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PERMEABILITY PROPERTIES OF PHOSPHOLIPID MEMBRANES: EFFECT OF CHOLESTEROL AND TEMPERATURE

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SUMMARY

1. The effect of cholesterol on the permeability and electrical properties of phospholipid membranes has been studied with unilamellar vesicles and with bilayers (black films). The presence of cholesterol produces a decrease in the permeability coefficients of phospholipid vesicles to Na^+, K^+, Cl^- and glucose; it also results in an increase in the electrical resistance and capacitance of phospholipid bilayers. The effect is apparent with negative, neutral as well as with positively charged membranes.

2. Temperature studies indicate that cholesterol has a considerable effect on the Arrhenius activation energies (E_a) for the diffusion of all the solutes studied. However the effect on the E_a for cation diffusion is a large decrease (from 27–30 kcal/mole to 13–14 kcal/mole) while the effect on the E_a for Cl^- and glucose is only a slight increase (from 14 to 17 kcal/mole and from 19 to 22 kcal/mole, respectively).

Values of free energies for activation (ΔF^+) are also evaluated and are in the range of 20–24 kcal/mole for all solutes studied, ΔF^+ being increased only by about 0.5 kcal/mole upon the introduction of cholesterol.

3. An analysis of the Arrhenius plots allowing for the energies of activation to be temperature dependent (a slight decrease with increasing temperature) indicates that linearization of the plot would result in erroneously large activation energies. Temperature-dependent structural changes have been observed by X-ray diffraction as thinning of the membrane with increasing temperature, and would probably be inhibited by the presence of cholesterol. This argument is offered as an explanation for the cholesterol effect on cation activation energies. Possible mechanisms for the cholesterol effect on the permeation of charged and uncharged species are outlined.

INTRODUCTION

Cholesterol is a major lipid constituent of many biological membranes. The cholesterol to phospholipid ratio varies considerably, with higher molar ratios (0.7 cholesterol to phospholipid) found in isolated plasma membranes compared to intracellular membranes[1]. Myelin membranes contain the highest (1.0) molar ratio[2].

Abbreviation: TES, *N*-tris(hydroxymethyl)methyl-2-aminoethane sulfonic acid.

The role of cholesterol in biological membranes is not well known, but recent evidence suggests that it plays an important role in the permeability and osmotic fragility of red cell membranes[3]. Such a role is quite consistent with the results of numerous studies on the effect of cholesterol on the physical properties of phospholipid membranes[4]. Studies with mixed monomolecular films have indicated that the presence of cholesterol reduces the area occupied by phospholipid molecules[5-7] (condensing effect). However the degree of condensation varies with the liquidity of the phospholipid films and it is observable only with films which are neither fully condensed nor fully expanded[8-10]. In studies with phospholipid suspensions in water, the addition of cholesterol restricts the motion of the CH_2 groups of the phospholipid hydrocarbon chains as shown by the broadening of the proton magnetic resonance spectrum[11], and by the restricted mobility of a spin label[12]. X-ray diffraction of phospholipid–cholesterol dispersions also indicates a smaller surface area per phospholipid molecule[13-15], as well as an increase in the thickness of the lipid layer[15]. In contrast to the above effects observed with phospholipids above their transition temperature (liquid-expanded state in monolayers), cholesterol tends to liquify phospholipid membranes which are normally below their crystalline to liquid–crystalline transition temperature. This effect can be observed by differential scanning calorimetry as the disappearance of the endothermic peak of the phospholipid at the transition temperature[16] and by X-ray diffraction as the appearance of a diffuse high-angle spacing at 4.45 Å, instead of a sharp 4.2 Å spacing[16]. In this case, the amount of bound water was observed to increase in the presence of cholesterol[16]. It thus appears that the addition of cholesterol to phospholipids modifies their molecular packing, and the mixed membranes are more condensed when compared to pure phospholipids above their transition temperature, and more fluid when compared to the pure phospholipids below their transition temperature.

Permeability studies with phospholipid vesicles and bilayers support and extend the above physical studies. It has been observed that cholesterol decreases the permeability of phospholipid vesicles to $^{36}Cl^-$ (ref. 17) and also to glucose[18] and other glycols and sugars[19,20]. Furthermore, it has been shown that bilayer membranes formed from solutions of phosphatidylcholine in decane containing cholesterol have decreased water permeability[21] and increased electrical capacitance[22,23] when compared to similar membranes without cholesterol.

The present report is a continuation of previous studies on the effect of cholesterol on electrical and permeability properties of phospholipid membranes[17,23]. This study involves the use of both vesicles and bilayers of different phospholipids with and without cholesterol. The measurements include electrical resistance, capacitance and also isotopic flux of Na^+, K^+, Cl^-, glucose and sucrose, at different temperatures. It is shown in Sections 1–3 that cholesterol decreases the permeability of the membranes to all solutes tested and also increases the electrical resistance as well as capacitance. The same effects can be observed with either neutral, negative or positively charged membranes. Furthermore, it is shown in Section 4, that cholesterol has a substantial effect on the Arrhenius activation energy for the ion diffusion rates. Section 5 deals with an analysis of Arrhenius plots allowing the energies of activation to be temperature dependent due to possible structural changes. Based on this analysis, a possible explanation of the effect of cholesterol on the activation energies is offered in terms of a slight membrane structure variation with temperature.

MATERIALS AND METHODS

Lipids

Most phospholipids used in this study were prepared and characterized in this laboratory. The isolation procedures were based on a combination of several methods and described in detail elsewhere[24]. All lipids were chromatographically pure and the fatty acid ester content was essentially similar to that in earlier studies[24]. Phosphatidylserine was isolated from beef brain. Some of the experiments with bilayers were done with phosphatidylserine samples purchased from Applied Science Labs. (State College, Pa.). Phosphatidylcholine was isolated from egg yolk, and phosphatidic acid by enzymatic hydrolysis of phosphatidylcholine by phospholipase D. Phosphatidylglycerol was prepared enzymatically from egg phosphatidylcholine by the method of Dawson[25]. Cholesterol (99 %) was obtained from Sigma Chemical Co. (St. Louis, Mo.) and recrystallized twice from methanol. Stearylamine was obtained from K and K laboratories (Planview, N.Y.). The later stages of the purification of phospholipids were performed under nitrogen in order to avoid air oxidation. The purified samples were stored under nitrogen in sealed ampoules at −50 °C. Each ampoule contained approx. 10–20 μmoles of phosphate (determined as P_i after $HClO_4$ digestion) in 1 ml of chloroform solution and was newly opened for each experiment.

Other chemicals

L-Histidine·HCl (Sigma Grade) and *N*-tris-(hydroxymethyl)methyl-2-aminoethane sulfonic acid (TES) were both obtained from Sigma Chemical Co. Sephadex G-50 (coarse) and Sepharose 4B were obtained from Pharmacia. $^{22}Na^+$, $^{36}Cl^-$, and [^{14}C]glucose and sucrose were obtained from New England Nuclear Corp. $^{42}K^+$ was obtained from Western New York Nuclear Center, as K_2CO_3 crystals. $^{86}Rb^+$ was obtained from ICN Corp. (Irvine, Calif.) as a solution in HCl. All other chemicals were reagent grade. Water was twice distilled, the second time in an all glass apparatus. The bilayer experiments were done in triple-distilled water, once over $KMnO_4$.

Preparation of phospholipid vesicles

The procedure was basically similar to that used previously[26,17]. The chloroform solution of either pure or mixed lipids was transferred from a newly opened ampoule to a glass tube which had been flushed with a stream of high purity nitrogen (Linde, H.P. Dry, 99.996 %). The chloroform was evaporated under vacuum while the tube was rotated by hand in a water bath at 22–24 °C. Nitrogen was then allowed to flow over the dried phospholipid, while 2.0–2.5 ml of a buffer solution containing the isotope (50–100 μCi per experiment) were pipetted into the tube. The buffer solution contained NaCl or KCl or RbCl (100 mM) and histidine (2 mM), TES (2 mM), EDTA (0.1 mM) adjusted to pH 7.4 by addition of 0.1 M NaOH. For the glucose or sucrose experiments, the solution contained also 20 mM of either compound. A small stream of nitrogen (previously saturated with water by passing through a gas-washing bottle) was passed through the tube during the rest of the procedure. The lipid was dispersed into the aqueous solution by mechanical (Vortex) shaking for 10 min. The suspension was then sonicated for 1 h by placing the tube in a small bath-type sonicator (Heat Systems, Model 5 × 5, 40 kHz) always under a stream of nitrogen. The temperature of the sonicating bath was maintained at approx. 22 °C by circulating

cold water. After the sonication, the suspension was almost clear ($A_{700\ nm} = 0.01\ mM^{-1} \cdot cm^{-1}$) and the ultraviolet spectrum showed no detectable oxidation[27]. The phospholipid was now in the form of small unilamellar vesicles[24,28] and was left at room temperature in a nitrogen atmosphere for 1 h before further processing. The sonication under the above conditions tends to minimize thermal and oxidative degradation and is preferable in our opinion to the use of probe-type sonicators.

