The Phospholipid–Cholesterol Interaction. Kinetics of Water Permeability in Liposomes†

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ABSTRACT: Stopped-flow light-scattering measurements were made on liposomes exposed to hypo- and hyperosmolar KCl solutions. The rapid recordings of changes in light transmission accompanying rapid changes in osmolarity represent liposomal water permeability. Equilibrium and kinetic data indicate that liposomes prepared with KCl in the osmotically active spaces obey the Boyle–van't Hoff law and behave as ideal osmometers. The initial rate of volume change is believed to provide an indication of the velocity of water diffusion through the outer lipid bilayer. Increasing unsaturation of the phospholipid species caused an increase in liposomal water permeability. The initial rate of water permeability of liposomes derived from saturated and unsaturated lecithins above their crystalline to liquid–crystalline transition temperatures decreased with increasing cholesterol concentration. On the other hand, cholesterol enhanced the rate of water permeability of liposomes derived from saturated lecithins below their transition temperatures. Diester and diether phosphate lecithins had the same rate of liposomal water permeability and were affected by cholesterol to the same extent. Evidence for hydrogen bonding between the cholesterol hydroxyl group and the negatively charged oxygen atom of the lecithin phosphate group was obtained from the effect of cholesterol on the initial rates of osmotic shrinking of liposomes derived from diether phosphate, phosphonate, and phosphinate analogs of lecithin. Studies made with 3β-thiocholestene (thiocholesterol) and 3α-hydroxycholestene (epicholesterol) support the involvement of hydrogen bonding between lecithin and cholesterol molecules in liposomes and show that attention must be given to the orientation of the hydroxyl group of cholesterol at the lecithin polar head group. Furthermore, propylphosphonylcholine and propylphosphinylcholine analogs of lecithin do not interact with cholesterol, whereas butylphosphonylcholine and butylphosphinylcholine analogs do interact with cholesterol. This suggests that strict demands at the lecithin polar head group must be met in order for the lecithin–cholesterol complex to form. The polyene antibiotic filipin III did not alter the kinetic or equilibrium permeability behavior of lecithin–cholesterol liposomes at a cholesterol to filipin III ratio of 16. The results are consistent with the hypothesis that the permeability of the bilayer is governed by the fluidity of the hydrocarbon chains and support the solubility-diffusion mechanism of water permeation.

Liposomes are closed, ordered, multilamellar structures that form spontaneously when phospholipids are dispersed in aqueous solution. These hydrated liquid crystals consist of concentric spheres of lipid bilayers, whose geometry depends on water content, temperature, ionic strength, and method of dispersion. The aqueous interstices between the bimolecular lamellae are separated by at least 45 Å and trap "markers," such as ions and nonelectrolytes (Bangham, 1968; Bangham *et al.*, 1965a; Sessa and Weissmann, 1968a; Papahadjopoulos and Miller, 1967). Liposomes exhibit permeability characteristics that qualitatively resemble those observed in natural biological membranes. For example, the effects of steroids, ionophores, polyenes, anesthetics, and lytic agents on the passage of entrapped marker ions or molecules from the aqueous compartments of these model membranes, through the bilayers, and into the surrounding medium are similar to those observed for simple diffusion across biological membranes (Weissmann *et al.*, 1966; De Gier *et al.*, 1970; Sessa and Weissmann, 1968b; Johnson and Bangham, 1969; Bangham *et al.*, 1965b). Moreover, the diffusional exchange of small molecules across the bilayers of liposomes depends on the degree of packing and thermal mobility of the hydrocarbon chains and on the charge of the polar head group of the phospholipid (Van Deenen, 1971). These studies have led to the view that the chemical composition of the lipids present at the interface may play an important role in regulating the permeability properties of the membrane.

The permeability of various solutes across intact cell suspensions and liposomes has been monitored spectrophotometrically under the assumption that the amount of light scattered by the particles is a function of their volume. Although in theory volume changes may not be inversely proportional to the first power of the relative light-scattering changes (Koch, 1961), it has been shown experimentally for mitochondria (Tedeschi and Harris, 1955, 1958), erythrocytes (Sidel and Solomon, 1957), microsomes (Kamino and Inouye, 1969), and liposomes (Bangham *et al.*, 1967) that volume changes are proportional to the reciprocal of the changes in absorbance, when the absorbance is due to light scattering only. For an ideal osmometer, *i.e.*, one permeable only to water and impermeable to the solute, the volume change resulting from a change in the osmotic strength of the suspending medium is given by the Boyle–van't Hoff law, $V = K(1/c) + b$, where V is the volume change (which is found to be inversely proportional to the change in absorbance), K is a constant independent of wavelength and osmometer concentration, c is the osmotic pressure gradient across the membrane in terms of concentration, and b is the osmotic dead space, *i.e.*, the volume occupied by the molecular species in the membrane and therefore unavailable for osmotic volume change. Volumetric determinations of pellet volumes, gravimetric determinations of water content, and packed mass measurements have shown that volume changes resulting

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from water movement in liposomes (Bangham *et al.*, 1967; Rendi, 1967), chloroplast grana (Gross and Packer, 1967), and microsomes (Kamino and Inouye, 1969) obey the Boyle–van't Hoff law. Since the net flow of water through the membrane per unit time is proportional to the area and the osmotic gradient, the large surface area of liposomes permits the initial rate of volume change, dV/dt, upon sudden change in the osmotic pressure of the suspending medium, to be measured conveniently from the change in absorbance. The validity of this technique to measure osmotic volume changes in liposomes resulting from changes in the concentrations of nonelectrolytes was confirmed by measurements of the volumes and water content of centrifuged pellets (Bangham *et al.*, 1967; Rendi, 1967).

