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Interaction of Filipin with Cholesterol in Vesicles of Saturated Phospholipids†

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ABSTRACT: Stopped-flow measurements were made of the initial rate of interaction of filipin with cholesterol in vesicles prepared from saturated phospholipids. The association process follows second-order kinetics (first order in each reactant) in vesicles prepared from mixtures of cholesterol and dimyristoyl-, dipalmitoyl-, or reduced egg phosphatidylcholine, or sphingomyelin. The initial rate increased with increasing temperature for association of filipin with vesicles prepared from these phospholipids, but decreased with increasing temperature for reaction with cholesterol-containing didecanoylphosphatidylcholine and dipalmitoylphosphatidylserine vesicles. The reaction order and magnitude of the initial rate at 30 °C were also markedly different for filipin association with cholesterol in vesicles prepared from the latter phospholipids compared with the other saturated phospholipids examined. The second-order rate constant for filipin–cholesterol complexation in dimyristoylphosphatidylcholine vesicles was essentially invariant when the mole percent of cholesterol was varied from 16 to 32%; a small increase in the second-order rate constant was noted at higher mole percent of cholesterol. No discontinuity in the Arrhenius activation energy was found at the lipid phase transition temperature. Incorporation of cholesterol oleate into dimyristoylphosphatidylcholine bilayers decreased the initial rate of filipin–cholesterol complexation without altering the reaction order. The differences in the kinetics of filipin and amphotericin B association with vesicles are discussed.

An impressive quantity of data has been gathered in recent years about the mode of action of the polyene antibiotics (cf. review by Norman et al., 1976). Some polyene antibiotics are used topically and parenterally to treat fungal infections. Recently, their membrane-perturbing activity has been used to probe various aspects of membrane structure and function. For example, amphotericin B and nystatin have been used to alter the membrane permeability of skeletal muscle fibers (Leung and Eisenberg, 1973), to modify the ion gradients of erythrocytes (Cass and Dalmark, 1973) and lens epithelium (Bentley and Candia, 1975), and to permit other antibiotics, which are normally impermeant to intact fungi, to inhibit cell synthetic mechanisms (e.g., Kobayashi et al., 1974; Beggs et al., 1976; cf. Huppert et al., 1976). Filipin has been used to establish the osmotic permeability properties of lipid-enveloped viral membranes (Bittman et al., 1976), the transport mechanism for entry of phosphate and succinate into spermatozoa (Babcock et al., 1975), the role of Ca^{2+} in flagellar activity of spermatozoa (Babcock et al., 1976), the mechanisms of vitamin D mediated intestinal uptake of Ca^{2+} (Wong and Norman, 1975), the relationship between hormone–receptor binding and adenylate cyclase activation in beef thyroid membranes (Moore and Wolff, 1974) and pigeon erythrocyte membranes (Puchwein et al., 1974), and the distribution of unesterified cholesterol between the outer and inner halves of the lipid bilayer of mycoplasma membranes (Bittman and Rottem, 1976).

The stoichiometry of filipin–cholesterol complexation in aqueous dispersions, phospholipid vesicles, and *Acholeplasma laidlawii* membranes (see references in Norman et al., 1976) and in vesicular stomatitis virion membranes (Majuk et al., 1977) has been found to be approximately 1 mol of cholesterol bound per mol of filipin. Several aspects of the mechanism of action of filipin are still not well defined. In order to obtain more information about the factors involved in complexation of this membrane-selective tool with cholesterol, we investigated the kinetics of filipin association with cholesterol in vesicles derived from saturated phospholipids.

Experimental Section

Materials. Lipids were purchased from the following sources: L-α-dimyristoylphosphatidylcholine (PC),[1] L-α-reduced egg PC, and L-α-didecanoyl-PC from Supelco, Inc.; L-α-dipalmitoyl-PC from General Biochemicals; L-α-distearoyl-PC from Calbiochemical Corp.; L-α-dipalmitoylphosphatidylserine (PS) from Serdary Research Lab.;

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[1] Abbreviations used: PC, phosphatidylcholine; PS, phosphatidylserine; ANS, 8-anilino-1-naphthalenesulfonic acid ammonium salt.

bovine-brain sphingomyelin from Lipid Products (Nutfield Ridge, England); cholesterol, cholesterol oleate, and dicetyl phosphoric acid from Sigma Chemical Co. Cholesterol was recrystallized from acetone. The lipids were found to be chromatographically pure on thin-layer analysis using silica gel G plates. Stock solutions of lipids in chloroform were stored at −20 °C under nitrogen. 8-Anilino-1-naphthalenesulfonic acid ammonium salt (ANS) was obtained from Sigma Chemical Co. Sodium thiocyanate and sodium salicylate were purchased from J. T. Baker Chemical Co. Other compounds were of reagent grade and were used without further purification.