Measurements of permeability

The procedure was essentially as described before[29,26] with minor modifications. The suspension of sonicated vesicles (or in some cases not sonicated, as specified in the text) was washed from the non-incorporated isotope by elution through a 2.7-g column of Sephadex (G-50, coarse) with the same buffer. The lipid peak, which was eluted with the void volume, was collected between 12 and 17 ml of eluting buffer. Aliquots of it (0.5 ml) containing 1–2 μmoles of phosphate, were transferred into small pre-washed dialysis bags (1.0 cm width, and approx. 6 cm length after tying). After a preliminary dialysis against 500 ml of buffer at 0 °C (either 1 h or overnight) the bags were transferred into individual tubes (1.2 cm internal diameter) filled with 10 ml of buffer at 10 different temperatures ranging from 0 to 52 °C. At specified time intervals, (1 h for Cl^- and sugars, 2 h for cations) the bags were transferred into new tubes at each temperature and the dialysis continued, usually for three consecutive intervals. The amount of radioactive tracer contained in each dialysate, and in the bag was determined in a liquid-scintillation counter. The results were expressed as the percentage of the total tracer present inside each bag at the beginning of the time period (% total). The equivalents of each solute diffusing per time per μmole of phospholipid (moles$\cdot h^{-1} \cdot$ mole^{-1}) were calculated on the basis of the percent total per time and the total equivalents captured per μmole of phospholipid. The flux in moles$\cdot cm^{-2} \cdot s^{-1}$ was then calculated on the basis of an area of 2000 cm^2/μmole of phospholipid (for pure phospholipids) and 2600 cm^2/μmole of phospholipid for 1:1 molar ratio of phospholipid to cholesterol. This calculation assumes: (a) that all the phospholipid is in unilamellar vesicles composed of bilayer membranes, (b) that the area per phospholipid molecule is 68 Å^2 (refs 15, 30, 31) and (c) that the combined area of one phospholipid *plus* one cholesterol molecule is 86.3 Å^2. The last assumption is based on a 25 % reduction of the phospholipid area in the presence of cholesterol, calculated from two separate sources[14,15]. No allowance is made for differences in number of molecules between the inside and outside face of the vesicle membrane[32]. The permeability coefficient in cm$\cdot s^{-1}$ is then obtained by dividing the flux values by the solute concentration in the bulk phase.

Measurements of conductivity and capacitance

The membrane forming solution was composed of 10 mg lipids dissolved in 0.5 ml of *n*-decane (Fluka, Switzerland, Purrum). In order to accelerate the mixing of cholesterol and phospholipids, the solution in decane was kept in a 35 °C bath for 0.5–1 h. The apparatus and technique of membrane formation were described previously[33]. The membrane resistance was obtained by measuring the current across the membrane during application of an external electrical potential. The results are expressed in units of $\Omega \cdot cm^2$ obtained by multiplying the membrane resistance by the area of the bilayer in cm^2. They are equivalent to the average resistivity multiplied

by the thickness of the membrane. The capacitance was measured with a universal bridge B221A, Wayne Kerr (frequency 10^4). The details for capacitance measurements are described in an earlier paper[23].

Calculation of E_a, ΔS^+, ΔF^+

From measurements at different temperatures it is possible to obtain activation energies for the permeation process, assuming that P, the permeability coefficient obeys an Arrhenius equation,

$$P = A \exp(-E_a/RT) \tag{1}$$

where A is some constant with respect to temperature, E_a is the activation energy, R is the gas constant, and T is the absolute temperature. By drawing log P *vs* $1/T$ one obtains a straight line, whose slope is $(-E_a/R)$. The observed linearity of the log P *vs* $1/T$ curve is thus both an indication that Eqn 1 is satisfied and a means to obtain the energy of activation. We determined E_a by using a linear least squares procedure which draws the best straight line from the point of view of minimizing the sum

$$\sum_{i=1}^{n} (Y_i - Y_{ic})^2 \tag{2}$$

where n is the number of data points, Y_i indicates a measured value, (of log P in our case) and Y_{ic} indicates a calculated value, *i.e.* the ordinate on the regression line. The results are given in Figs 4–7 and in Table I. We also include in our calculations the following statistical measures[34]:

$$\text{R.M.S.E.} = \left(\frac{\sum_{i=1}^{n} (Y_i - Y_{ic})^2}{n-2} \right)^{\frac{1}{2}} \tag{3}$$

$$\Delta(\text{slope}) = \frac{\text{R.M.S.E.}}{\left(\sum_{i=1}^{n} (X_i - \bar{X})^2 \right)^{\frac{1}{2}}} \tag{4}$$

$$\Delta(\text{intercept}) = \Delta(\text{slope}) \left(\sum_{i=1}^{n} X_i^2/n \right)^{\frac{1}{2}} \tag{5}$$

in which X_i stands for $1/T_i$ and $\bar{X}$ is the average of $1/T$ in the given range. R.M.S.E., Δ (slope) and Δ (intercept) are statistical evaluations of the deviations of the ordinates of points from the straight line, and of the errors in the slope and intercept, respectively. The estimated error in E_a is twice that of Δ (slope) in units of cal/mole, due to the R in the exponent in Eqn 1.

We tried to extend the information gained from the experiments by also calculating the entropy ΔS^+ and free energy of activation ΔF^+. In doing this we are however, forced to introduce some simplifying assumptions. The permeability coefficient is regarded[35] to be given by

$$P = KD/l \tag{6}$$

in which K is the partition coefficient, D is the diffusion coefficient for diffusion within the membrane, and l is the membrane thickness.

In employing Eqn 6 it is implicitly assumed that there is an equilibrium distribution of the solute between the membrane and external phases. This need not be a bad assumption in our case where we consider tracer fluxes at very low solute concentrations, assuming Fickian behavior. The approximation in which both the diffusion coefficient and partition coefficient are regarded as independent of position along the vesicle radius may be regarded as more critical due to the inhomogeneity along the axis perpendicular to the membrane plane[36,37], but so far there is no treatment which takes inhomogeneity into account. Therefore, K and D should be regarded as "average" quantitities.

We then apply an expression from the absolute reaction rate theory[38] for the diffusion coefficient,

$$D = \lambda^2(\boldsymbol{k}T/h) \exp(-\Delta F_d^+/RT) \tag{7}$$

in which λ is the distance between two equilibrium positions in the direction of the diffusional motion, $\boldsymbol{k}$ is Boltzmann's constant, h is Plank's constant and $\Delta F_d{}^+$ is the free energy for diffusion. K is given simply by

$$K = \exp(-\Delta F_s^+/RT) \tag{8}$$

where $\Delta F_s{}^+$ is the free energy change for the solubility process.
From Eqns 6–8 we obtain

$$P = \frac{\lambda^2 \boldsymbol{k}T}{lh} \exp(-\Delta F^+/RT) = \frac{\lambda^2 \boldsymbol{k}T}{lh} \exp(\Delta S^+/R) \exp(-\Delta H^+/RT) \tag{9}$$

in which,

$$\Delta S^+ = \Delta S_d^+ + \Delta S_s^+$$

$$\Delta H^+ = \Delta H_d^+ + \Delta H_s^+$$

and

$$\Delta F^+ = \Delta F_d^+ + \Delta F_s^+ \tag{10}$$

Since ΔS^+ appears in an exponential, an error by a factor of e = 2.78 in the evaluation of the term exp $(\Delta S^+/R)$, due to an error in λ, will bring in an error of only two entropy units, *etc.* Hence we used the same value of λ^2 for all diffusants and determined ΔS^+ from the intercept of the Arrhenius plot. For calculations of ΔS^+ and ΔF^+ we used the following values: l = 60 Å, λ = 3 Å, and T = 300 °K.