The present study demonstrates that the kinetics of water permeability in liposomes can be studied by stopped-flow light-scattering measurements and provides evidence that seemingly subtle modifications at the 3 position of cholesterol produce marked changes in the initial rates of water permeability in liposomes derived from lecithin and sterols. The initial rate of water permeability through the bilayer was found to increase with increasing unsaturation of the phospholipid species and to decrease with increasing concentration of cholesterol. However, cholesterol increased the permeability of saturated lecithins below their transition temperatures. These findings support the hypothesis that the rate of diffusion through the bilayer is controlled by the fluidity of the apolar chains. Studies with synthetic diether phosphate, phosphonate, and phosphinate analogs of lecithin indicate that in liposomes the cholesterol hydroxyl group is hydrogen bonded to the negatively charged, unsubstituted oxygen atom of lecithin.

Experimental Section

Materials. Egg lecithin was isolated and purified from hen egg yolk by the method of Singleton *et al.* (1965). The purity was determined by thin-layer chromatography on silica gel plates using a solvent system consisting (by volumes) of chloroform–acetone–methanol–acetic acid–water (3:4:1:1:0.5) (Rouser *et al.*, 1969). Dipalmitoyl-L-α-lecithin and dioleoyl-L-α-lecithin were purchased from Supelco, Inc., and used without further purification. The fatty acid distribution was not analyzed. Dihexadecyl-L-α-lecithin, $ROCH_2CH(OR)CH_2OP(O)(O^-)OCH_2CH_2N^+(CH_3)_3$ ($R = C_{16}H_{33}$), was purchased from Schwarz-Mann BioResearch. Infrared analysis (in KBr pellet and chloroform solution) showed that no ester was present. Samples of the following lecithin analogs were kindly provided by Dr. A. F. Rosenthal (Long Island Jewish Medical Center, New Hyde Park, N. Y.): DL-2-hexadecoxy-3-octadecoxypropylphosphonylcholine (I), 2-(trimethylammonium)ethyl DL-2-hexadecoxy-3-octadecoxypropylphosphinate (II), DL-3,4-dioctadecoxybutylphosphonylcholine (III), and 3-(trimethylammonium)ethyl L-3,4-dioctadecoxybutylphosphinate (IV). All of the lecithin samples were analyzed by thin-layer chromatography on silica gel G coated plates in a solvent system consisting (by volumes) of chloroform–methanol–water (65:25:4) (Rouser *et al.*, 1969). Visualization was by molybdenum blue reagent (Dittmer and Lester, 1964) and by iodine vapor. Chromatograms developed from lysolecithin showed that the thin-layer chromatography method was sufficiently sensitive to detect and separate hydrolysis products of lecithins; however, no lysophosphatides were detected in the lecithin samples used.

Cholest-5-en-3β-ol (cholesterol) was purchased from Sigma Chemical Co., cholest-5-en-3α-ol (3α-hydroxycholestene or epicholesterol) from Schwarz-Mann BioResearch, and 3β-thiocholest-5-ene (3β-thiocholestene or thiocholesterol) from Aldrich Chemical Co. The sterols were recrystallized from acetone and the purity was checked by thin-layer chromatography on silica gel plates using solvent systems consisting (by volumes) of benzene–methanol (92:8) and chloroform–acetone (98:3.5). The melting points of the sterols were cholesterol 147.5–148°, epicholesterol 141.5, and thiocholesterol 97–98°.

Dicetyl phosphate and Tris were purchased from Sigma Chemical Co. D_2O (99.88 mol %) was purchased from Bio-Rad Laboratories. All other chemicals were of analytical grade.

Filipin III was generously supplied by Dr. G. B. Whitfield of the Upjohn Co., Kalamazoo, Mich. Stock solutions were prepared in dimethylformamide. Aliquots of the stock solution were added to 0.01 M Tris–0.06 M KCl solution (pH 7.2) to give the desired filipin concentration and a final concentration of dimethylformamide of 1.0%. Based on a molecular weight of 670, the molar extinction coefficient of filipin III in 100% dimethylformamide was found to be 5.0×10^4 M^{-1} cm^{-1} at 361 nm; in 0.01 M Tris–0.06 M KCl solution (pH 7.2) containing 1.0% dimethylformamide the molar extinction coefficient at 358 nm, immediately after preparation of the solution, was found to be 5.1×10^4 M^{-1} cm^{-1}. The ratio of the absorption peaks of filipin was checked before the solution was used. Verification that such preparations retained activity on interaction with liposomes containing cholesterol came from spectral (alteration of the peak ratios in the uv absorption spectrum, enhancement of the circular dichroic bands of filipin, increase in fluorescence polarization) and electron micrographic (disruption of the ordered bilayer structure) studies (Bittman and Reddy, 1971; R. Bittman *et al.*, unpublished results).

Preparation of Liposomes. Liposomes were prepared by pipetting into 20-ml vials the desired amounts of lecithin, dicetyl phosphate, and cholesterol from stock solutions prepared in chloroform. The chloroform was removed under nitrogen and the lipids were evaporated to dryness *in vacuo*. The thin, dry lipid film was suspended in 0.01 M Tris buffer containing the desired concentration of KCl adjusted to pH 7.2. (Since Tris may participate in hydrogen bonding and is known to disrupt liposomes at high concentrations, in some preparations the buffer was omitted. The results were unaltered by the omission of the buffer, demonstrating that Tris does not interfere with or play a role in the interaction between lecithin and cholesterol.) The molar ratios of the lipids in the liposomes were calculated from the input concentrations. To prepare liposomes from egg lecithin or from a mixture of dipalmitoyl- and dioleoyllecithin a glass bead was added and the lipids were dispersed at 25° by agitation on a Vortex mixer for 60 sec. To prepare liposomes from saturated phospholipids one glass bead was added per milliliter of suspending KCl solution. The lipids were dispersed at 25° (except dipalmitoyl- and dihexadecyllecithin, which were dispersed at 38°) by agitation on the Vortex mixer for 60 sec. The suspension was then ultrasonically irradiated with a 20-kHz Branson Sonifier (Model S-110) fitted with a solid tap horn at power level 4 for 5 or 10 sec (as indicated in the figure captions). All liposomes contained 4 mol % dicetyl phosphate, which was added to confer a net charge on the bilayers and allow salt to be trapped in the aqueous interstices.