Filipin complex (lot no. 8393 DEG-11-8, Upjohn Co., Kalamazoo, Mich.) was purified by filtration of the filipin complex from a concentrated slurry in chloroform as described before (Whitfield et al., 1955). The purity of the preparation was ~80% as determined by comparison of molar absorbancy of the resulting material with that of a pure sample (5.0×10^4 M^{-1} cm^{-1} at 338 nm). Stock solutions of the filipin were prepared in dimethylformamide and aliquots were added to 0.06 M KCl solution. The final concentration of dimethylformamide was 0.3% (v/v).

Preparation of Vesicles. Vesicles were prepared as reported previously (Bittman et al., 1974). The vesicles were sonicated to constant absorbance at 600 nm. For cholesterol-containing phospholipid vesicles, the absorbance was 0.08 ± 0.02; for vesicles containing cholesterol oleate and cholesterol, the absorbance was 0.19. All vesicles contained 4 mol % dicetyl phosphoric acid. Vesicles were prepared in 0.06 M KCl solution, and after sonication dimethylformamide was added to a concentration of 0.3% (v/v).

Characterization of Vesicles. The sonicated vesicles were found by negative-staining electron microscopy to be predominantly unilamellar, as described previously (Bittman et al., 1974). Vesicle diameters were estimated by measuring a total of 600 vesicles in at least five areas of the grid for each preparation. About 80% of the dimyristoyl-PC–cholesterol (3:1 mol ratio) vesicles obtained by sonication for 2 min had an average diameter of 300 ± 80 Å. Vesicle size decreased with increasing sonication time. After 10 min of sonication, the percentages of dimyristoyl-PC–cholesterol (3:1 mol ratio) vesicles present with the following diameters were: 50%, 250 ± 20 Å; 25%, 350 ± 20 Å; 20%, <230 Å; 5%, >370 Å. Vesicles that were eluted from a Sepharose 4B column (2.5 × 40 cm) in the ascending portion of the peak had the following distribution of diameters: 50%, 250 ± 20 Å; 40%, 350 ± 20 Å; 10%, >370 Å. All of the vesicles that eluted in the descending portion of the peak had diameters of 230 Å or less.

To further characterize the vesicles prepared from different phospholipids, the average vesicle size was estimated by measurement of the trapped water volume. Vesicles were prepared by dispersing the lipids (phospholipid, cholesterol, and dicetyl phosphoric acid in a molar ratio of 3:1:0.16) in 0.50 M $K_3Fe(CN)_6$ solution and sonicating for 2 min. Vesicles containing $K_3Fe(CN)_6$ were separated from the free ferricyanide on a Sephadex G-50 (fine) column (1.0 × 25 cm). The absorbance at 420 nm was measured after the vesicles were treated with 1-propanol, and the trapped volume was determined as described by Newman and Huang (1975). Phospholipid concentrations were measured by digesting 0.2-mL aliquots of vesicle suspensions with 0.2 mL of concentrated H_2SO_4 at 240 °C for 15 min, followed by redigestion for 5 min after the addition of a few drops of 30% H_2O_2. After the excess acid was neutralized with 10% NaOH, inorganic phosphate was determined as described by Taussky and Shorr (1953). For each preparation of vesicles, volume and phospholipid measurements were made in triplicate. The volumes of trapped water (L per mol of lipid) were as follows. (The number of determinations of different vesicle preparations is shown in parentheses.) Dimyristoyl-PC:cholesterol 5:1 molar ratio, 0.42 ± 0.03 (3); 3:1, 0.19 ± 0.03 (4); 2:1, 0.56 ± 0.05 (2); 1:1, 0.78 ± 0.06 (3). Dimyristoyl-PC:cholesterol:cholesterol oleate, 3:1:0.46 molar ratio, 0.40 ± 0.08 (2). The following volumes were determined using vesicles containing 3:1 molar ratios of phospholipid to cholesterol: didecanoyl-PC, 0.30 ± 0.04 (2); dipalmitoyl-PC, 0.55 ± 0.02 (4); distearoyl-PC, 0.86 ± 0.01 (2); reduced egg PC, 0.52 ± 0.03 (2); sphingomyelin, 0.66 (2); dipalmitoyl-PS, 0.53 ± 0.12 (2).