The evaluation of the error in ΔS^+ is given by Δ (intercept) × 2 in entropy units. When the temperature dependence is that of Eqn 7, E_a exceeds ΔH^+ by an amount of RT, *i.e.* 0.6 kcal/mole in our case (ref. 38). From a knowledge of E_a, we obtain $\Delta F^+ = E_a - T\Delta S^+ - RT$.

RESULTS

(1) *Effect of cholesterol on resistance and capacitance:*

The d.c. resistance of phosphatidylcholine–cholesterol and phosphatidylserine–cholesterol membranes formed in 0.1 M NaCl, with respect to the concentration (w/w

%) of the cholesterol in the membrane forming solution is shown in Fig. 1. It should be noted that the concentration of cholesterol in the bilayer membrane proper is not known, and it might not be the same as in the membrane forming solution. The d.c. resistance of phosphatidylcholine membranes increases with increasing amounts of cholesterol. The maximal value obtained with 60 % cholesterol ($4 \cdot 10^7\ \Omega \cdot cm^2$) is approx. 10 times higher than that of pure phosphatidylcholine membranes. The results are identical with or without EDTA (0.01 mM) in the aqueous phase. The resistance decreases as the concentration of cholesterol increases from 60 to 80 %. It was found difficult to form stable membranes from a solution containing more than 85 % cholesterol.

The behaviour of phosphatidylserine membranes is quite different, depending on the presence or absence of EDTA. When phosphatidylserine membranes are formed in 0.1 M NaCl without EDTA, the addition of cholesterol (up to 65 %) has a small effect, usually decreasing the resistance. However, if phosphatidylserine membranes are formed in 0.1 M NaCl with EDTA (0.01 mM) the effect of cholesterol is quite different and similar to that with phosphatidylcholine membranes. Thus, as the concentration of cholesterol is increased, the membrane resistance increases gradually from $3.5 \cdot 10^6$ to $1.2 \cdot 10^8\ \Omega\ cm^2$ at 65 % cholesterol concentration in the membrane solution.

The effect of cholesterol on the capacitance of phosphatidylserine and phosphatidylcholine membranes is shown in Fig. 2. With phosphatidylcholine and phosphatidylserine (with EDTA) membranes the capacitance increases gradually with increased cholesterol concentration, reaching a plateau at approx. 65 %. In the absence of EDTA, phosphatidylserine membranes respond again quite differently to cholesterol.

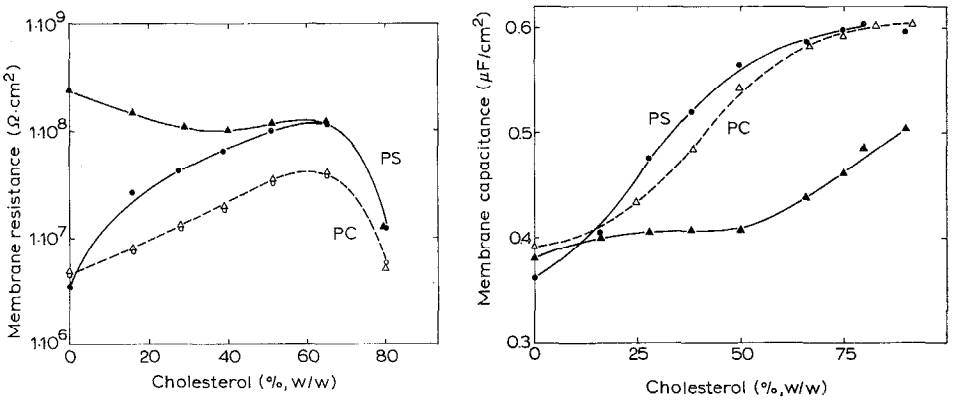

Fig. 1. Electrical resistance of phospholipid bilayers with different amounts of cholesterol in the membrane-forming solution. Aqueous phase: 100 mM NaCl, with or without 0.01 mM EDTA. Phosphatidylserine membranes, closed symbols: ●, with EDTA; ▲, without EDTA. Phosphatidylcholine membranes, open symbols: ○, with EDTA; △, without EDTA. The cholesterol concentration is expressed as a percentage of the total weight of phospholipid *plus* cholesterol in the decane solution.

Fig. 2. Electrical capacitance of phospholipid bilayers with different amounts of cholesterol in the membrane-forming solution. Aqueous phase: 100 mM NaCl, with or without 0.01 mM EDTA. Phosphatidylserine membranes, closed symbols: ●, with EDTA; ▲, without EDTA. Phosphatidylcholine membranes, open symbols: △, with and without EDTA.

There is very little increase in capacitance up to 50 % cholesterol concentration, and a rapid increase at higher concentrations.

The increase in membrane resistance with cholesterol indicates a "tighter" membrane. This result corresponds to the inhibition of CH_2 motion evidenced by proton magnetic resonance[11]; to the condensation effect of cholesterol on phosphatidylcholine monolayers; to the decrease in water permeability[21], and to the decrease in ion flux discussed below. The increase in capacitance confirms earlier observations[22,23]. Since work on X-ray diffraction of egg phosphatidylcholine membranes indicates only a small increase in the thickness of the lipid lamellae in the presence of cholesterol[15,37], the increased capacitance could only be attributed to an increase in the dielectric constant of the membrane. Calculations with cholesterol membranes (in the presence of hexadecylmethylammonium bromide gave an average value of 4.0 for the dielectric constant of a predominantly cholesterol membrane. Estimates for the dielectric constant of phospholipids range from 2.07 (ref. 39) to 2.3 (ref. 40). Thus the ratio of the dielectric constants of phospholipids and cholesterol corresponds approximately, to the ratio of the capacitances of the membranes formed with and without cholesterol as given in Fig. 2. Although the assumptions involved in the estimates of dielectric constants make a direct comparison with capacitance values rather tentative[39,40], the conclusions seem to be in the right direction. The effect of EDTA on phosphatidylserine membranes indicates that even small amounts of divalent metals, present either in the sample or in the salts used in making solutions, have a strong influence on resistance although much less on the capacitance (Figs 1 and 2). The lack of cholesterol effect in the absence of EDTA could indicate either that the cholesterol is not present in the area of the bilayer under these conditions, or that bivalent metal ions are released as cholesterol concentration increases.

(2) *Gel filtration of phospholipid vesicles*

In order to establish the degree of homogeneity and size distribution of the phospholipid vesicles used for permeability studies, the phospholipid suspensions were eluted through a Sepharose 4B column before and after sonication. The results are shown in Fig. 3. The phosphatidylserine suspension was prepared in a NaCl buffer containing tracer $^{22}Na^+$, as described in the Materials and Methods section. The elution was followed by estimation of total phosphate, and the amount of $^{22}Na^+$ present in each fraction. When the suspension was passed through the column before sonication, practically all the phosphate appeared with the void volume (Tubes 10–13, Fig. 3A). A small fraction of the $^{22}Na^+$ added initially to the buffer accompanied the phosphate peak. This is presumably the $^{22}Na^+$ incorporated inside the phosphatidylserine liquid crystals (liposomes). The non-incorporated $^{22}Na^+$ appeared much later (Tubes 31–39) as with the usual elution of the phosphatidylserine vesicles through Sephadex G-50. The ratio of the amount of $^{22}Na^+$ per μmole of phosphate is given in triangles in the same figure and it varies considerably along the phospholipid peak, indicating heterogeneity. Fig. 3B shows the results obtained with the same phospholipid eluted through the same column after 1 h of sonication. In this case the main phosphate peak appears in Tube 20 which is twice the void volume. The shoulder appearing in Tubes 11–14 represents approx. 7 % of the total phosphate. The ratio $^{22}Na^+$ per μmole of phosphate, also indicated in Fig. 3B is fairly constant along the main phosphate peak and much lower than the ratios

obtained with unsonicated suspsenions (Fig. 3A). The comparatively higher ratio found in the early part of the phosphate peak in this elution pattern probably reflects the presence of a small amount of larger vesicles or vesicles with more than one lamella. However, since the shoulder in the phosphate elution peak represents only a small percentage of the total phospholipid phosphorus, we consider in our calculations of the surface area per μmole of phospholipid that all the material is in the form of unilamellar vesicles. Fig. 3C shows the elution pattern of a sonicated suspension of phosphatidylserine containing cholesterol (1:1 molar ratio). The results are substantially similar to those obtained with pure phosphatidylserine, and the bulk of the phosphate eluted with a peak centered at Tube 19. The ratio of captured $^{22}Na^+$ per μmole of phospholipid was also fairly constant along the phosphate peak, with slightly higher ratios obtained in the area of the early shoulder (Tubes 11–13, fig. 3C).