Assay of Osmotic Permeability. In kinetic experiments liposomes dispersed in 0.01 M Tris–KCl solution (pH 7.2) were mixed rapidly (less than 3 msec) with an equal volume

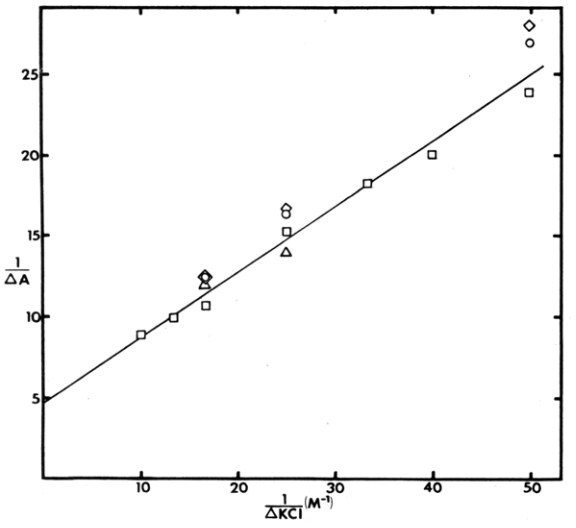

FIGURE 1: Relationship at equilibrium between the reciprocals of absorbance changes at 600 nm and the reciprocals of the change in KCl concentrations after osmotic shrinking. The change in KCl concentration is the difference between the final KCl concentration in the solution to which the liposome was added and that in which it was prepared. Liposomes were prepared in 0.06 M KCl solution from (□) egg lecithin, (◇) egg lecithin and cholesterol in a 2:1 molar ratio, and (Δ) dioleoyllecithin and dipalmitoyllecithin in a 1:1 molar ratio. The final lipid concentration in the liposomes was 1.0×10^{-3} M. The circles (○) indicate data obtained from the stopped-flow apparatus for the mixing of egg lecithin liposomes with KCl solutions; the total (final minus initial) changes in transmittance observed on the oscilloscope were converted to $1/\Delta A$.

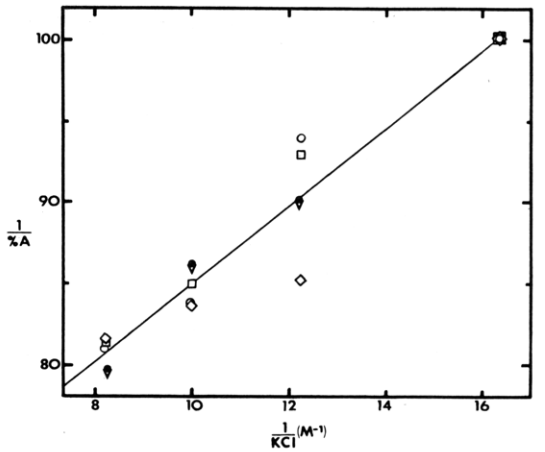

FIGURE 2: Relationship at equilibrium between the reciprocal of the per cent absorbance increase at 600 nm of liposomes prepared in 0.06 M KCl solution and the reciprocal of the final concentration of KCl in the solution after osmotic shrinking. Liposomes from (◇) dioleoyllecithin and from a mixture of dioleoyl- and dipalmitoyllecithin in (○) 1:1 and (□) 1:2 molar ratio were prepared at 25°, and the absorbances were measured at 25°. Liposomes from (▽) dipalmitoyllecithin and from (●) dihexadecyllecithin were prepared at 38°, and the absorbances were measured at that temperature. The final lipid concentration in the liposomes was 1.0×10^{-3} M.

of buffered hyper- or hypotonic KCl solution in a stopped-flow apparatus (Durrum Instrument Co., Palo Alto, Calif.). The initial change in transmittance at 600 nm was monitored on a Tektronix storage oscilloscope equipped with a Polaroid camera. A slit width of 1.0 mm was used. The temperature was maintained at 25° unless otherwise indicated. The initial rates of shrinking or swelling were determined by converting the change in transmittance per second to dA/dt. The results of a given shrinking or swelling experiment represent the analysis of at least three separate photographs, and in general seven to ten separate photographs were analyzed. For liposomes derived from egg lecithin or from a mixture of dipalmitoyl- and dioleoyllecithin, the rates were examined between approximately 0.15 and 1.0 sec after stopping of the flow; however, for liposomes derived from saturated lecithins the initial rate measurements were extended to longer times. Initial rates were determined from the slope of the linear portion of the relaxation curve. A disturbance was reproducibly observed within the first 0.15 sec. This prevented measurements of volume change to be made at very fast times.

Swelling rates of liposomes upon exposure to isotonic glycerol solutions were measured on the stopped-flow apparatus and a Cary 14 spectrophotometer.

In equilibrium measurements the total change in absorbance resulting from osmotic shrinking or swelling was determined on a Cary 14 spectrophotometer at 600 nm and ~25°, unless otherwise noted.

Results

Figures 1 and 2 show that liposomes prepared with KCl as the osmotically active species give linear Boyle–van't Hoff plots for osmotic shrinking when exposed to an osmotic pressure gradient. The linear dependence observed for liposomes derived from natural as well as synthetic lecithins and for lecithin–cholesterol liposomes indicates that they are ideal osmometers under these conditions and are completely impermeable to solute. Also to be noted is the good agreement between the absorbance change at equilibrium measured on a Cary 14 spectrophotometer and that calculated from transmittance changes on the stopped-flow apparatus (Figure 1). Liposomes derived from diester and diether phosphate lecithins (dipalmitoyl- and dihexadecyllecithin, respectively) underwent identical per cent absorbance increases after osmotic shrinking (Figure 2).

Figures 3 and 4 show examples of the experimental results for the initial rates of osmotic shrinking of liposomes prepared from lecithin and lecithin–cholesterol in a 2:1 molar ratio. The initial rates of shrinking of lecithin and lecithin–cholesterol liposomes when exposed to hyperosmolar KCl solutions show a linear dependence on the osmotic pressure gradient (Figure 4). The marked effect of cholesterol on decreasing the initial rate of volume change is discussed below.