Kinetic Measurements. Rapid mixing of filipin solutions with vesicles was performed in a stopped-flow apparatus (Durrum Instrument Corp., Palo Alto, Calif.). The changes in transmittance at 360 nm were monitored on a Tektronix storage oscilloscope equipped with a Polaroid camera. A slit width of 1 mm was used. The photomultiplier supply voltage was adjusted so that a signal of 1600 mV on the oscilloscope corresponded to 100% transmission. The initial transmittance (T_i) of the reaction was measured, and the initial absorbance was calculated from $A_1 = -\log (T_i/1600)$; in the same way the absorbance at a later time was calculated. The initial rate of absorbance change per s, dA/dt, was converted to the initial rate of disappearance of free filipin, $d[F]/dt$, by dividing dA/dt by the pathlength of the cell in the stopped-flow spectrophotometer (2.0 cm) and by the molar absorbancy of filipin at 360 nm (4.5×10^4 M^{-1} cm^{-1}). Aqueous filipin solutions were prepared freshly every day. Solutions having A_{323}/A_{358} less than 0.9 were used. The experiments were carried out at 30.0 ± 0.2 °C unless otherwise stated.

Measurements of the Crystalline–Liquid Crystalline Phase Transition. The phase transition temperatures of reduced egg PC and reduced egg PC–cholesterol dispersion were determined in the presence of about 2×10^{-6} M ANS (Sackmann and Träuble, 1972) at a total lipid concentration of 0.38 mM. Changes in fluorescence intensity as a function of temperature were recorded with a Hitachi Perkin-Elmer Model MPF-2A spectrofluorometer. The excitation and emission wavelengths were 380 and 480 nm, respectively. The temperature change at which the largest change in fluorescence intensity occurred was taken as the phase transition temperature. The phase transition temperature of reduced egg PC is 45–46 °C and that of reduced egg PC–cholesterol (molar ratio of 3:1) is 43–44 °C.

Results

The rate of reaction of filipin with cholesterol-containing vesicles was examined by stopped-flow measurements of initial rates rather than of the full reaction profiles because reactions occurring after the initial association showed poor reproducibility. This irreproducibility in rate at long reaction times (20 s) may be related to the well-known ability of filipin to disrupt the lipid organization of cholesterol-containing membranes. Therefore, initial rates were determined from the slopes of transmittance changes generally in the time range of 0 to 50 ms, and in no cases longer than 150 ms after mixing of the reactants.

Interaction of Filipin with Externally Oriented Cholesterol in Vesicles. The initial rates measured here appear to represent filipin binding to cholesterol on the outer surface of the bilayer. We have previously reported that the initial rate of filipin–cholesterol association is sensitive to the state of lipid packing in vesicles (Bittman et al., 1974). The rate is slower when vesicles that have previously been subjected to osmotic shrinking are mixed in the stopped-flow apparatus with filipin

TABLE I: Effect of Cholesterol Concentration and Mole % Cholesterol in the Bilayer on Initial Rate of Association and Second-Order Rate Constant of the Reaction of Filipin with Cholesterol-Containing Dimyristoyl-PC Vesicles.[a]

Concn of cholesterol (μM)	d[F]/dt (μM s^{-1})			
	16 mol %	24 mol %	32 mol %	48 mol %
10	1.1		1.7	2.1
20	2.3	2.4	2.9	3.9
30	3.8	4.2		
40	5.1	5.8	5.3	6.7
60	7.0	7.7	8.2	9.1
$10^{-4}k_2$ (L mol^{-1} s^{-1})[b]	1.7 ± 0.1	1.9 ± 0.1	2.1 ± 0.2	2.6 ± 0.3

[a] The filipin concentration was 7.0 μM. [b] k_2 is the average second-order rate constant.