The elution volume (two void volumes) obtained here with sonicated vesicles is identical to that reported earlier[41,32]. This indicates that either method tends to produce vesicles of similar size although there are some differences in terms of homogeneity. Thus egg phosphatidylcholine sonicated with probe type sonicators was eluted from Sepharose 4B in two separate peaks as reported by Huang[41], or in one delayed peak with variable ratio of captured volume per μmole of phospholipid as

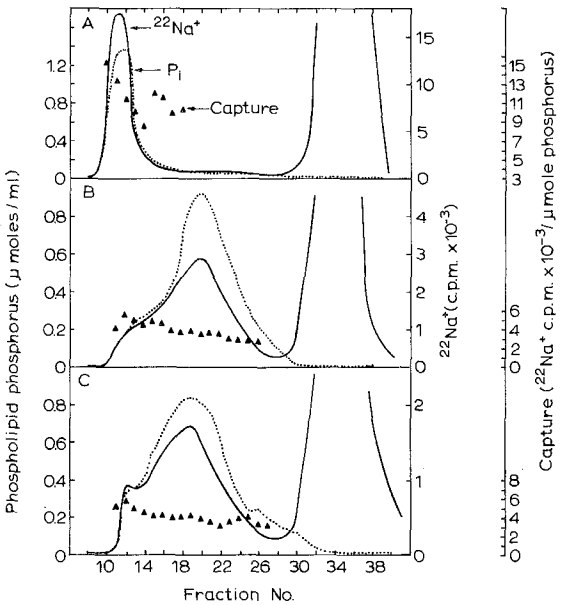

Fig. 3. Elution of phospholipid vesicles through Sepharose 4B columns. Aqueous phase: 100 mM NaCl, 2 mM histidine, 2 mM TES, 0.1 mM EDTA (pH 7.4) containing $^{22}Na^+$ tracer. Column dimensions: 1.5 cm × 29 cm. Equilibrated and eluted at room temperature (22–24 °C) by gravity. Sample volume: 2.0 ml. Amount of phosphate: 11 μmoles per sample. Fraction volume: 1.1 ml. Flow rate: 3 min per fraction. Recovery of applied phosphate: 70–80%. A. Phosphatidylserine suspended by mechanical agitation. B. Phosphatidylserine suspended by sonication (1 h) as described in Materials and Methods. C. Equimolar mixture of phosphatidylserine and cholesterol suspended by sonication as above. ———, elution profile of $^{22}Na^+$; ....., elution profile of total phosphorus; ▲, ratio of $^{22}Na^+$ per μmole of phosphorus, indicating in a comparative fashion the amount of isotope captured within the vesicles. The amount of isotopic Na^+ ($^{22}Na^+$) is expressed as cpm × 10^{-3}.

reported by Kornberg and McConnell[32]. In our case over 90 % of the phospholipid was eluted in the delayed peak, with a fairly constant capture per μmole of phospholipid. The difference is perhaps due to the absence of an excess negative charge in phosphatidylcholine which might render this phospholipid more difficult to sonicate to the minimal size vesicles. It has been reported that the presence of salts increases the time for complete sonication of phosphatidylcholine (ref. 42). In any case there seems to be a general agreement concerning the size of the small vesicles, which seem to be spheroidal particles of 250–500 Å outside diameter[17, 41, 43]. The captured volumes of such vesicles can be calculated assuming a spherical shape and a membrane thickness of 50 Å. Thus, for an inside radius of 75 Å (corresponding to 250 Å outside diameter) the internal volume is $1.77 \cdot 10^6$ Å^3 and for a radius of 100 Å (300 Å outside diameter), the volume is $4.2 \cdot 10^6$ Å^3 per vesicle. Assuming a particle weight of $2 \cdot 10^6$ daltons (ref. 41) and a molecular weight of 770, the number of phospholipid molecules per vesicle is approx. $2.6 \cdot 10^3$ or $4.3 \cdot 10^{-15}$ μmole. It then follows that the captured volume for each μmole of phospholipid should be 0.4 μl for a 250 Å diameter sphere and 1.0 μl for a 300 Å diameter sphere. The captured volume of phosphatidylserine vesicles used in this study was estimated as 1.0 μl on the basis of [^{14}C]sucrose capture experiments (0.02 μmole sucrose/μmole in a solution containing 20 μmoles of sucrose per ml). This calculation assumes that there is no adsorption of sucrose on the surface of the vesicles which is supported by equilibrium dialysis experiments. Although there is a good agreement between the expected and estimated captured volume for small spheres (300 Å outside diameter), the same captured volume could correspond to larger diameter spheroids of flattened or perhaps biconcave shape.

The captured volume depends greatly on the excess fixed charge of the phospholipids. Thus the captured volume for disaccharides for sonicated phosphatidylcholine vesicles was found[32] to be only 0.1 μl/μmole, a value which is in substantial agreement with results obtained in this laboratory with the same phospholipid. A flattened biconcave sphere has been proposed in order to accommodate this small captured volume[32]. The amount of cations captured by phosphatidylserine vesicles in the present study is approx. 0.2 μmole/μmole of phospholipid; the same for Na^+, K^+ and Rb^+. The capture for anions is much smaller, 0.018 μmole/μmole, slightly less than the capture for sucrose. The cation capture is of course much higher compared to anions and uncharged molecules since they (cations) participate as counterions in the double layer of the anionic phospholipid surface.

(3) Effect of cholesterol on isotopic flux of Na^+, K^+, Cl^- and glucose

In all cases we have studied so far, cholesterol substantially reduces the diffusion rate of cations, anions and glucose. The magnitude of the decrease varies with temperature as will be discussed later. Table I gives the diffusion rates at 36 °C for phosphatidylserine vesicles with and without cholesterol (1:1 molar ratio). In general, the diffusion rates for glucose, Cl^- and Na^+ in presence of cholesterol are approximately half the rates with pure phosphatidylserine. Although most of the studies with vesicles were done with phosphatidylserine, the effect of cholesterol on Na^+ diffusion rates was studied with other phospholipids also, for comparative purposes. For studies with phosphatidylcholine, vesicles were made with either 4 % phosphatidic acid or 2 % stearylamine in phosphatidylcholine, with or without cholesterol. As