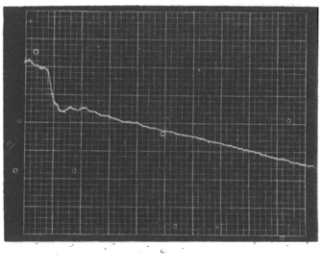

FIGURE 3: Typical oscilloscope trace for the initial change in liposome permeability upon exposure to a hyperosmolar KCl solution in the stopped-flow apparatus. Liposomes were prepared in 0.06 M KCl solution from egg lecithin and cholesterol in a 2:1 molar ratio and mixed with 0.18 M KCl solution. The transmittance was monitored at 600 nm. Each large vertical division corresponds to 20 mV. Each large horizontal division corresponds to 100 msec. The disturbance in the first 1.5 divisions precluded measurement of volume change in the initial 150 msec following mixing.

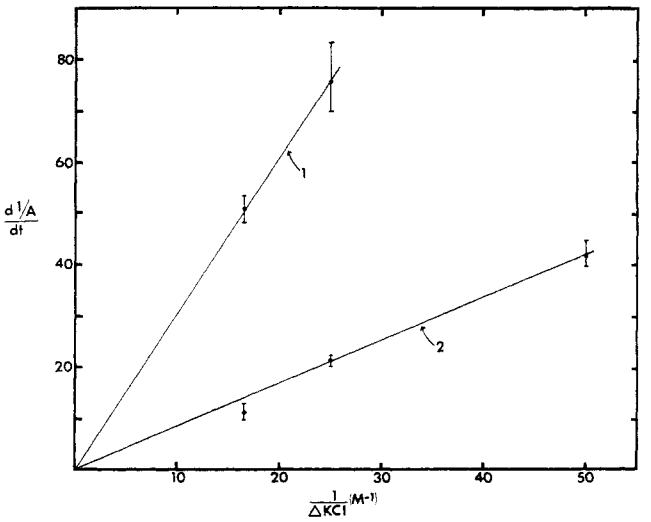

FIGURE 4: Plots of initial shrinking rates *vs.* the reciprocal of the osmotic difference of KCl for (1) lecithin liposomes and (2) a 2:1 molar ratio of lecithin–cholesterol liposomes. Liposomes were prepared in 0.06 M KCl solution and mixed in the stopped-flow apparatus with an equal volume of varying concentrations of KCl. The final lipid concentration in the liposomes was 1.0×10^{-3} M.

The rates correspond to permeation of water. Table I shows that the rate of entry of glycerol into liposomes is nearly two orders of magnitude slower than the rate of osmotic shrinking observed on exposing the same liposomes to a hyperosmolar KCl solution. Moreover, liposomes having NaCl entrapped in the osmotically active spaces gave the same initial rate of volume change as those containing KCl (Table I). Further evidence for water movement through the bilayers is shown in Table II. The initial rate of swelling is slower when D_2O enters the liposomes compared to H_2O. Although the magnitude of the difference is surprising, it is clear that this is not entirely a kinetic phenomenon; the absorbance change at 600 nm at equilibrium is also smaller for D_2O penetration than for H_2O penetration (0.080 and 0.105, respectively).

The initial rates of volume change of liposomes derived from dipalmitoyl- and dioleoyllecithins are presented in Table III. For a given osmotic pressure gradient, the permeability of the liposomes to water decreases as the degree of unsaturation decreases.

The presence of cholesterol lowers the initial rate of water penetration, and the rate decreases linearly as the lecithin to cholesterol ratio is varied from 1:0 to 1:1 (Table IV). However, epicholesterol and thiocholesterol do not cause the marked decrease in initial rate of shrinking that cholesterol itself causes. Therefore, the nature and stereochemistry of the group at the 3 position of the sterol play a significant role in controlling the diffusion of water through the bilayer.

Table V shows that the diester and diether phosphate lecithins have the same initial rates of shrinking. Therefore, the carbonyl group of each fatty acyl chain in natural lecithins makes a negligible contribution to the initial rate of water penetration, assuming that both lecithins are in the crystalline phase at 38°. The permeability of dipalmitoyllecithin liposomes increases dramatically on going from 38 to 45°. Since the crystalline to liquid-crystalline phase transition causes an increase in the mobility of the hydrocarbon chains (Ladbrooke and Chapman, 1969), the large enhancement in permeability demonstrates that liposomal water transport is sensitive to the conformational changes that occur on passing through the lipid phase transition of dipalmitoyllecithin ($T_t \approx 41°$). It is interesting to note that the initial rate of volume change of dipalmitoyllecithin liposomes at 45° is identical with that of egg lecithin liposomes at 25°. In contrast to the decrease in permeability of egg lecithin liposomes

TABLE II: Initial Swelling Rates of Lecithin:Cholesterol Liposomes in H_2O and D_2O.[a]

Osmotic Stress	dA/dt (sec^{-1})
H_2O	0.029 ± 0.003
D_2O	0.018 ± 0.003

[a] Liposomes of egg lecithin and cholesterol in 2:1 molar ratio were formed in buffered 0.18 M KCl solution and mixed with an equal volume of buffer dissolved in H_2O or D_2O. The final lipid concentration in the liposomes was 1.0×10^{-3} M. The initial rates of swelling were measured at 600 nm.

TABLE I: Initial Rates of Permeability Behavior in Lecithin:Cholesterol Liposomes.[a]

Type of Expt	Salt Entrapped in Osmotically Active Spaces (0.06 M)	Osmotic Stress	$dA/dt \times 10^3$ (sec^{-1})
Swelling	KCl	0.12 M glycerol	0.37 ± 0.03
Shrinking	KCl	0.18 M KCl	28 ± 1
Shrinking	NaCl	0.18 M NaCl	28 ± 5

[a] Liposomes of egg lecithin and cholesterol in 2:1 molar ratio were formed in the 0.06 M salt solution indicated and mixed with an equal volume of isotonic glycerol or 0.18 M KCl or NaCl solution. The final lipid concentration in the liposomes was 1.0×10^{-3} M. The initial rates of osmotic swelling and shrinking were measured at 600 nm.