TABLE II: Effect of Filipin Concentration and Mole % Cholesterol in the Bilayer on Initial Rate of Association and Second-Order Rate Constant of the Reaction of Filipin with Cholesterol-Containing Dimyristoyl-PC Vesicles.[a]

Concn of filipin (μM)	d[F]/dt (μM s^{-1})			
	16 mol %	24 mol %	32 mol %	48 mol %
3.0	3.4	3.2	3.7	3.6
5.0	5.3	5.6	5.8	5.9
7.0	7.0	7.7	8.3	9.0
10.6	9.4		10.7	17.5
$10^{-4}k_2$ (L mol^{-1} s^{-1})[b]	1.7 ± 0.2	1.8 ± 0.1	1.9 ± 0.2	2.2 ± 0.3

[a] The cholesterol concentration was 60 μM. [b] k_2 is the average second-order rate constant.

than when vesicles that have been diluted with isotonic medium or subjected to osmotic swelling conditions are used. When vesicles suspended in 0.06 M KCl are simultaneously subjected to osmotic shrinking conditions and to filipin (dissolved in 0.24 M KCl) in the stopped-flow apparatus, the initial rate of filipin binding (8.6 μM s^{-1}) is very similar to that measured in 0.06 M KCl solution, where no change in osmolarity is applied (9.2 μM s^{-1}). (Vesicles were prepared with a dimyristoyl-PC: cholesterol molar ratio of 2:1. The total lipid concentration was 0.19 mM, and the filipin concentration was 8.1 μM.) The coincidence of rates suggests that osmotic water movement across the bilayer did not occur within the very short time range used to monitor the initial rate of filipin–cholesterol association (0 to 30 ms); therefore, it is likely that no significant penetration of filipin (a much larger molecule than water) into the inner bilayer surface occurred. Indeed, independent stopped-flow measurements of the initial rate of water permeation across the outermost bilayer of liposomes were made at longer time ranges (150 ms to 2 s) (Bittman and Blau, 1972).

Initial Rates as a Function of Filipin and Cholesterol Concentrations. The initial rate of association of filipin with cholesterol-containing vesicles is a function of the concentrations of cholesterol (Table I) and filipin (Table II) and of the mole percent of cholesterol in the bilayer (Tables I and II). At a fixed mole percent of cholesterol, the initial rate is linearly dependent on the absolute concentration of cholesterol in the concentration range of 10 to 60 μM. The reaction of filipin with cholesterol in dimyristoyl-PC vesicles was found to obey second-order kinetics (see below), and the second-order rate constant is invariant to changes in the mole percent of cholesterol up to at least 32% or in PC to cholesterol mole ratios up to at least 2:1; the small increase in k_2 at 48 mol % cholesterol (Tables I and II) is within the error limits of the values obtained at lower sterol composition. It appears unlikely that the slight increase at a 1:1 molar ratio of dimyristoyl-PC to cholesterol arises from the increase in vesicle size that accompanies incorporation of cholesterol above 30 mol % (see data in Experimental Section; Johnson, 1973; Gent and Prestegard, 1974; Newman and Huang, 1975; de Kruijff et al., 1976); data presented in Table III suggest that no correlation exists between k_2 and size when the molecular composition of the vesicles is altered.

The initial rates of filipin binding to cholesterol in vesicles prepared from various saturated phospholipids are plotted in Figure 1 as a function of sterol concentration. The dependence of the initial rates on filipin concentration is presented in Figure 2. The slopes of log–log plots of initial rate vs. cholesterol concentration or vs. filipin concentration give the reaction orders with respect to cholesterol and filipin. In vesicles prepared from dimyristoyl-PC, dimyristoyl-PC and cholesterol oleate, dipalmitoyl-PC, distearoyl-PC, reduced egg PC, and sphingomyelin, the order with respect to cholesterol is 1.00 ± 0.10; the order with respect to filipin is 1.00 ± 0.15. In vesicles prepared from didecanoyl-PC and dipalmitoyl-PS fractional orders with respect to both reactants are found. The order with respect to cholesterol in these vesicles is 0.62 ± 0.05, and the order with respect to filipin is 2.5 ± 0.3. Thus the rate of filipin–cholesterol complexation in didecanoyl-PC and dipalmitoyl-PS bilayers is more complex than the simple behavior found in bilayers of the other chemically defined saturated phospholipids reported here.

