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Acid-induced fusion of liposomes: studies with 2,3-seco-5α-cholestan-2,3-dioic acid

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The effect of 2,3-seco-5α-cholestan-2,3-dioic acid on the bilayer to hexagonal phase transition temperature of dielaidoylphosphatidylethanolamine is markedly dependent on pH. Above pH 6.56, the 2,3-seco-5α-cholestan-2,3-dioic acid raises the temperature of this transition, i.e., it stabilizes the bilayer phase. At pH 6.56 there is little effect of this sterol derivative on the bilayer to hexagonal phase transition temperature of dielaidoylphosphatidylethanolamine. However, below pH 6.56, the 2,3-seco-5α-cholestan-2,3-dioic acid markedly lowers the temperature of this transition. The promotion of hexagonal phase formation increases both with increasing mol fraction of this sterol derivative and with lower pH, particularly in the range between pH 6.56 and pH 5.0. Below about pH 6, 2,3-seco-5α-cholestan-2,3-dioic acid also induces vesicle fusion as measured both by lipid mixing as well as by mixing of aqueous contents. For these assays vesicles made of phosphatidylethanolamine (made from egg phosphatidylcholine) and extruded through 0.2 μm pore membranes were used. At higher concentrations or at lower pH the 2,3-seco-5α-cholestan-2,3-dioic acid induces some leakage of the contents of these vesicles. Nevertheless, with vesicles containing only 2 weight% sterol derivative, it was possible to demonstrate substantial mixing of aqueous contents of the vesicles over the pH range 3.5 to 5.5. Several of the properties of 2,3-seco-5α-cholestan-2,3-dioic acid indicate that this compound may be useful in sensitizing vesicles to acid-induced fusion for the purpose of endocytic drug delivery.

Introduction

There are at least two mechanisms by which membrane-entrapped material can be delivered to the cytoplasm of a cell. One is by fusion of a liposome or encapsulated virus with the plasma membrane of the recipient cell and the other is by endocytosis of the intact particle, followed by acid-induced fusion with an endosomal membrane. There is interest in using these mechanisms to introduce into cells, polar materials to which the cell surface membrane is impermeable. Negatively charged liposomes are internalized by mam-

Abbreviations: SCD, 2,3-seco-5α-cholestan-2,3-dioic acid; DEPE, dielaidoylphosphatidylethanolamine; PE, phosphatidylethanolamine made from egg phosphatidylcholine; DSC, differential scanning calorimetry; EDTA, ethylenediaminetetraacetic acid; TLC, thin-layer chromatography; ANTS, 1-amino-3,6,8-naphthalenetrisulfonic acid; DPX, *N,N'-p*-xylylene-bis(pyridinium bromide); NBD-PE, *N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)phosphatidylethanolamine; Rh-PE, *N*-(lissamine Rhodamine B sulfonyl)phosphatidylethanolamine; Tes, *N*-[tris(hydroxymethyl)methyl]-2-aminoethanesulfonic acid; Pipes, piperazine-*N,N'*-bis(2-ethanesulfonic acid).

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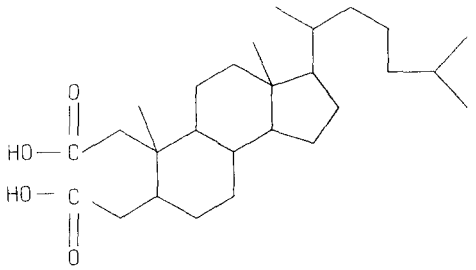

Fig. 1. 2,3-Seco-5α-cholestan-2,3-dioic acid (SCD).

malian cells primarily through the endocytic-lysosome fusion pathway [1]. The efficiency of delivery of drugs into the cytosol by this pathway is expected to be greater if the liposome undergoes acid-induced fusion so that the contents of the liposome will be released into the cytoplasm as a result of membrane fusion with endosomal membranes. There have been several recent demonstrations using oleic acid [2], cholesterol hemisuccinate [3], double-chain amphiphiles [4] an amphipathic peptide [5] or succinylated melittin [6] as agents to sensitize liposomes to acid-induced fusion. It has been demonstrated that pH-sensitive liposomes can deliver encapsulated materials to the cytosol of living cells [7,8]. In the present work, we demonstrate that a sterol derivative, 2,3-seco-5α-cholestan-2,3-dioic acid (SCD) is potent in sensitizing vesicles made of phosphatidylethanolamine to acid-induced fusion and that the pH dependence of its effects is related to its promotion of hexagonal phase structures. The structure of SCD is shown in Fig. 1.