TABLE III: Initial Shrinking Rates of Dipalmitoyllecithin–Dioleoyllecithin Liposomes.[a]

Concn of Dipalmitoyllecithin × 10^3 (M)	Concn of Dioleoyllecithin × 10^3 (M)	dA/dt (sec^{-1}) 0.08 M KCl	0.10 M KCl	0.12 M KCl
0.00	0.96	0.0435	0.0557	0.0990
0.48	0.48	0.0270	0.0285	0.0347
0.64	0.32	0.0151	0.0198	0.0331
0.96	0.00			0.0019[b]

[a] Liposomes were formed in 0.06 M KCl and mixed with an equal volume of hyperosmolar KCl solution. The concentrations of lipid and KCl shown are those obtained after mixing. [b] Liposomes were prepared at 38° and ultrasonically irradiated for 5 sec. The initial shrinking rate was measured at 38°.

TABLE IV: Effect of Modifications at the 3 Position of Cholesterol on the Initial Shrinking Rates of Lecithin–Sterol Liposomes.[a]

Concn of Lecithin × 10^3 (M)	Concn of Cholesterol × 10^3 (M)	dA/dt (sec^{-1})		
		3β-Hydroxy	3α-Hydroxy	3β-Thio
0.96	0.00	0.048 ± 0.004	0.045 ± 0.003	0.043 ± 0.003
0.77	0.19	0.034 ± 0.002	0.041 ± 0.003	0.048 ± 0.004
0.64	0.32	0.028 ± 0.001	0.035 ± 0.003	0.036 ± 0.004
0.57	0.39	0.021 ± 0.001	0.031 ± 0.003	0.028 ± 0.002
0.48	0.48	0.018 ± 0.003	0.024 ± 0.003	0.018 ± 0.002

[a] Liposomes of egg lecithin and varying concentrations of 3β-hydroxy-, 3α-hydroxy-, or 3β-thiocholestene were formed in 0.06 M KCl solution and mixed with an equal volume of 0.18 M KCl solution. The lipid concentrations shown are those obtained after mixing. The average values and the error limits of the initial rates shown for each experiment were obtained from analyses of several oscilloscope traces of the same preparation. The average values and error limits obtained from different preparations of egg lecithin liposomes in separate experiments are shown.

TABLE V: Effect of Cholesterol on the Initial Shrinking Rates of Liposomes from Diester Phosphate and Diether Phosphate Lecithins below and above the Transition Temperatures.[a]

Di-16: 0 Lecithin	Concn of Lecithin × 10^3 (M)	Concn of Cholesterol × 10^3 (M)	dA/dt (sec^{-1})	
			38°	45°
Diester[b]	0.96	0.00	0.0019 ± 0.0005	0.044 ± 0.004
Diester[b]	0.64	0.32	0.0067 ± 0.0020	0.020 ± 0.002
Diether[c]	0.96	0.00	0.0021 ± 0.0006	
Diether[c]	0.64	0.32	0.0082 ± 0.0018	

[a] Liposomes were formed in 0.06 M KCl solution at 38°. The suspensions, after having been shaken on the Vortex mixer for 1 min, were ultrasonically irradiated for 5 sec. The initial rates of shrinking, obtained on mixing liposomes with an equal volume of 0.18 M KCl solution, were measured at 38 or 45°, as indicated. [b] Liposomes were formed from dipalmitoyllecithin. [c] Liposomes were formed from dihexadecyllecithin.

caused by the presence of cholesterol (Table IV), cholesterol increases the initial rate of volume change of liposomes derived from lecithins below their transition temperatures. The permeability of liposomes from the diester and diether phosphate lecithins are affected by cholesterol to the same extent, within experimental error. Above the transition temperature, the permeability of dipalmitoyllecithin liposomes decreases on addition of cholesterol.

Cholesterol does not alter the permeability of liposomes prepared from the diether phosphonate lecithin I and the diether phosphinate lecithin II (Table VI). In view of the finding that cholesterol affects the permeability of diether phosphate lecithin liposomes (Table V), studies were made to determine whether the absence of interaction of the phosphonate and phosphinate lecithins with cholesterol represents an electronic or steric effect. The data in Table VI show that the diether lecithin analog III, which is isosteric with natural lecithin in its ionic moiety ($-CH_2-P$ substituted for the glycerphosphoryl $-O-P$), does interact with cholesterol. Similarly, the permeability of liposomes derived from the 4-carbon (dialkoxybutyl) derivative IV, which is isosteric with natural lecithin on one side of the phosphorus function, is affected by the presence of cholesterol.

At low concentrations the polyene antibiotic filipin appears to interact specifically with cholesterol in membranes and lead to the formation of pores through the membrane (Kinsky, 1970). In an effort to gain insight into the mechanism of water permeability in liposomes, the initial rate of shrinking and the total absorbance change were measured in the presence of filipin III, the principal component of the filipin complex (Bergy and Eble, 1968). Table VII shows that at a ratio of cholesterol to filipin of 16 the kinetic and equilibrium data for water permeability are not altered by filipin III. Under these conditions, uv absorption, fluorescence polarization, and circular dichroism studies show that filipin III is bound (R. Bittman *et al.*, unpublished results) and electron micrographic studies show that some disruption of the liposomes has occurred (R. Bittman, O. R. Anderson, and W. C. Chen, unpublished results). The results summarized in Table VII may appear to be at variance with other studies of the effect of filipin on permeability in liposomes until one considers the conditions of the experiments. In experiments designed to test the specificity of the antibiotic for cholesterol, one report showed that at a cholesterol to filipin ratio of less than 1, the initial rate and the total amount of glucose released from liposomes containing varying concentrations of cholesterol was greater than from those lacking cholesterol (Kinsky *et al.*, 1968); however, it is not known whether glucose release would have been enhanced at higher cholesterol to filipin ratios. Another report described release of trapped chromate ion from liposomes containing 70 mol % egg lecithin, 20 mol % dicetyl phosphate, and 12.5 mol % cholesterol; the release