TABLE III: Second-Order Rate Constants and Activation Parameters of the Association of Filipin with Cholesterol in Vesicles Prepared from Different Phospholipids.

Lipid composition of vesicles[a]	$10^{-4}k_2$ (L mol^{-1} s^{-1})	E_a (kcal mol^{-1})	$\Delta S^{\ddagger}$[b] (cal deg^{-1} mol^{-1})
Didecanoyl-PC	0.25 ± 0.02		
Dimyristoyl-PC	1.9 ± 0.1	14.3	6.2
Dimyristoyl-PC and cholesterol oleate	1.1 ± 0.1	14.4	5.4
Dipalmitoyl-PC	1.2 ± 0.1	11.3	−4.6
Distearoyl-PC	6.5 ± 0.5[c]		
Reduced egg PC	1.6 ± 0.2	6.8	−18.9
Sphingomyelin	1.3 ± 0.2		

[a] Each vesicle contained 24 mol % cholesterol. [b] The error in $\Delta S^{\ddagger}$ was ±5.0 cal deg^{-1} mol^{-1}. [c] Value is dependent on the temperature of sonication. Vesicles prepared by sonication at 30 to 60 °C were smaller (0.40 ± 0.05 L per mol of lipid) than those sonicated at 0 °C, and gave smaller k_2 values (1.1–2.8 × 10^4 L mol^{-1} s^{-1}).

Second-Order Rate Constants and Activation Parameters. Figures 1 and 2 show that the initial rate of filipin–cholesterol association is sensitive to lipid composition, becoming slower relative to pure dimyristoyl-PC bilayers when cholesterol oleate is incorporated, or when visicles are prepared from a short-chain phospholipid and investigated at 30 °C. To allow quantitative comparisons of rates on interaction with vesicles of varying composition, rate constants were calculated for reactions that were found to be first order in filipin and in cholesterol concentration. The second-order rate constants are listed in Table III.

In order to determine whether the initial rates of filipin–cholesterol association depend on the size distribution of the

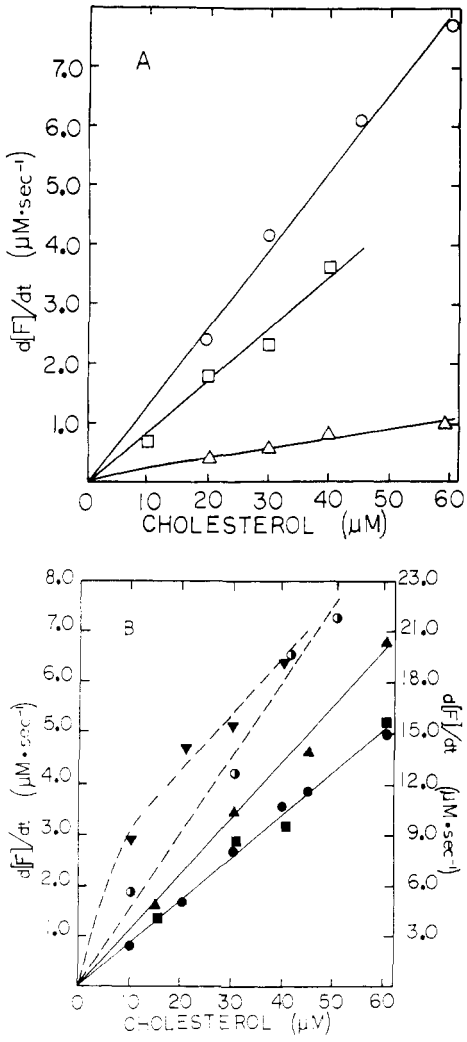

FIGURE 1: Effect of cholesterol concentration on the initial rates of association of filipin with phosphilipid-cholesterol vesicles. The filipin concentration was 7.0 μM. The phospholipid-free cholesterol molar ratio was 3:1. The phospholipids were: (A) (○) dimyristoyl-PC, (□) dimyristoyl-PC and cholesterol oleate, (△) didecanovl PC; (B) (▲) reduced egg PC, (■) sphingomyelin, (●) dipalmitoyl-PC, (▼) dipalmitoyl-PS, (◑) distearoyl-PC. The right-hand ordinate refers to the rates of binding to dipalmitoyl-PS and distearoyl-PC vesicles (dashed curves).