TABLE I

SELF-DIFFUSION RATES AND ACTIVATION ENERGIES FOR VARIOUS SOLUTES THROUGH PHOSPHOLIPID VESICLES

Lipid *	*Solute* **	*Self-diffusion rate at 36 °C (%/h)*	*P at 36 °C (cm/s) × 10⁻¹²*	*Temp. range (°C)*	E_a *(kcal/mole)* ***	ΔS^+ *(Cal/degree/mole)* ***	ΔF^+ *(300 °K) (kcal/mole)* ***	*R.M.S.E.* ***
PS	Glucose (1)	53.5	41.3	12–42	19.6 ± 0.6	− 2.6 ± 2	19.9 ± 1.1	0.08
PS–Chol	Glucose (1)	13.5	17.5	24–52	22.5 ± 0.3	5.0 ± 1.1	20.5 ± 0.7	0.4
PS	Cl^- (2)	25.1	6.5	0–36	13.6 ± 0.2	−26.0 ± 0.6	20.8 ± 0.4	0.04
PS–Chol	Cl^- (2)	11.0	3.7	6–42	16.9 ± 0.5	−16.6 ± 1.8	21.3 ± 1.1	0.1
PS	K^+ (3)	0.3	0.91	30–52	30.4 ± 2.0	24.3 ± 6.5	22.5 ± 3.9	0.3
PS–Chol	K^+ (3)	0.045	0.047	12–42	12.8 ± 0.6	−38.2 ± 2.0	23.7 ± 1.2	0.1
PS	Na^+ (4)	0.058	0.16	30–52	27.0 ± 1.4	9.6 ± 4.5	23.5 ± 2.7	0.2
PS–Chol	Na^+ (4)	0.018	0.05	18–52	14.0 ± 2.4	−34.6 ± 7.9	23.7 ± 4.8	0.3
PS	Rb^+ (5)	0.26		23–47	29 ± 1			
PS	Sucrose (6)	0.22	0.3	36–52	25 ± 3			
PA–PC	Na^+ (7)	0.17	0.11	23–42	16.2			
				36–51	24.4			
PA–PC–Chol	Na^+ (7)	0.07	0.10	25–47	16.0			
Stam–PC	Na^+ (7)	0.12	0.05	25–52	21 ± 2			
Stam–PC–Chol	Na^+ (7)	0.08	0.07	25–52	14.5 ± 2			
PG	Na^+ (4)	0.46	1.5	24–52	26 ± 1			
PG–Chol	Na^+ (7)	0.07	0.2	24–47	18.5 ± 2			

* Abbreviations: PS, phosphatidylserine; Chol, cholesterol; PA, phosphatidic acid; PC, phosphatidylcholine; Stam, stearylamine; PG, phosphatidylglycerol.

** The figures in parentheses represent the following: (1) Average of two experiments. 1-h dialysis period at each temperature, immediately following an initial 1-h dialysis at 0 °C. Individual points shown in Fig. 7. (2) Results for phosphatidylserine are averages of three experiments. Results for phosphatidylserine/cholesterol are averages of two experiments. In both cases, the dialysis period was 1 h, immediately following an initial overnight dialysis at 0 °C under nitrogen. Individual points shown in Fig. 6. (3) Average of two experiments. 2-h dialysis period at each temperature. Preliminary dialysis: overnight at 0 °C under nitrogen and 2 h equilibration at each temperature. Individual points shown in Fig. 5. (4) Results for phosphatidylserine are averages of three experiments. Results for phosphatidylserine/cholesterol are averages of two experiments. In both cases, 2-h dialysis period at each temperature. Preliminary dialysis: overnight at 0 °C under nitrogen and 2 h equilibration at each temperature. Individual points shown in Fig. 4. (5) Single experiment. 2-h dialysis period at each temperature (3 points). Preliminary dialysis: overnight at 0 °C under nitrogen and 2 h equilibration at each temperature. (6) Average of two experiments. 2-h dialysis period at each temperature. Preliminary dialysis: 1 h at 0 °C. Four temperature points within the average shown. (7) Single experiments. 2-h dialysis period at each temperature. Preliminary dialysis: overnight at 0 °C under nitrogen and 2 h equilibration at each temperature. Six temperature points within the range shown.

*** The errors are defined by Eqns 3–5.

shown in Table I, cholesterol had qualitatively the same effect (decrease) as with phosphatidylserine. Similar results were also obtained with pure phosphatidylglycerol with and without cholesterol as indicated in Table I. It thus appears that the effect of cholesterol in decreasing the permeability is independent of surface charge and particular head groups since it is observed with various negatively (phosphatidic acid/phosphatidylcholine, phosphatidylserine, phosphatidylglycerol) as well as positively (stearylamine/phosphatidylcholine) charged membranes.

The above results correlate well with the effect of cholesterol in decreasing electrical conductance of phospholipid bilayers discussed in Section 1. The actual diffusion rates at 36 °C are of considerable interest and several useful comparisons can be made: (a) The diffsion rates of all solutes studied (cations, Cl^- and glucose) are much lower to comparable values from biological membranes[44]. (b) The permeability coefficient for glucose diffusion through phosphatidylserine vesicles is close or even lower than the values obtained with bilayer membranes[45,46]. (c) The permeability coefficient for Na^+ through phosphatidylserine vesicles ($1.6 \cdot 10^{-13}$ cm/s) and 4 % phosphatidic acid in phosphaditylcholine vesicles ($1.1 \cdot 10^{-13}$ cm/s) obtained in this study compares well with values obtained[47] for K^+ through similar vesicles ($3.3 \cdot 10^{-13}$ cm/s). (d) There is a considerable difference between the permeability of Na^+ and K^+ through phosphatidylserine vesicles. This phenomenon was reported recently in detail[48] and it appears that of several phospholipids studied so far, only phosphatidylserine and phosphatidylglycerol exhibit substantial difference in permeability rates in favor of K^+ over Na^+. Previous reports indicating lack of Na^+-K^+ discrimination[17,49] could be rationalized as the result of either autooxidation or use of mixtures of phospholipids (or both).

Using the treatment of Hodgkin[50] relating ionic flux to conductance, one can estimate a value of the electrical resistance across the vesicle membranes. Assuming that the current is carried predominantly by cations, the resistance across phosphatidylserine vesicles would be $1.7 \cdot 10^{10}\ \Omega \cdot cm^2$ for Na^+ and $2.7 \cdot 10^9\ \Omega \cdot cm^2$ for K^+. On the other hand if the charge carrying ion is Cl^- the resistance would be much lower, $4 \cdot 10^8\ \Omega \cdot cm^2$. However the transference numbers for conductance across phospholipid bilayer indicate that cations are the predominant charge carrying species[49,51]. This apparent discrepancy between isotopic fluxes with vesicles indicating higher anionic flux, and bilayers indicating cation permselectivity was resolved by Pagano and Thompson[51] who demonstrated that the Cl^- flux through phospholipid bilayer membranes is electrically silent. The actual molecular species involved during Cl^- diffusion is still not known and our reference to $^{36}Cl^-$ fluxes does not imply that $^{36}Cl^-$ is transported as Cl^-.

The calculated electrical resistance for phosphatidylserine vesicles based on cation flux (10^9–$10^{10}\ \Omega \cdot cm^2$) is considerably higher than the bilayer resistance reported here for the same phospholipid and also to those obtained by other laboratories[52]. However it is worth noting the careful study by Hanai *et al.*[53] which indicated that the true bilayer resistance, (not including border "leaks") is $10^9\ \Omega \cdot cm^2$ or more. In any case it is possible that the measured electrical resistance of bilayers includes other charge carrying species, *i.e.* H^+, OH^- or e^-. The electrical resistance through phosphatidylglycerol vesicles, calculated similarly from isotopic flux of Na^+ in this study is $6 \cdot 10^8\ \Omega \cdot cm^2$. This value compares favorably with the resistance of phosphatidylglycerol bilayers in NaCl (10^8–$10^9\ \Omega \cdot cm^2$)[54].

(4) Temperature studies on phospholipid vesicle permeability

The permeability of phosphatidylserine and other phospholipid vesicles for Na^+, K^+, Cl^-, glucose, and sucrose was studied at several temperatures within the range of 0–52 °C. The results are shown in Figs 4–7, where the logarithm of the permeability coefficient is plotted against the inverse absolute temperature (Arrhenius plots). Values of the activation energy (E_a) for different systems are given in Table I. The two sets of points in each figure represent permeability coefficients with and without cholesterol. It is immediately apparent that cholesterol has very different effect on the activation energy of glucose and Cl^- on one hand, and the cations on the other. Thus, only a slight increase in E_a is observed for glucose or Cl^- through phosphatidylserine vesicles, from 19.6 to 22.5 kcal/mole and from 13.6 to 16.9 kcal/mole, respectively. In contrast, the presence of cholesterol decreases the E_a for K^+ and Na^+ from 30.4 and 27.0 kcal/mole to 12.8 and 14.0 kcal/mole, respectively. This substantial decrease in activation energy for cation permeability was also observed with other phospholipid vesicles such as 4 % phosphatidic acid in phosphatidylcholine and 2 % stearylamine in phosphatidylcholine as shown also in Table I. Although all the above systems were sonicated, substantially similar results were