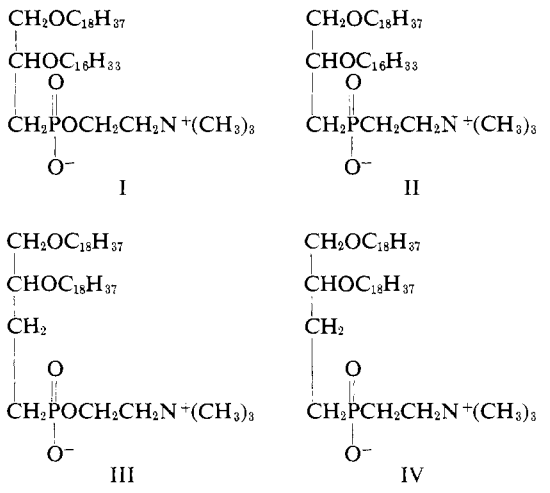

FIGURE 5: Structural formulas of (I) DL-2-hexadecoxy-3-octadecoxypropylphosphonylcholine, (II) 2-(trimethylammonium)ethyl DL-2-hexadecoxy-3-octadecoxypropylphosphinate, (III) DL-3,4-dioctadecoxybutylphosphonylcholine, and (IV) 3-(trimethylammonium)ethyl L-3,4-dioctadecoxybutylphosphinate.

became marked when the cholesterol to filipin III ratio reached about 30, and became more extensive as the ratio decreased (Sessa and Weissmann, 1968b). It is not known what ratio of cholesterol to filipin would be required to cause significant leakage from liposomes containing higher cholesterol and lower dicetyl phosphate concentrations. Table VII shows that at a cholesterol to filipin III ratio of 16 the initial rates and total amount of water diffusion in liposomes containing 33.3 mol % cholesterol are unaltered by the presence of the antibiotic, even though a significant fraction of the antibiotic is bound, and the same observations have been made for glycerol penetration under these conditions (R. Bittman and L. Blau, unpublished results). Although considerably higher concentrations of filipin III do lead to enhanced initial rates of volume change, these conditions do not seem to be reasonable for understanding the molecular basis of permeability in liposomes. Interpretation of the results shown in Table VII is deferred to the discussion section.

TABLE VI: Effect of Cholesterol on the Initial Shrinking Rate of Liposomes from Diether Phosphonate and Phosphinate Lecithin Analogs.[a]

Compound	Concn of Lecithin Analog $\times 10^3$ (M)	Concn of Cholesterol $\times 10^3$ (M)	dA/dt (sec^{-1})
I	0.96	0.00	0.0055 ± 0.0010
I	0.64	0.32	0.0053 ± 0.0017
II	0.96	0.00	0.0016 ± 0.0005
II	0.64	0.32	0.0014 ± 0.0005
III	0.96	0.00	0.0020 ± 0.0009
III	0.64	0.32	0.0075 ± 0.0010
IV	0.96	0.00	0.000096 ± 0.000032
IV	0.64	0.32	0.00045 ± 0.00014

[a] Liposomes of the lecithin analog, with or without cholesterol, were formed in 0.06 M KCl solution. Suspensions from compounds I and III, after having been shaken on the Vortex mixer for 1 min, were ultrasonically irradiated for 10 sec; those from compounds II and IV were ultrasonically irradiated for 5 sec. Liposomes were mixed with an equal volume of 0.18 M KCl solution.

TABLE VII: Effect of Filipin III on Initial Shrinkage Rates and Equilibrium Absorbance Changes of Lecithin–Cholesterol Liposomes.[a]

Concn of KCl (M)	Without Filipin III		With Filipin III	
	dA/dt (sec^{-1})	ΔA	dA/dt (sec^{-1})	ΔA
0.08	0.0097 ± 0.0015	0.073	0.0071 ± 0.0011	0.066
0.10	0.0167 ± 0.0008	0.103	0.0178 ± 0.0014	0.103
0.12	0.0268 ± 0.0012	0.135	0.0266 ± 0.0025	0.132

[a] Liposomes of egg lecithin and cholesterol in 2:1 molar ratio were formed in 0.06 M KCl solution and mixed with an equal volume of hyperosmolar KCl solution. The concentrations of KCl indicated are those obtained after mixing. The final lipid concentration in the liposomes was 0.50×10^{-3} M. All solutions contained 1% dimethylformamide. Filipin III was incubated with the liposomes at a concentration of 1.0×10^{-5} M for 2 hr. The concentration of filipin III and dimethylformamide in the osmotic pulsing medium was the same as that in the solution in which the liposomes were incubated.

Discussion

Evidence has been presented to confirm the assumption that the initial rapid changes in light scattering produced by the osmotic gradient represent initial volume changes resulting from water diffusion in the closed, semipermeable membranes of liposomes. This is supported by the findings that the initial rate of osmotic shrinking is independent of whether KCl or NaCl occupies the osmotically active spaces (Table I), the initial swelling rate due to D_2O permeation is lower than that due to H_2O (Table II), the initial rate of glycerol permeation is lower than that of water (Tables I and II), and the initial rate of water permeability depends on the osmotic gradient (Tables III and VII). The initial rate of volume change presumably provides a measure of the velocity of water transport through the outer lipid bilayer of the liposomes, since initially the KCl concentration gradient exists across the first barrier only.

The marked increase in the initial rates of water permeability that occur on introduction of double bonds into the paraffin chains (Table III) are consistent with the findings that the initial rate of penetration of nonelectrolytes such as glycerol and erythritol into liposomes and into intact cells increases as the degree of unsaturation of the lecithin increases (De Gier *et al.*, 1968; McElhaney *et al.*, 1970). Furthermore, the rate of release of glucose and the valinomycin-induced leakage of Rb^+ from liposomes are enhanced when the unsaturation of the fatty acids constituents of the phospholipids increases (Demel *et al.*, 1968; De Gier *et al.*, 1970). The enhancement in permeability can be explained in terms of the increased fluidity of the hydrocarbon chains caused by the introduction of double bonds. That looser packing of the apolar chains occurs in the bilayer, which constitutes the

barrier to permeability in the liposome, is confirmed by the observation that in monolayers the molecular area of lecithins increases when double bonds are introduced (Demel *et al.*, 1967).