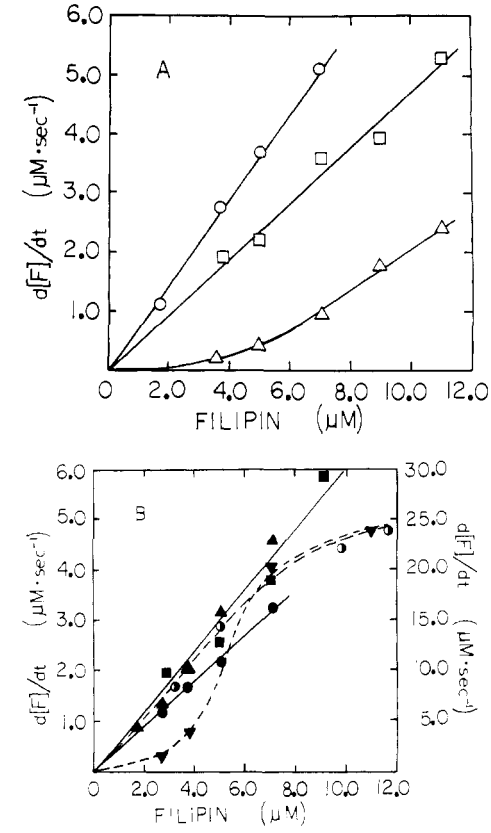

FIGURE 2: Effect of filipin concentration on the initial rates of association of filipin with phospholipid-cholesterol vesicles. The cholesterol concentration was 40 μM. The total lipid concentration was 0.25 mM. The phospholipid:free cholesterol molar ratio was 3:1. The phospholipids were: (A) (○) dimyristoyl-PC, (□) dimyristoyl-PC and cholesterol oleate, (△) didecanoyl-PC; (B) (▲) reduced egg PC, (■) sphingomyelin, (●) dipalmitoyl-PC, (▼) dipalmitoyl-PS, (◑) distearoyl-PC. The right-hand ordinate refers to the rates of binding to dipalmitoyl-PS and distearoyl-PC vesicles (dashed curves).

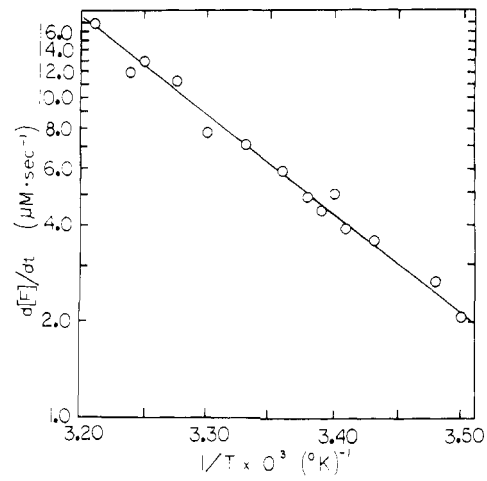

FIGURE 3: Plot of ln d[F]/dt as a function of the reciprocal of the absolute temperature. Vesicles were prepared from dimyristoyl-PC and cholesterol, at a PC to sterol molar ratio of 3:1. The total lipid concentration was 0.25 mM. The filipin concentration was 7.0 μM.

vesicles, measurements were made of the interaction of 7 μM filipin with cholesterol (40 μM) in unfractionated and Sepharose 4B fractionated dimyristoyl-PC vesicles. The following d[F]/dt values were found at 30 °C: unfractionated vesicles, 5.3 μM s^{-1}; vesicles in ascending portion of peak, 4.9 μM s^{-1}; vesicles in descending portion of peak, 6.0 μM s^{-1}. Since the difference in initial rates was only slightly greater than the experimental error, we measured the kinetics of filipin-cholesterol association in unfractionated vesicles.