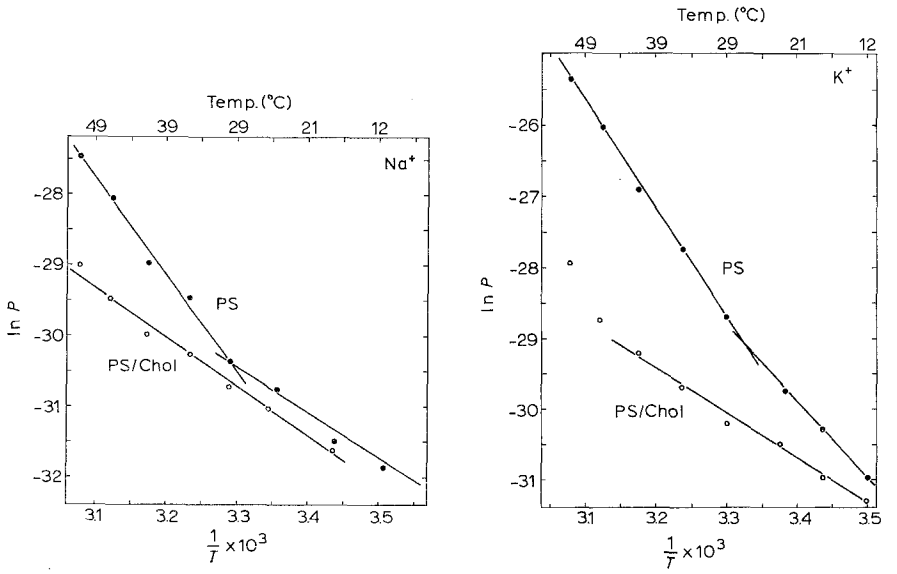

Fig. 4. Arrhenius plots for Na^+ permeability coefficients (P) through phosphatidylserine vesicles with and without cholesterol. Both systems were suspended in 100 mM NaCl solution as in the legend for Fig. 3, and sonicated for 1 h as in Materials and Methods. Points represent averages of two or three separate experiments and a 2-h dialysis period. Preliminary dialysis included an overnight period at 0 °C under nitrogen and an initial 2-h equilibration at each temperature. ●, pure phosphatidylserine; ○, equimolar mixture of phosphatidylserine with cholesterol. P in cm/s.

Fig. 5. Arrhenius plots for K^+ permeability coefficients (P) through phosphatidylserine vesicles with and without cholesterol. Both systems were suspended in 100 mM KCl solution containing buffer and EDTA as in Fig. 3, and sonicated for 1 h as in Materials and Methods. Points represent averages of two experiments and a 2-h dialysis period. Preliminary dialysis included an overnight period at 0 °C under nitrogen and an initial equilibration of 2 h at each temperature. ●, pure phosphatidylserine; ○, equimolar mixture of phosphatidylserine with cholesterol. P in cm/s.

obtained for Na^+ with non-sonicated phosphatidylserine and phosphatidylserine/cholesterol multilamellar vesicles.

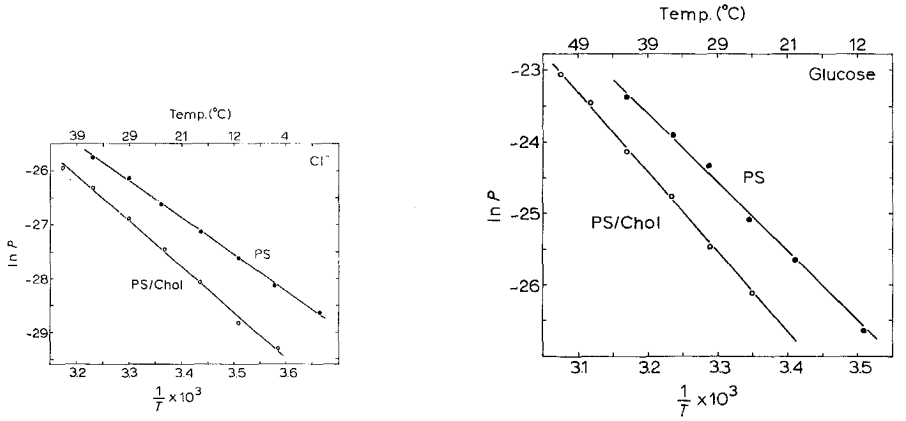

Fig. 6. Arrhenius plots of Cl^- permeability coefficients (P) through phosphatidylserine vesicles with and without cholesterol. Both systems were suspended in 100 mM NaCl solution as in Fig. 3 and sonicated for 1 h as in Materials and Methods. Points represent averages of two or three experiments and a 1-h dialysis period, immediately following an initial overnight dialysis at 0 °C under nitrogen. ●, pure phosphatidylserine; ○, equimolar mixture of phosphatidylserine with cholesterol. P in cm/s.

Fig. 7. Arrhenius plots of glucose permeability coefficients (P) through phosphatidylserine vesicles with and without cholesterol. Both systems were suspended in 100 mM NaCl containing 20 mM glucose, 2 mM histidine, 2 mM TES and 0.1 mM EDTA at pH 7.4. Sonicated for 1 h as in Materials and Methods. Points represent averages of two experiments and a 1-h dialysis period, immediately following an initial 1-h dialysis at 0 °C. ●, pure phosphatidylserine; ○, equimolar mixture of phosphatidylserine with cholesterol. P in cm/s.

The decrease in E_a for cation diffusion in the presence of cholesterol is an interesting and intriguing phenomenon for the following reasons:

(a) It is not observed with Cl^- or glucose, indicating a basic difference in the mechanism for diffusion between cations and uncharged molecules or anions. This point will be further elaborated in the following section.

(b) A decrease in the energy of activation accompanying a decrease in permeability (the effect of cholesterol on cations) corresponds to a large decrease in the entropy of activation, as shown in Table I. This can be interpreted as the result of a more "ordered" structure produced by cholesterol, which is in reasonable agreement with proton magnetic resonance and monolayer studies. However, the intriguing point is that cholesterol does not show the same effect with Cl^- and glucose. De Gier *et al.*[55] have recently observed that the E_a for the diffusion of several non-electrolyte species through multilamellar phospholipid vesicles was substantially the same in saturated, unsaturated, or cholesterol containing system.

(c) The true rate-limiting step for the diffusion across interfaces is characterized by the energy of activation (ΔF^+) which can be calculated from the values of ΔS^+ and E_a. As shown in Table I, the ΔF^+ for either permeant species is only slightly affected by the presence of cholesterol and is in all cases 20–23 kcal/mole. It is thus reasonable to conclude that the apparent high E_a for cation diffusion is due to structural changes within the membrane. As will be discussed in the following section, it is

possible that small, reversible, temperature-dependent changes, such as "thinning" of the lipid bilayer[6,7,31], could account for the steep slopes (large E_a values) obtained at higher temperatures, and for the deviation from linearity in the Arrhenius plots for cation diffusion. The presence of cholesterol would then produce a decrease in the apparent E_a by inhibiting such temperature-dependent structural changes.

(d) It is worth noting here that the Arrhenius plots for the cation diffusion show an inflection at approx. 30 °C. Furthermore, the slope at higher temperatures is dependent on time, becoming steeper with each 1-h time interval. This is not the case for the slopes for Cl^- and glucose with or without cholesterol, and for the slopes for cations with cholesterol. Fig. 8 shows the changes with time for K^+ and Na^+ permeability through phosphatidylserine vesicles. The data presented in Table I and Figs 4–7 concerning cation diffusion, represent the results obtained after an initial 2-hour interval at these temperatures, when the differences in slope between pure phospholipids and phospholipid–cholesterol mixtures are maximized. The activation energies (E_a) were calculated from the linear part of the curves starting from 30 to 52 °C. The points between 0 and 30 °C follow a more shallow line and were not considered in the calculations.

We intentionally stressed the structural changes which seem to be more pronounced at higher temperatures by drawing two lines with an inflection point at approx. 30 °C. It should be emphasized that the appearance of a sharp inflection between the lines might be the result of having only a discrete number of points. The structural changes as reflected by the increase in the slope at higher temperatures seem to be time dependent. Thus, the inflexion is more difficult to detect at the initial time intervals of dialysis and particularly in the system of 4 % phosphatidic acid in phosphatidylcholine. Johnson and Bangham[47] reported no inflexion with such vesicles for K^+ diffusion. However, similar inflexion points were reported earlier for the diffusion of Na^+ and K^+ through phosphatidylserine membranes[56].