Materials and Methods

Materials

Dielaidoylphosphatidylethanolamine (DEPE) and phosphatidylethanolamine prepared from egg yolk phosphatidylcholine by transphosphatidylation (PE) were purchased from Avanti Polar Lipids and SCD from Steraloids, Wilton, NH. These compounds were shown to be pure by TLC using *n*-hexane/ diethyl ether/ glacial acetic acid (70 : 30 : 1, v/v) as the developing solvent. 1-Aminonaphthalene-3,6,8-trisulfonic acid (ANTS) and *N,N'-p*-xylylene bis(pyridinium bromide) (DPX) were purchased from Molecular Probes. *N*-(7-nitrobenz-2-oxa-1,2-diazol-4-yl) phosphatidylethanolamine (NBD-PE) and *N*-(lissamine Rhodamine B sulfonyl)phosphatidylethanolamine (Rh-PE) were purchased from Avanti Polar Lipids.

Sample preparation

Phospholipid and SCD, as well as fluorescently labeled lipid where indicated, were dissolved together in a chloroform/ methanol (2 : 1, v/v) solution. The solvent was evaporated with a stream of dry nitrogen so as to deposit the lipid as a film on the walls of a glass test tube. Last traces of solvent were removed into a liquid nitrogen trap by placing the samples in a vacuum oven at 40 °C. The apparatus was maintained under a high vacuum for at least 90 min. The lipid film was then suspended in aqueous solution by warming the tube to about 45 °C and vortexing vigorously for about 30 s.

Differential scanning calorimetry (DSC)

Lipid suspension of DEPE with varying amounts of SCD was loaded into the sample cell and buffer into the reference cell of an MC-2 high-sensitivity scanning calorimeter (Microcal Co., Amherst, MA). A scan of 39 K/h was employed. Second heating scans on the same sample were similar to the first scan. The transition enthalpy of DEPE was 5.5 ± 1 kcal/mol for the gel to liquid crystalline transition and was 480 ± 100 cal/mol for the bilayer to hexagonal phase transition. These values are independent of the mol fraction of SCD.

^{31}P-NMR

NMR spectra were recorded on a Bruker AM-500 spectrometer operating at 202.45 MHz. The probe temperature was maintained to within ± 1 C° by a Bruker B-VT 1000 variable temperature unit. Temperatures were checked by thermocouple measurements. A 10 nm broad band probe was used. A 40 kHz sweep width was employed with an acquisition time of 0.28 s (16 K data points). The 90° pulse width was 16.6 μs with composite pulse proton decoupling. FID's were processed using exponential multiplication (line broadening 15.0 Hz). Chemical shifts are expressed in ppm from an external reference of 85% phosphoric acid in ^2H_2O.

Vesicle leakage

Leakage was measured by entrapping fluorescent ANTS together with the quencher DPX in liposomes. As the entrapped material in the liposomes leak out, the DPX quenching of ANTS is lost [9]. Two μmoles of PE with or without added SCD were suspended in 3 ml of a mixture of 12.5 mM ANTS and 45 mM DPX in 10 mM Tes, 150 mM NaCl, 0.1 mM EDTA at pH 7.4. Suspension of the lipid was aided by a brief sonication of a few seconds to remove the lipid from the walls of the test tube. The lipid suspension was then extruded through 0.2 μm pore diameter Unipore (Bio-Rad) polycarbonate membrane filters to form oligolamellar vesicles [2,10]. The vesicles were then passed through a 1.5×18 cm column of Sephadex G-75 to remove non-entrapped material. The column was eluted with 150 mM NaCl, 10 mM Tes, 0.1 mM EDTA (pH 7.4) and the ANTS/DPX-containing vesicles were collected in the void volume.