Initial rate data were obtained over a temperature range of 18 to 44 °C, which includes temperatures below and above the phase transition temperature of dimyristoyl-PC. A plot of ln d[F]/dt vs. the reciprocal absolute temperature (Figure 3), which was made to calculate the Arrhenius activation energy, reveals no discontinuity in rate around the transition temperature. From the data shown in Table IV, the energies and entropies of activation were calculated for filipin binding to vesicles made from various saturated phospholipids (Table III). The temperature dependence of the initial rates of filipin association with cholesterol in vesicles made from didecanoyl-PC and dipalmitoyl-PS was the reverse from the expected trend displayed by vesicles derived from the other phospholipids (Table IV). This unusual temperature dependence of d[F]/dt suggests that the filipin-cholesterol interfacial association may contain a larger number of hydrophobic contacts than present in vesicles having the normal temperature dependence.

Salt Effects on Initial Rates of Filipin-Cholesterol Association. In an attempt to determine whether the activated complex is less solvated than the isolated reactants, we ex-

TABLE IV: Effect of Temperature on the Initial Rate of Association of Filipin with Cholesterol-Containing Vesicles.[a]

	d[F]/dt (μM s^{-1})					
Temp (°C)	Dimyristoyl-PC[b]	Dimyristoyl-PC[b] and cholesterol oleate	Dipalmitoyl-PC[b]	Reduced egg PC[b]	Didecanoyl-PC[c]	Dipalmitoyl-PS[c]
18	3.6	2.7			7.6	
20	3.9					26.2
22	4.4		2.7			
24	5.9	4.2		5.9	1.2	
27	7.1		4.7			
30	7.7	5.4	5.0	7.0	1.0	20.1
35	11.7	10.5	7.0	9.2		
39	16.7		9.0	11.2		
44			14.2	12.8		12.2

[a] The filipin concentration was 7.0 μM. The phospholipid:cholesterol molar ratio was 3:1. The cholesterol concentration was as follows. [b] 60 μM. [c] 40 μM.

amined the effects of anions differing in charge density on the initial rates. Stopped-flow kinetic experiments were performed with filipin (7.0 μM) and dimyristoyl-PC–cholesterol vesicles (250 μM total lipid concentration, PC:cholesterol molar ratio of 3:1). Vesicle suspensions and antibiotic solutions were prepared in 0.02 M sodium phosphate buffer, pH 7.0, and the sodium salt of Cl^-, SCN^-, or salicylate was added. The initial rates of association were: 8.9 μM s^{-1} in 1.0 M Cl^-; 0.8 μM s^{-1} in 1.0 M SCN^-; and ~0.02 μM s^{-1} in 1.0 M salicylate. Thus anions with low charge density (SCN^-, salicylate) lower the rate. For reactions involving desolvation at the transition state, anions having high charge density, such as Cl^-, are expected to enhance the reaction rate relative to anions having low charge density; Cl^- will bind solvent water molecules more effectively than SCN^- or salicylate, facilitating displacement of water molecules from the solvation spheres of the reactants.

Discussion

The initial rates of association of filipin with vesicles prepared from phospholipid alone were at least 50 to 100 times slower than those with vesicles derived from mixtures of phospholipid and cholesterol. With vesicles prepared from some phospholipids, no rapid reaction was detected upon stopped-flow mixing with filipin. It is therefore justified to assume that the initial rates measured in this study represent association of filipin with cholesterol. Since initial rates were measured within 60 ms after mixing of the reactants, it may be assumed that the absorbance decrease arises only from the reaction of filipin with cholesterol. No filipin-induced transmittance changes in cholesterol-containing phospholipid vesicles were detected at 600 nm in the time range of 0 to 200 ms. Since filipin does not absorb at 600 nm, and since this wavelength is sensitive to structural changes in vesicle structure, the rapid transmittance changes monitored at 360 nm do not reflect vesicle disruption.