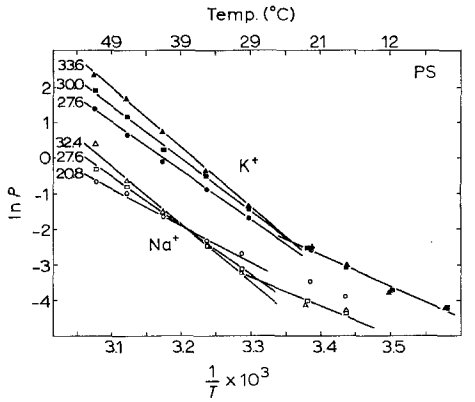

Fig. 8. Arrhenius plots for Na^+ and K^+ self-diffusion rates through phosphatidylserine vesicles at different time intervals. Both systems were suspended in 100 mM NaCl or KCl containing buffer and EDTA as in Fig. 3. Sonicated for 1 h as in Materials and Methods and dialysed overnight at 0 °C under nitrogen. Each set of points represents an individual experiment, and three consecutive 2-h dialysis periods. Open symbols Na^+, and closed symbols K^+. ● and ○, first time interval; ■ and □, second time interval; ▲ and △, third time interval. Self diffusion rates were expressed as natural logarithms of the percent diffusion at each 2-h interval. The numbers adjacent to the lines represent the activation energies (E_a) obtained from the slope of each line between 29 and 52 °C.

Prolonged dialysis at elevated temperatures raises the possibility of irreversible structural changes due to auto-oxidation. In order to investigate this possibility we decreased the temperature of a phosphatidylserine/Na^+ system after an initial dialysis of 6 h at 52 °C down to 0 °C. Following this treatment, these vesicles were exhibiting Na^+ permeability identical to other vesicles that were dialysing at 0 °C from the beginning of the experiment. It thus appears that no irreversible changes in permeability were occurring during the experimental times even at the highest temperature studied, indicating no irreversible degradative structural changes.

(5) *Effect of slight temperature dependence of the E_a on analysis of the Arrhenius plots*

In the previous section, the Arrhenius equation, Eqn 1, was used to calculate the energy of activation (E_a) assuming that E_a is temperature independent. The linear fit of the experimental results is usually taken as evidence that there are no phase transitions or structural changes within the given temperature range and that the slope represents the activation energy for permeation. As will be shown in this section, a slight temperature dependence of the true E_a for permeation produces quite misleading results without affecting substantially the linearity of the Arrhenius plot. The following treatment applies to any process described by Eqn 1, although in presenting the analysis we have in mind its applicability to the transport process through bilayer phospholipid membranes.

It has been shown that minor structural changes with temperature occur in phospholipid membranes at temperatures above their transition point from solid to liquid-crystalline. These structural changes are indicated by "thinning" of the hydrocarbon layer and a more liquid-like behaviour of the membranes[57,31]. It would be expected that such changes may lead to a slight decrease in the enthalpy of permeation with temperature. We will show that, paradoxically, the experimentalist who tries to obtain the enthalpy of activation (or E_a) from the Arrhenius plot, may obtain high, unrealistic values, although his experimental points may seem to be giving a good linear fit.

When E_a in Eqn 1 is temperature dependent, it follows that

$$\frac{\mathrm{d}\log P}{\mathrm{d}(1/T)} = -\frac{E_a}{R} + \frac{T}{R}\frac{\mathrm{d}E_a}{\mathrm{d}T}$$

$$= -\frac{1}{R}\left(E_a - T\frac{\mathrm{d}E_a}{\mathrm{d}T}\right) \qquad (11)$$

Thus, the absolute magnitude of the slope of the log P *vs* $1/T$ curve is smaller or greater than E_a/R, depending on whether the value of $\mathrm{d}E_a/\mathrm{d}T$ is positive or negative, respectively. For a slight decrease of E_a with increasing T (as may be the case for phospholipid bilayer membranes) the increase in the absolute magnitude of the slope over E_a/R may be quite significant.

For a specific example, let E_a decrease by 1 kcal/mole over a 40 °C temperature interval between 280 and 320 °K. The value of $|\mathrm{d}E/\mathrm{d}T|$ is then 0.025 kcal·mole^{-1}·degree^{-1}. For T = 300 °K the term $-T(\mathrm{d}E/\mathrm{d}T)$ equals 7.5 kcal/mole. If the value of E_a at 300 °K is around 10 kcal/mole, the E_a values obtained from a linearization

of log P *vs* $1/T$ plots would be in this case almost twice the real E_a values in this temperature range.

In order to investigate the problem numerically, a program was written for computer analysis. The program first calculates according to Eqn 1 values of P and log P at a given value of $1/T$ for an arbitrary value of A (which is of no relevance) and a given functional form of $E_a(T)$. In the second stage a least squares procedure is applied, which results in the slope and intercept of the straight line which fits best the curve of log P *vs* $1/T$. The output also includes the deviations of the points from the straight line and some statistical measures which are included in Table II, (see Eqns 3–5).

The results listed in Table II are based on 40 points. E_a values are considered to decrease linearly in the given range of temperature and the number of subintervals for the E_a is also given in Table II. Thus, when the number of subintervals is 20 and E_a decreases from 10 to 9 kcal/mole over the range of 40 °C, the decrease per subinterval is 0.05 kcal/mole. The results are in accord with the analysis of Eqn 11. For instance, when E_a decreases by 1 kcal/mole (from 10 to 9 kcal/mole) the resulting E_a value from the linearization of the log P *vs* $1/T$ plot turns out to be 16.99 kcal/mole, *i.e.* approx. 7.5 kcal/mole higher than the value of 9.5 kcal/mole at 300 °C

It is instructive to compare the goodness of the fit of the linearized calculated plots as listed in Table II with the corresponding experimental plots for diffusion through phospholipid vesicles (Table I). For a decrease of E_a by less than 1 kcal/mole in the range of 280–320 °K the computed plots (Table II) show better linear fit than the experimental curves shown in Table I. It is interesting to point out that the statistical measure Δ(slope) $\times$ 2 (0.05 kcal/mole) is very close to the uncertainty of the E_a in the interval (0.05 kcal/mole per subinterval).

In the above analysis we have ignored the temperature dependence of A in Eqn 1. It has been pointed out[38] that when A is proportional to T, the activation energy obtained from the plot of log P *vs* $1/T$ exceeds ΔH, the enthalpy of activation by the amount RT, which is 0.6 kcal/mole for $T = 300$ °K. Thus, the exact temperature dependence of A, the preexponential factor is only of minor importance (numerically) in this analysis.

In summary it is remarkable that the activation energy values obtained from the computed Arrhenius plots are substantially higher than the real values, in accord with the prediction of Eqn 11. Our analysis of the Arrhenius plots predicts the occurrence of paradoxically high values of E_a for liquid-like membranes, substantially higher than in the case of more rigid membranes. In view of this possibility, experimentally obtained E_a values or enthalpies of permeability coefficients for diffusion through bilayer and perhaps biological membranes should be critically examined.

For the sake of generality, it should be mentioned that ΔS^+ might also be temperature dependent. When such an effect is to be taken into account the slope of the log P *vs* $1/T$ curve will include an additional term $-T^2/R\ \mathrm{d}(\Delta S^+)/\mathrm{d}T$.

When any one of the quantities ΔS^+ or ΔH^+ is temperature dependent, a correlation between them may be expected. However, a study of such correlation may be performed only when much more data are available.

DISCUSSION

(1) Permeability coefficients for the membranes studied here are very sensitive

TABLE II

APPARENT ACTIVATION ENERGIES OBTAINED FROM A LINEARIZATION OF log P *vs* $1/T$ PLOT WHEN ACTIVATION ENERGIES DECREASE WITH TEMPERATURE

Details, as in text (Section 5 of Results).