The vesicles were diluted 1:4 with 150 mM NaCl, 10 mM Tes, 0.1 mM EDTA in a 1 cm^2 cuvette. The appearance of fluorescence was measured as a function of time using a Perkin-Elmer MPF-44 spectrofluorimeter. The excitation monochrometer was set at 384 nm and emission at 512 nm was recorded using slit widths of 4 nm. The temperature of the cuvette in this and subsequent fluorescence experiments was maintained at 25 °C. To measure the effect of pH on the rate of leakage, the solution in the cuvette was acidified by adding a small volume of 1 M citric acid or alternatively the lipid film was originally suspended in a more acidic buffer and eluted from the G-75 column at a lower pH. The final pH of the solution in the fluorimeter cuvette was always measured. The amount of leakage was calculated using the fluorescence at zero time as zero leakage and measuring 100% leakage by rupturing the vesicles in 0.5% Triton X-100 followed by 1 minute of sonication.

Lipid mixing

Lipid mixing was used as a criterion of membrane fusion. Dilution of NBD-PE and Rh-PE from vesicles in which they were undergoing energy transfer to unlabelled vesicles was monitored [11]. Vesicles containing PE and 2% (w/w) SCD (mol fraction 0.036) either with or without added NBD-PE and Rh-PE at 1 mol% each, were prepared by extrusion through polycarbonate filters as described above for vesicle leakage.

The extruded vesicles were diluted into buffer in a fluorimeter cuvette. The final concentration of labeled vesicles was about 25 μM and of unlabeled vesicles, 230 μM in the Tes buffer at pH 7.4. The fluorescence emission spectrum was measured at various time intervals using an excitation wavelength of 450 nm and 4 nm slit widths. To measure lipid mixing at lower pH the solution in the cuvette was acidified with small volumes of 1 M citric acid and the spectra measured at various time intervals. The ratio of the intensities of the emission peaks at 530 and 591 nm was measured. This ratio was converted into C_0/C, where C_0 is the initial fluorophore in the membrane and C is the fluorophore concentration in the membrane after an interval of time. For this purpose a standard curve of C_0/C vs. the ratio of fluorescence intensity at 530 and 591 nm was established by mixing the labeled lipid with varying amounts of unlabeled lipid and SCD in chloroform/methanol. These lipid films were then suspended in buffer and extruded through polycarbonate filters. Standard curves were constructed for several different pH values both with and without SCD. The values of C_0/C were used to calculate the % lipid mixing according to the formula:

$$\% \text{ Fusion} = \frac{(C_0/C) - 1}{D - 1} \cdot 100$$

where D is the maximum possible dilution (10 in the present case). Lipid exchange and partial fusion without mixing of aqueous contents are also measured as fusion by this assay.

Mixing of aqueous contents

A more stringent test of vesicle fusion is the mixing of the aqueous contents of two vesicles without exposure to the extravesicular fluid. A fluorescence assay to monitor this process and suitable for measurements at acidic pH has been designed by Ellens et al. [12]. Vesicles were prepared as described for vesicle leakage except that 12.5 mM ANTS and 45 mM DPX were each entrapped in two separate populations of vesicles at pH 7.4. After gel filtration, 300 μl of each of

the two vesicle populations was mixed with 2.0 ml of the Tes buffer. The time dependence of the fluorescence at 512 nm was measured before and after acidification with small aliquots of 1 M citric acid.

