sterols), in the hydrophobic environment of the POPC monolayer (Franks, 1976; Worcester and Franks, 1976). Finally, it is possible that the three sterols used in this study have different affinities for 2OHpβCyD, and if so, this could affect the measured desorption rates. Affinity studies on sterol binding to 2OHpβCyD suggest that increased hydrophobicity results in higher affinity of sterol binding to 2OHpβCyD. The association constant for cholic acid (sodium salt) binding to 2OHpβCyD is $2.5 \cdot 10^3$ per M, whereas it is $4.4 \cdot 10^3$ per M for deoxycholic acid (sodium salt; Ollila and Slotte, unpublished observations), and $19 \cdot 10^3$ per M for cholesterol binding (Frijlink et al., 1991; Breslow and Zhang, 1996). These results are in agreement with several other affinity studies on the influence of bile acid hydrophobicity on sterol/CyD affinity (Tan and Lindenbaum, 1991; Comini et al., 1994; Yang and Breslow, 1997). Based on these observations, one can assume that the fluorescent sterols had lower affinities for 2OHpβCyD than cholesterol because of their more hydrophilic character, and consequently the faster desorption rates determined for the fluorescent sterols could not be explained by a difference in the CyD/sterol affinity parameter. The monolayer desorption data therefore suggest very strongly that both dehydroergosterol and cholestatrienol differed markedly from cholesterol in both their interfacial behavior and in their desorption properties.

Nemecz and co-workers (Nemecz et al., 1988; Nemecz and Schroeder, 1988) have measured kinetics of dehydroergosterol and cholestatrienol exchange between donor and acceptor vesicles. According to their results, the desorption rate of dehydroergosterol (35 mol% in POPC donor vesicles) was similar to that of [^{14}C]cholesterol (Nemecz et al., 1988; Nemecz and Schroeder, 1988), whereas they observed that the desorption of cholestatrienol was slightly slower than the rate measured for dehydroergosterol. We have not compared desorption rates of cholesterol and the fluorescent sterols in a vesicular assay system, but the rates clearly differed in a monolayer system. Using extruded small unilamellar vesicles of different sizes (50, 100 and 200 nm), we consistently observed that cholestatrienol desorbed with a markedly faster rate than dehydroergosterol, irrespective of the phospholipid matrix. The desorption properties of the two fluorescent sterols were qualitatively similar in our vesicle and monolayer systems.

The cyclodextrin concentration (2.5 mM) that we used in our experiments with vesicles was at a saturating level, so that the desorption of sterols from the bilayer membrane to cyclodextrin was rate limiting (see also Yancey et al., 1996; Rothblat et al., 1999). The total stoichiometry of available sterols and CyDs was very high (about 1–6300). Clearly much less CyD is needed to solubilize cholesterol, maybe 10 CyD molecules per a cholesterol molecule is enough (Gimpl et al., 1995). It is possible that the high molar excess of CyD needed in the desorption assays reflects some aggregation of CyDs, which may render them unavailable for the desorption process.

Several earlier studies have shown that cholesterol desorption from donor vesicles is highly retarded by sphingomyelin, as compared to phosphatidylcholine (Clejan and Bittman, 1984a; Fugler et al., 1985; Bhuvaneswaran and Mitropoulos, 1986; Thomas and Poznansky, 1988a; Kan et al., 1991). Our qualitative results showing that dehydroergosterol and cholestatrienol desorption was faster from POPC vesicles

as compared to *N*-O-SM vesicles agree well with the idea that sterols interact more favorably with sphingomyelins than they do with phosphatidylcholines (Ohvo and Slotte, 1996; Ohvo et al., 1997). The surface curvature of the donor vesicles also affects the rate of cholesterol exchange, with higher rates observed in small donor species than in large donor species (McLean and Phillips, 1984; Fugler et al., 1985; Thomas and Poznansky, 1988b). Looser packing of cholesterol molecules in highly curved small vesicles do not allow a parallel distribution of the lipids, which weakens the lipid intermolecular forces and leads to a higher desorption rate. Our desorption data for dehydroergosterol and cholestatrienol in *N*-O-SM vesicles showed faster desorption rates for smaller vesicles, but this was not evident with POPC vesicles. Desorption rates were very fast from ethanol-injected vesicles as compared to extruded vesicles (a $\sim$150-fold increase for *N*-O-SM vesicles). This large difference in sterol desorption rates can not be explained by differences in vesicle size, since the size of ethanol-injected vesicles is expected to be the same or a little less than for the smallest extruded vesicles. Ethanol (0.35 mM) has been shown to stimulate the rate of cholesterol desorption from donor membranes by 30–40% (Daniels and Goldstein, 1982). This effect cannot explain the large difference seen in sterol desorption rates between the ethanol-injected and extruded vesicles, since the ethanol concentration in our experiments was very low (0.28 vol% or 0.05 mM). It is not clear why ethanol-injected vesicles show such fast desorption kinetics. It is possible that their sterol desorption rates would slow down somewhat if they were allowed to age (to reach an equilibrium state). However, their use in sterol (or lipid) desorption studies is clearly not recommended based on our present results.

Despite findings that dehydroergosterol and cholestatrienol are nontoxic to cultured cells (Schroeder, 1981; Hale and Schroeder, 1982), and that dehydroergosterol can replace desmosterol in LM fibroblasts (Hale and Schroeder, 1982), this study has indicated that the fluorescent sterols are not similar to cholesterol in their membrane properties. It is well known that the presence or absence of double bonds in the sterol ring structure crucially affects for instance how sterols are taken up into membranes (Wharton and Green, 1982; Child and Kuksis, 1983). Changing the double bond position in a sterol molecule has also been shown to affect how efficiently the sterol analogue is esterified by acyl-coenzyme A: cholesterol acyltransferase (Tavani et al., 1982; Billheimer, 1985). In addition, lathosterol (Δ^7) and cholesterol (Δ^5) differ markedly in their capacity to regulate the activation of cytidylyl transferase in sterol-enriched cells (Leppimäki, Mattinen and Slotte, submitted). Furthermore, sterol/dipalmitoyl phosphatidylcholine interactions have been shown to be sensitive to the position of the double bond of the sterol molecule (Slotte, 1995). Taking these results into account, it is really not surprising that both dehydroergosterol and cholestatrienol differ markedly from cholesterol in their behavior in membranes.

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