The kinetic properties of filipin association with vesicles derived from chemically defined phospholipids differ from those of amphotericin B. Stopped-flow kinetic measurements of the initial rate of association of amphotericin B with vesicles indicated that amphotericin–lipid interactions compete with lipid–lipid interactions (Chen and Bittman, 1977). Unlike the situation in amphotericin–vesicle association, filipin reacts rapidly only with cholesterol-containing vesicles. The initial rate of association of filipin with vesicle-bound cholesterol is considerably faster than that of amphotericin B; for example, with vesicles made from dimyristoyl-PC and cholesterol (molar ratio of 5:1), d[F]/dt is approximately 20 times faster than d[amphotericin]/dt.

The second-order rate constant for filipin–cholesterol complexation in dimyristoyl-PC bilayers is not markedly affected by the mole percent of cholesterol. A larger variation in initial rate as a function of mole percent cholesterol at a given absolute sterol concentration was observed in egg PC bilayers (Bittman et al., 1974). A comparison of the k_2 values reported in Table III with the trapped volumes given in the Experimental Section indicates that in unilamellar vesicles of varying molecular composition k_2 is not size dependent. Given the variation in k_2 with fatty acyl chain length, estimation of the relative concentrations of cholesterol in the inner and outer halves of cell membranes by measurements of the initial rates of filipin–cholesterol association (Bittman and Rottem, 1976) requires a symmetric distribution of phospholipid fatty acyl composition in the transverse plane. Evidence presently available for erythrocyte membranes indicates that the phospholipid fatty acyl composition with the same polar head group is the same on the external and cytoplasmic sides of the membrane (Renooij et al., 1974, 1976).

With dipalmitoyl-PS–cholesterol bilayers, the initial rate of filipin–cholesterol complexation is faster (above a filipin concentration of ~6 μM) than with any other bilayer composition we investigated. This comparison is limited to the temperature range of 18 to 39 °C because the initial rate of reaction with dipalmitoyl-PS–cholesterol vesicles decreases with increasing temperature (Table IV). Comparison of the data obtained with cholesterol-containing dipalmitoyl-PC and dipalmitoyl-PS vesicles suggests that the nature of the phospholipid head group has an important influence on the magnitude of the reaction rate, on the order of the reaction with respect to the reactants, and on the temperature dependence of the initial rate. Therefore, it appears that this change in charge properties of the phospholipid head group brings about a change in reaction mechanism.

The Arrhenius activation energies for the association of filipin with cholesterol in dimyristoyl-PC and dipalmitoyl-PC vesicles are similar. In bilayers of reduced egg PC, which has a heterogeneous fatty acid composition, a lower activation energy was found. Since the phase transition temperatures of dipalmitoyl-PC and reduced egg PC are above 40 °C, whereas that of dimyristoyl-PC is 23 °C, the difference in activation energy (Table III) is not related to the difference in physical state (gel vs. liquid crystal) of the phospholipids. Furthermore, no change in activation energy was observed for dimyristoyl-PC above and below its transition temperature (Figure

3). Insertion of filipin into the bilayer may account for the unusually high activation energies found for this noncovalent complex formation. The value of activation entropy found in reduced egg PC–cholesterol vesicles (−18.9 eu) is not unexpected for a bimolecular reaction (Laidler, 1965); considerably more negative activation entropies for bimolecular reactions have been reported (Page and Jencks, 1971; Bittman and Blau, 1975), indicative of a higher degree of restriction to freedom of motion in the transition state. The more positive entropies of activation found for filipin–cholesterol association in vesicles derived from dimyristoyl-PC and dipalmitoyl-PC (Table III) are consistent with the hypothesis that some displacement of water molecules occurs in going from the isolated reactants to the transition state. The observation that the initial rate of filipin binding to cholesterol–dimyristoyl-PC vesicles was decreased in the presence of 1.0 M SCN^- and salicylate ion by 10- and 300-fold, respectively, was also interpreted in terms of transition-state desolvation. However, it is recognized that a chaotropic ion such as SCN^- tends to weaken hydrophobic interactions; therefore, the decrease in association rate could arise from weakening of hydrophobic contacts involved in the formation of the activated complex. Since SCN^- and salicylate anion are absorbed to thin lipid membranes (Singer, 1973; McLaughlin et al., 1975), the possibility that they lower the reaction rate by modifying the surface charge of the vesicle cannot be ruled out.

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