Results

Phase transitions

It has been demonstrated that the bilayer to hexagonal phase transition temperature is sensitive to the presence of small amounts of certain additives [13]. The lipid DEPE was used as a model to study the variation of the bilayer to hexagonal transition temperature under different conditions since this lipid exhibits a sharp endothermic transition with little hystesis [14]. The effect of SCD on the bilayer to hexagonal phase transition temperature of DEPE was markedly dependent on pH (Fig. 2). Between pH 5 and 8 the bilayer to hexagonal phase transition temperature of pure DEPE shows little variation. The effect of adding 0.060 mol fraction of SCD was to markedly lower this phase transition temperature at acidic pH but to raise it at alkaline pH (Fig. 2). Above pH 8 the bilayer to hexagonal phase transition could not be detected even for the pure lipid. The extent of the shift in the bilayer to hexagonal phase transition temperature is proportional to the mol fraction of SCD added, at least at low mol fractions of SCD (Fig. 3). Even though this additive has a marked effect on the transition temperature, a pH can be chosen, i.e., pH 6.56, where the effect of this additive is negligible (Fig. 3).

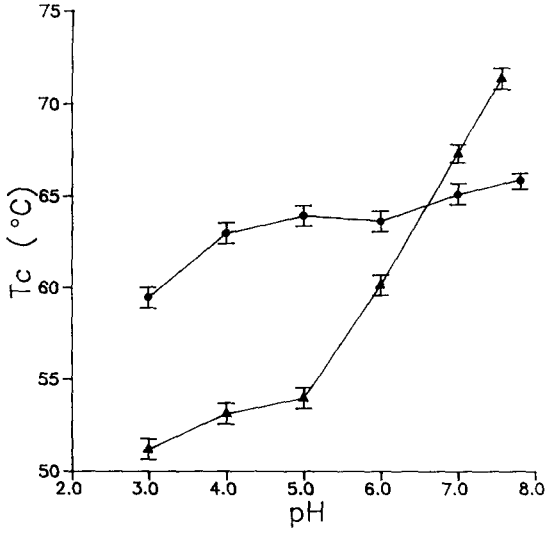

Fig. 2. pH dependence of the bilayer to hexagonal phase transition temperature of DEPE (●) and DEPE admixed with 0.060 mol fraction SCD (▲). Data from DSC heating curves of lipid suspensions in 20 mM citrate, 150 mM NaCl, adjusted to the desired pH with NaOH.

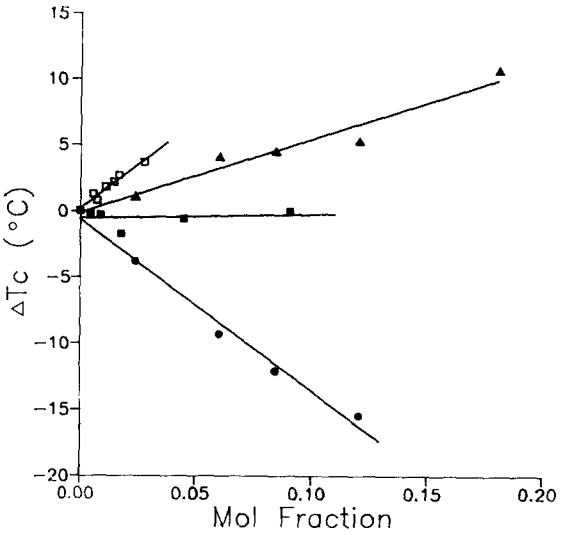

Fig. 3. Effect of SCD and pH on the bilayer to hexagonal phase transition temperature of DEPE. ΔT_c is the difference between the transition temperature of DEPE-SCD mixtures and that for pure DEPE at pH 4.0 (●), pH 6.56 (■), pH 7.0 (▲) and pH 8.0 (□). Buffers used are 20 mM sodium citrate, 150 mM NaCl for pH 4.0 and 6.56 and 50 mM Hepes, 150 mM NaCl, 1 mM EDTA for pH 7.0 and 8.0.

The effect of SCD on the gel to liquid crystalline transition was more complex. At higher mol fractions of SCD this transition clearly split into two peaks. The transition was analyzed by deconvolution into two van't Hoff components. As the mol fraction of SCD increases, the size of the low temperature peak increases (Fig. 4) although the total enthalpy of the transition (sum of high and low temperature peaks) does not change. The transition temperature of the lower melting component at a particular pH is invariant with the amount of SCD added but the higher temperature transition gradually decreases with added SCD (Fig. 5). The multiple peaks may result from a morphological heterogeneity of the sample since the gel to liquid crystalline transition of DEPE is apparently very sensitive to bilayer curvature [15].