The initial rate of water permeability of liposomes derived from egg lecithin decreases as the concentration of cholesterol is increased (Table IV). The phospholipid–cholesterol interaction has been the subject of considerable study. Many investigations have been made concerning the functional changes in biological membranes induced by cholesterol. At temperatures above the liquid–crystal transition temperature the initial rates of penetration of glycerol are diminished when cholesterol is incorporated into liposomes and into intact cells of *Acholeplasma laidlawii* B (De Gier *et al.*, 1968; McElhaney *et al.*, 1970). Cholesterol-enriched mitochondria are more resistant to swelling than control mitochondria (Graham and Green, 1970) and cholesterol-depleted erythrocyte membranes are more permeable to glycerol than the untreated membranes (Bruckdorfer *et al.*, 1969). The release of trapped glucose, chromate ion, and chloride ion from liposomes decrease when cholesterol is present (Demel *et al.*, 1968; Weissmann and Sessa, 1967; Papahadjopoulos and Watkins, 1967), as does the leakage of certain metabolites from intact cells of *Phythium mycelium* grown in the presence of cholesterol (Child *et al.*, 1969). Furthermore, the water conductivity of lipid films of single bimolecular membranes formed across the aperture of a diaphragm separating two aqueous solutions decreases with increasing cholesterol content (Finkelstein and Cass, 1967) and the permeability of indoles in such single lipid bilayers is also reduced by cholesterol (Bean *et al.*, 1968). The results shown in Table IV are consistent with these reports, but not with the finding that water conductivity in red cell membranes is independent of membrane cholesterol content (Sha'afi *et al.*, 1968).

Studies of structural changes in biological membranes support the idea that when the paraffin chains are in the liquid state, cholesterol limits the mobility by hindering the chain motion. Evidence to support this hypothesis comes from studies of monomolecular films (Demel *et al.*, 1967; Chapman, 1968; Chapman *et al.*, 1969), electron spin resonance (esr) spectra of oriented multilamellar films of lipid spin labels (Hsia *et al.*, 1970; Oldfield and Chapman, 1971; Hsia and Williams, 1970; Butler *et al.*, 1970), and nuclear magnetic resonance (nmr) spectra of sonicated and unsonicated codispersions (Chapman and Penkett, 1966; Darke *et al.*, 1972). The findings that cholesterol does not produce significant changes in the bilayer thickness of dispersions (Lecuyer and Dervichian, 1969) and single bimolecular membranes (Cherry *et al.*, 1971) suggest that the decrease in liposomal water permeability reported in Table IV results from restricted motional freedom of the paraffin chains of egg lecithin and not from an increase in membrane thickness caused by spacing out of the polar groups of lecithin.

Cholesterol appears to increase the fluidity of lecithins containing only saturated paraffin chains at 38° (Table V) and 25° (Table VI). Considerable evidence has been accumulated in support of this interpretation. For example, the observations that on addition of cholesterol to long-chain saturated lecithins the X-ray long spacings are decreased (Ladbrooke *et al.*, 1968; Rand and Luzzati, 1968), the crystalline–liquid phase transition observed in calorimetric measurements and Raman spectra are broadened (Ladbrooke *et al.*, 1968; Lippert and Peticolas, 1971), the surface viscosity in multilayers is reduced (Joos, 1970), condensation effects in monolayers are absent (Demel *et al.*, 1967), and the mobility of an esr spin probe is increased (Oldfield and Chapman, 1971) all lead to the conclusion that cholesterol decreases the hydrophobic interactions between adjacent hydrocarbon chains of saturated lecithins. Thus, the cholesterol-induced increases in the initial rates of volume change (Tables V and VI) are consistent with the contention that cholesterol increases the fluidity of crystalline, saturated paraffin chains. Above the lipid phase transition temperature, however, cholesterol causes a decrease in the initial rate of volume change (Table V). Therefore, the modification of the molecular packing of phospholipids caused by cholesterol is dependent on the crystallinity or liquidity of the hydrocarbon chains of the phospholipid.

Many of the structural studies stress the importance of van der Waals forces in stabilizing the phospholipid–cholesterol complex(es). X-Ray measurements of phospholipid–cholesterol liquid crystals indicate that the steroid nucleus penetrates into the fatty acid region of the bilayer, and the hydroxyl group is likely to be oriented in the aqueous phase (Rand and Luzzati, 1968; Lecuyer and Dervichian, 1969). Some evidence for hydrogen bonding has been presented in dry films of lecithin and cholesterol (Zull *et al.*, 1968; Long *et al.*, 1970). In comparison to cholesterol, 3α-hydroxysterols showed diminished effects on the crystalline–liquid crystalline phase transition of 1-oleoyl-2-stearoyllecithin and on the permeability of liposomes derived from *A. laidlawii* cells (De Kruyff *et al.*, 1972). Table IV shows that liposomes containing thiocholesterol and epicholesterol have higher initial rates of water permeability than those containing cholesterol, until an equimolar ratio of lecithin to sterol is achieved, which is the maximum possible in a dispersion (Bourges *et al.*, 1967). Assuming that the heterogeneity in the distribution of cholesterol molecules in the bilayer is similar to that of thiocholesterol and epicholesterol at comparable molar ratios, the results obtained with thiocholesterol support the involvement of hydrogen bonds in stabilizing the lecithin–cholesterol complex. Since sulfur is less electronegative than oxygen, hydrogen bonded to a sulfur atom does not participate in hydrogen bonding as extensively as hydrogen bonded to an oxygen atom. Therefore, the association of lecithin and thiocholesterol may be weaker, or the lifetime of the complex may be shorter than that formed between lecithin and cholesterol itself. The results obtained with epicholesterol indicate that in addition to the role of apolar forces between the steroid nucleus and the fatty acid constituents of lecithin in stabilizing the complex, attention must be given to the stereochemical orientation of the hydroxyl group at the polar regions of lecithin.