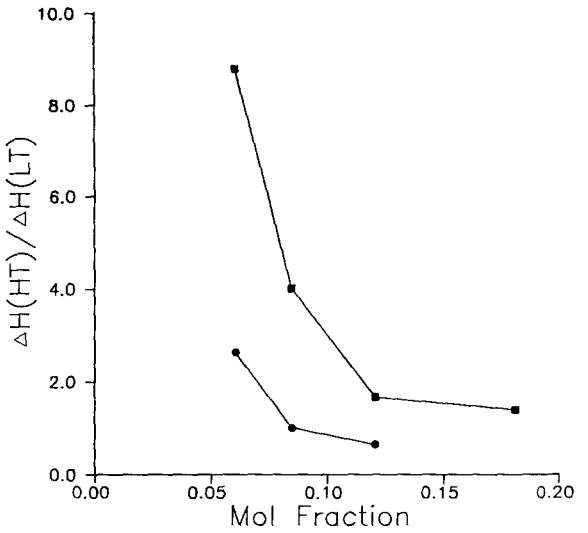

Fig. 4. Ratio of the enthalpy of the higher melting component [ΔH(HT)] to that of the lower melting component [ΔH(LT)] for the gel to liquid crystalline transition of DEPE as a function of the mol fraction of SCD added. Buffers for pH 4.0 (●) and for pH 7.0 (■) as for Fig. 3.

It is possible that SCD causes smaller vesicular structures to form. The effect of pH on the slope of the high temperature component of the gel to liquid crystalline transition is markedly different

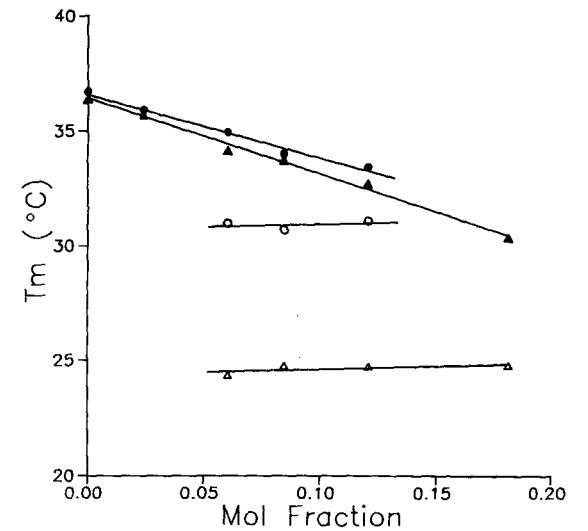

Fig. 5. The gel to liquid crystalline transition of DEPE/SCD mixtures resolved into two van't Hoff components as a function of the mol fraction of SCD added. Buffers for pH 4.0 (● and ○) and pH 7.0 (▲ and △) as for Fig. 3.

TABLE I

EFFECT OF pH ON THE SENSITIVITY OF THE TRANSITION TEMPERATURE TO SCD CONCENTRATION

Slopes are obtained from plots of mol fraction of SCD vs transition temperature using DSC curves of DEPE/SCD mixtures. A negative slope indicates a lowering of the transition temperature with increasing mol fractions of SCD.

pH	Slope	
	$L_\beta \rightarrow L_\alpha$ (high-temperature component)	$L_\alpha \rightarrow H_{II}$
4.0	−28±3	−129±12
6.56	−30±2	3± 3
7.0	−33±2	56± 8
8.0	−36±1	132± 3

from its effect on the slope of the transition to the hexagonal phase (Table I).

^{31}P-NMR

The bilayer to hexagonal phase transition was also monitored by ^{31}P-NMR (Fig. 6). Two identical films were prepared containing DEPE admixed with 0.060 mol fraction SCD. Each film was suspended either in buffer at pH 5 or pH 8. The difference in bilayer stability is dramatically demonstrated by ^{31}P-NMR. At pH 5, the conversion to the hexagonal phase occurs below 50 °C while at pH 8, it occurs at about 70 °C. At pH 8, there is also a very minor contribution from an isotropic signal above 70 °C. The ^{31}P spectra confirm the morphological assignments of the phases.

Vesicle leakage

In order to measure vesicle fusion by mixing of aqueous contents, conditions had to be chosen in which the membrane maintained a permeability barrier. Vesicles prepared at pH 7.4 and containing 0 or 1.9 weight% SCD exhibited only 5–10% of the maximal fluorescence determined by Triton-bursting of the vesicles. Vesicles at pH 7.4 with 5 weight% SCD showed 20% of the maximal fluorescence. None of these three vesicle preparations showed any increase in fluorescence as a function of time over 10–15 min at pH 7.4. This fluorescence is presumably is a result of vesicle leakage between the time the vesicles are placed

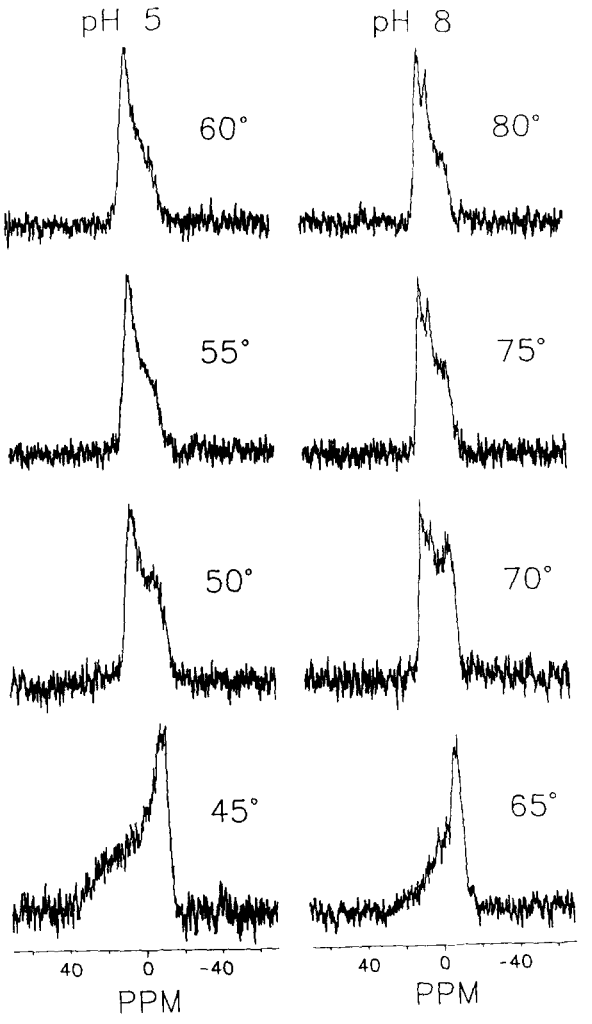

Fig. 6. ^{31}P-NMR spectra as a function of temperature for DEPE admixed with 0.060 mol fraction SCD. The chemical shift is given with external 85% phosphoric acid in $^{2}H_2O$ as reference. Lipid suspended in 20 mM sodium citrate, 150 mM NaCl (pH 5.0) (left) or 50 mM Hepes, 150 mM NaCl, 1 mM EDTA (pH 8) (right).

on the column and the time they are examined in the fluorimeter. Upon acidification with citrate there is an additional rapid increase in fluorescence signal over one minute or less, followed by undetectable leakage over the next 10 min. This rapid burst of fluorescence above the value at pH 7.4 is expressed as the percentage of maximal fluorescence determined by the addition of Triton minus the fluorescence at pH 7.4 (Table II).