In order to gain insight into the nature of the hydrogen acceptor groups in the lecithin molecule, the effects of cholesterol on the permeability of liposomes prepared from glycerol and butyl diether phosphonate and phosphinate lecithins were examined (Table VI). Cholesterol interacts with both the butylphosphonylcholine (III) and butylphosphinylcholine (IV) compounds. This suggests that the cholesterol hydroxyl group is hydrogen bonded to the negatively charged oxygen atom of the lecithin phosphate group. That no interaction occurs with the glycerol diether phosphonate and phosphinate lecithins (I and II) thus appears to be a steric and not an electronic effect. This is supported by the finding that cholesterol interacts with the diether phosphate lecithin shown in Table V.

The mechanisms of water permeation in single lipid bilayer membranes formed across an aperture has attracted considerable attention and it is of interest to speculate here about the mechanism operative in liposomes. In single bimolecular lipid

membranes and in erythrocyte membranes suggestions have been made that water and other low molecular weight molecules permeate by diffusion through transient pores or water-filled channels within the membrane (Huang and Thompson, 1966; Thompson and Huang, 1966; Paganelli and Solomon, 1957) or by a solubility-diffusion mechanism in which water partitions into the hydrocarbon region of the bilayer, which is believed to have considerable fluidity above the liquid–crystal transition temperature, and diffuses through by jumping into mobile pockets of free volume created by thermal fluctuations of the hydrocarbon chains (Traüble, 1971; Lieb and Stein, 1969; Hanai and Haydon, 1966; Price and Thompson, 1969; Finkelstein and Cass, 1968; Reeves and Dowben, 1970). It has been proposed that the enhanced water, ion, and nonelectrolyte permeabilities that have been observed in single bimolecular membranes containing cholesterol after addition of polyene antibiotics may be due to the formation of aqueous pores induced by the polyenes, although the mechanism of transport is unknown (Van Zutphen *et al.*, 1971; Holz and Finkelstein, 1970; Cass *et al.*, 1970). The finding that filipin does not alter the water permeability of liposomes containing cholesterol under the conditions shown in Table VII suggests that if filipin does cause formation of aqueous pores in liposomes they are too few in number or they close too rapidly to permit diffusion of water. Therefore, the results described in this paper appear to be consistent with the solubility-diffusion mechanism for passive diffusion of water across the outer bilayer of liposomes. Cholesterol is expected to lower the diffusion coefficient of water permeation by rigidizing the liquid-crystalline hydrocarbon chains and limiting the formation of mobile structural defects resulting from conformational changes in the hydrophobic core of the bilayers. On the other hand, increasing unsaturation liquifies the hydrocarbon region and facilitates nonspecific permeation through the bilayer.

The data shown in Table V indicate that the carbonyl groups of the fatty acyl chains of dipalmitoyllecithin play a negligible role in the packing of lecithin molecules in the bilayer. The phospholipids of extremely halophilic bacteria, which require 4 M NaCl for optimal growth, are unique in that they consist almost entirely of derivatives of a glycerol diether rather than of a fatty acid diester (Joo and Kates, 1969). Although the lipids of moderately halophilic and non-halophilic bacteria do not contain ether-linked alkyl groups, one may speculate that a major determinant of chain mobility and membrane permeability is the degree of branching or unsaturation in the paraffin chains.

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Direct Incorporation of Fatty Acids into the Halosulfatides of *Ochromonas danica*†

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ABSTRACT: The lipids of the phytoflagellate, *Ochromonas danica*, contain large amounts of docosane 1,14-disulfates and tetracosane 1,15-disulfates with from 0 to 6 hydrogens on the chain replaced with chlorine atoms. [^{14}C]Acetate, [^{14}C]octanoate, [^{14}C]laurate, [^{14}C]palmitate, [^{14}C]stearate, and [^{14}C]oleate were incorporated into these sulfatides from the media. Each of these precursors was utilized for the biosynthesis of the fourteen disulfates investigated with a virtually identical labeling pattern. Incorporation was confirmed by degradation of the product. Palmitate was most efficiently incorporated into the sulfatide fraction and stearate was least efficiently utilized for sulfatide biosynthesis. These data show that (1) the carbon chain is biosynthesized using the normal fatty acid pathway; (2) the secondary hydroxyl group is put on the chain after the chain is fully synthesized *via* the hydration of a cis double bond; (3) the fatty acid chains are chlorinated after the chains are fully synthesized.

A unique series of lipids has been characterized in the photosynthetic protozoan, *Ochromonas danica*. These polar lipids are polychlorodocosane 1,14-disulfates and tetracosane 1,15-disulfates with from 0 to 6 chlorine atoms replacing hydrogens on the aliphatic chain (Elovson and Vagelos, 1969, 1970; Haines, 1965, 1971; Haines *et al.*, 1969; Mayers and Haines, 1967; Mayers *et al.*, 1969; M. Pousada, B. Das, and T. H. Haines, personal communication). The lipids are unique as polar lipids because they (a) are alkyl sulfates, (b) contain polar (charged) groups at both ends of the molecule, and (c) contain up to six chloro groups on the chain. Each series may be considered as derivative of the hexachlorosulfatides 2,2,11,13,15,16-hexachloro-1,14-docosanediol 1,14-disulfate (Elovson and Vagelos, 1970) and 2,2,12,14,16,17-hexachloro-1,15-tetracosanediol 1,15-disulfate (Haines, 1971). Other compounds in the series have been shown to contain chlorine atoms on the respective alkyl disulfates in various combinations of the positions described above (Haines, 1971).

The chlorosulfolipids constitute approximately 3% of the dry weight of the cells (Elovson and Vagelos, 1969) or 10–20% of the lipids. The occurrence of such large amounts of a polar lipid in a microbe suggests that the lipid is present in membrane. The location of these compounds in the cell and their function is as yet unknown. The study of the biosynthesis

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