Leakage was also measured from vesicles prepared in buffers of different pH values and eluted from the G-75 column at the same pH. The kinetics of ANTS release by this protocol was very different from that described above in which the external fluid around the vesicles was abruptly lowered from pH 7.4. With vesicles containing 2 weight% SCD there was no leakage at pH 7.4, but at pH 5 or 3.5 there was a linear increase in fluorescence with time amounting to 0.5% release per min.

TABLE II

VESICLE LYSIS RESULTING FROM ACIDIFICATION

Weight% SCD	pH	% vesicles burst
0 [a]	5	1.5
	3.3	4.0
2	5.3	0
	4.6	34
	3.8	32
5	6	2
	5.1	48
	3.3	64

[a] Pure PE.

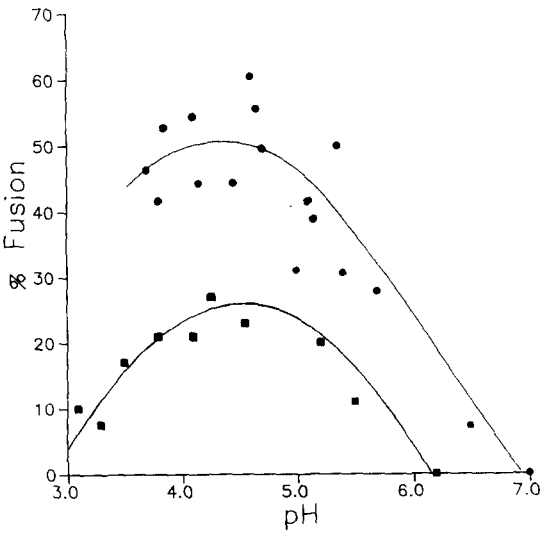

Fig. 7. Vesicle fusion as a function of pH as measured by lipid mixing (●) or by mixing of aqueous contents (■). Vesicles composed of PE with 2 weight% SCD were prepared at pH 7.4 and acidified to the indicated pH with citrate. See Materials and Methods for experimental details.

Vesicle fusion

Vesicle fusion was measured by lipid mixing for pure PE vesicles and for PE with 2 weight% SCD. Lipid mixing was negligible at all pH values between 3.5 and 7.4 for the pure PE vesicles. For the vesicles with 2% SCD, mixing was negligible at pH 7.4 but increased rapidly upon acidification of the vesicle suspension. The apparent rate of fusion was rapid during the first several minutes after acidification followed by a 10–100-fold slower rate over the next 30 minutes. The results of the lipid mixing experiment are summarized by plotting the % fusion measured after 2 minutes as a function of the final pH after acidification (Fig. 7).

Pure PE vesicles also did not exhibit any acid-induced fusion as measured by the mixing of aqueous contents. Vesicles with PE plus 2 weight% SCD showed no mixing of aqueous contents over 10 minutes at pH 7.4. However, upon acidification the SCD-containing vesicles did exhibit mixing of aqueous contents. The kinetics of the mixing of aqueous contents shows a rapid initial phase followed at lower pH by a gradual loss of quenching, probably due to leakage (Fig. 8). The loss of fluorescence quenching is much slower at pH 5.2 (Fig. 8) where the vesicles are less leaky (Table II). To summarize results such as those presented in Fig. 8, the maximum observed fusion (i.e. the maximum % quenching) is presented as a function of pH (Fig. 7). The apparent decrease in fusion below pH 4 probably results from an increased rate of leakage rather than a decreased rate of vesicle fusion.

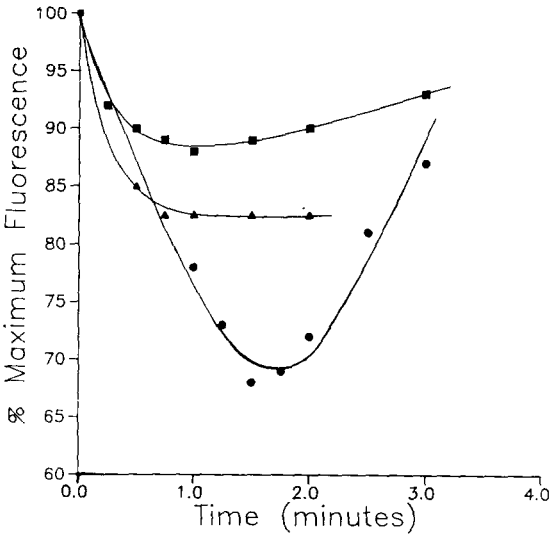

Fig. 8. Examples of the time course for mixing of the contents of ANTS-containing and DPX-containing vesicles. Vesicles composed of PE with 2 weight% SCD were prepared at pH 7.4 with either ANTS or DPX entrapped and then acidified to pH 5.2 (▲), pH 4.25 (●) or pH 3.3 (■) with citrate. At pH 7.4 there was no change in fluorescence intensity with time (not shown).

Discussion

The bilayer to hexagonal phase transition temperature is very sensitive to the presence of certain additives in the membrane [13]. The effect of SCD on this transition is highly dependent on pH. At higher pH values where the carboxyl groups of SCD are ionized this sterol becomes more polar and stabilizes the bilayer phase. At acidic pH, the uncharged SCD has less effect in increasing the polarity and hydration of the membrane surface and it acts more like a hydrocarbon to induce hexagonal phase formation.

The general features of the effect of pH on the bilayer to hexagonal phase transition temperature are also observed in vesicle fusion. Bilayer destabilization increases rapidly between pH 6.6 and pH 5 (Fig. 2). This is about the same pH range in which the fusion efficiency greatly increases (Fig. 7). This does not imply that the hexagonal phase is necessarily required for fusion. The PE used for the fusion studies does not form a hexagonal phase at 25 °C. In addition, Leventis et al. [4] recently reported that one of the double-chain amphiphiles (compound 1d) that promotes acid-induced fusion has little effect on the bilayer to hexagonal phase transition temperature of DEPE. However, in general substances which promote hexagonal phase formation also promote fusion, probably by making the bilayer surface more hydrophobic. In the case of SCD, the protonation of the carboxyl groups will increase the hydrophobicity of the membrane surface and cause vesicle fusion.

Although SCD causes some vesicle leakage, it is small enough not to prevent our observing mixing of aqueous contents of fusing vesicles (Fig. 8). The initial rate of leakage is much greater when the vesicles are acidified subsequent to gel filtration

compared to experiments in which the vesicles are prepared at lower pH. In the latter case, the vesicles with entrapped material probably aggregate and fuse during preparation and gel filtration. In this state they may be more resistant to leakage than sonicated vesicles prepared at neutral pH. However, upon acidification of the vesicles at neutral pH there is initially a pH gradient between the internal contents at pH 7.4 and the extravesicular buffer at lower pH. Since SCD will promote opposite changes in the intrinsic curvature of the bilayer [16] at neutral and acidic pH, this may be particularly perturbing to the maintenance of a permeability barrier. Since a substantial fraction of the vesicles burst instantaneously when acidified (Table II), only a fraction of the initial population of vesicles still contain entrapped ANTS. Thus the fraction of remaining vesicles which can undergo mixing of aqueous contents below pH 5 is even higher than that calculated for the total vesicles population (Fig. 7). This vesicle bursting is probably the major factor causing the % fusion calculated from the mixing of aqueous contents to be lower than that calculated from lipid mixing (Fig. 7). In addition, lipid mixing also counts lipid exchange and partially fused vesicles as fusion while mixing of aqueous contents does not.

Thus, SCD has a number of features which make it potentially very useful for sensitizing liposomes to acid-induced fusion for drug delivery. The pH below which SCD begins to destabilize bilayers, i.e., pH 6.56, is high enough to be suitable for fusion to endocytic vesicles. In addition, SCD does not induce hexagonal phase formation at pH 7.4 and is not fusogenic at this pH. It may even have some stabilizing effects on liposomes at neutral pH. Finally, the introduction of only a small percent of SCD into a liposome is sufficient to sensitize the liposome to fusion at low pH.

Acknowledgements

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