E_a (T_1) (*kcal/mole*)	E_a (T_2) (*kcal/mole*)	*Computed* E_a (*kcal/mole*)	*R.M.S.E.* *	Δ (*slope*) × 2 (*kcal/mole*) **	T_1 (°K)	T_2 (°K)	*Number of sub-intervals*	*Max. decrease of* E_a *per sub-interval* (*kcal/mole*)
10	9.9	10.74	0.007	0.009	280	320	20	0.005
10	9.5	13.49	0.02	0.025	280	320	20	0.025
10	9.0	16.99	0.04	0.05	280	320	20	0.05
10	8.0	23.98	0.08	0.1	280	320	20	0.1
15	5.0	109.5	2.2	3	280	315	7	2

* See Eqn 3.
** See Eqn 4.

to the charge of the ion, being larger for the case of the anion Cl^- by more than an order of magnitude compared to monovalent cations. As has been shown elsewhere[51] the $^{36}Cl^-$ flux through phosphatidylcholine bilayers is electrically silent, indicating that Cl^- is diffusing through the membrane uncharged or as ion-pair. It has been proposed[51] that Cl^- could be diffusing through the bilayer lipid membranes as a complex with lecithin and a heavy metal. In a very recent review[68], Bangham discusses the different possiblilities and concludes that the $^{36}Cl^-$ fluxes could involve the diffusion of either HCl or of some Cl^- complex. Therefore any remark in this paper concerning $^{36}Cl^-$ fluxes is not meant to imply that $^{36}Cl^-$ crosses the membranes as Cl^-. In any event our observations in this paper indicate that the permeability properties of Cl^- are more in accord to those of glucose than of the monovalent cations. It should be noted, however, that membranes composed of phosphatidic acid or phosphatidylinositol do show higher permeability to cations (K^+ or Na^+) compared to Cl^- (ref 17).

(2) A comparison between glucose and sucrose permeability indicates that the smaller molecule (glucose) diffuses faster by two orders of magnitude. This follows the general trend observed with many uncharged molecules through both biological and artificial membranes[44,58,59]. The activation energy of sucrose diffusion is considerably larger than that of glucose through the same membranes.

(3) A comparison between glucose and ion diffusion shows that although glucose is a larger molecule, its permeability coefficient is approx. 6, 40, and 250 times larger than that of Cl^-, K^+ and Na^+, respectively. Several other studies also indicate that the permeability coefficients of uncharged molecules are much larger than those of charged molecules of similar molecular weight[61,62]. It is reasonable to consider that the diffusion process includes two stages: one, dissolution into the membrane matrix and two, diffusion through the membrane[60,55]. It is also reasonable to assume that the second step would depend mostly on the size of the diffusant if the diffusional process does not involve specific chemical interactions. Therefore, in order to rationalize the differences in the permeability coefficients between glucose and Cl^- (or Na^+) it can be stated that the first step (which involves the partition coefficient) is the rate limiting step for the process of diffusion. The same conclusion was reached, on independent arguments, for the diffusion of K^+ (ref. 60) and several glycols[55] through phospholipid vesicles.

(4) Upon the addition of cholesterol to phospholipid membranes (1:1 molar ratio) the permeability coefficients and electrical conductivity tend to decrease. This is in agreement with earlier studies indicating a corresponding drop in permeability to water[21], Cl^- (ref. 17), glucose[18], and other uncharged molecules[19,20]. This effect is compatible with the decrease in molecular motion (observed by proton magnetic resonance[11]) and an increase in packing (observed with monolayers) produced by cholesterol. However, as shown in the present study, the permeability at 36 °C decreases by a factor of 4–18 for the cations, and only by a factor of 2 for glucose and Cl^-. In view of the conclusion in Section 3, we may also conclude that cholesterol affects the first step (dissolution into the membrane) more than the second. If the second step (diffusion through the membrane matrix) was critically affected by the introduction of cholesterol, we would expect a larger decrease in the permeability coefficient of the larger molecules, *e.g.* glucose, than for the cations. This argument is strengthened by considering the effect of cholesterol on the permeability of glucose and two other uncharged molecules, water[21] and glycerol[19]. Again we observe that

the decrease in permeability is larger in the case of the smaller molecules (water by a factor of 4 and glycerol by a factor of 5–6). Therefore, the effect can be rationalized as due primarily to a decrease in the partition coefficient.

The decrease in conductivity (approximately one order of magnitude, see Fig. 1) is numerically similar to the decrease in cation permeability. Consistent with the argument elaborated above, it would appear that both phenomena may be explained in terms of a drop in the cation partition coefficient brought about by the presence of cholesterol.

(5) Cations (Na^+, K^+, Rb^+) show an apparent paradoxical temperature behavior with phospholipid membranes; the activation energies being very high, in the range of 27–30 kcal/mole. These values are much smaller in the presence of cholesterol whereas the permeability coefficients in this case also are smaller. A plausible explanation to this has been given in Section 4 and can be summarized as follows: liquid–crystalline phospholipid membranes undergo slight structural changes with increasing temperature which have been observed as "thinning" of the hydrocarbon layer by X-ray diffraction[31,57]. These structural changes could result in a small gradual decrease in the activation energy with temperature. However, as shown in Section 5, a small gradual decrease shows up as a big increase in the value of the activation energy obtained from the linearization of the log P *vs* $1/T$ plot. Cholesterol would tend to inhibit such temperature-dependent structural changes, (as it inhibits phase transitions from solid to liquid–crystalline[4]). Consequently the activation energy obtained from the same plot in the presence of cholesterol is substantially lower and perhaps closer to its true value.

It has already been shown by X-ray diffraction that phospholipid bilayer membranes containing cholesterol are thicker at room temperature[13–15] compared to pure phospholipid membranes. The thinning of the bilayers with temperature (up to 40 °C) is also observed in the presence of cholesterol but to a smaller extent. (P. Rand, private communication). It is conceivable that at higher temperatures, structural changes may be quite pronounced with cholesterol-containing membranes as well.

(6) The free energy for permeation, *i.e.* $\Delta F^+ = \Delta H^+ - T\Delta S^+$ can be considered as the true rate limiting function. The Arrhenius activation energy discussed above is numerically near to the enthalpy of activation (ΔH^+). In all cases studied here, the values of free energy for permeation are in the range of 20.0–23.7 kcal/mole. They show a slight increase of 0.2–0.5 kcal/mole for each permeant upon introduction of cholesterol. The values of ΔF^+ shown in Table I are monotonically decreasing functions of the permeability coefficients at a fixed temperature (36 °C). Values for the entropy of activation (ΔS^+) also shown in Table I, are negative in most cases except for the diffusion of cations through pure phosphatidylserine membranes. The positive values obtained in this case are probably the result of the slight gradual decrease of activation energy with temperature, which gives fictitiously high Arrhenius activation energy upon linearization of the plot.

(7) In contrast to the effect of cholesterol in lowering the Arrhenius activation energy for the diffusion of cations, the presence of cholesterol produces a slight increase in the case of Cl^- and also glucose. If we accept the suggestion made earlier, that temperature-dependent structural changes are responsible for the high apparent activation energy for cation diffusion, it follows that these structural changes do not

have an appreciable effect on Cl^- or glucose diffusion. Since the mechanism for either cation or anion diffusion is not well understood, it is difficult to rationalize the above differences in molecular terms. However, we have encountered the following two possibilities which might help in the interpretation.

(a) It is possible that uncharged molecules can pass through a bilayer by first dissolving and then diffusing across by following the fluctuations (kinks) in molecular packing[59,63]. In this case, Cl^- might be following the same route as an uncharged species. This mechanism, which involves a displacement from a medium of high dielectric constant (external solution) to a medium of a low dielectric constant is unfavorable for charged species in general[64,65]. The diffusion of such permeants would then depend to a certain extent on the occurrence of a statistically infrequent pore[66] whose interior is lined by the polar head groups of the phospholipid molecules. Such pores of short duration might be the result of thermal fluctuations which can be identified as a fast lateral movement of the phospholipid molecules on the surface of the membrane, and a slow flip-flop reorientation between the two sides of the bilayer[32,36].

Such effects would become statistically more significant with raising temperature and would be inhibited by cholesterol, which tends to make the membranes more rigid. It should be noted that cholesterol seems to increase the dielectric constant of the phospholipid bilayers, which in itself would tend to increase the permeability for charged species, an effect contrary to the one observed.

(b) The other possibility involves the effect of dipole orientation at the water–lipid interface. As Danielli[67] has pointed out in relation to the energy barriers for ion diffusion through a bimolecular leaflet, the alignment of the permanent dipoles at the interface could be more important than formal charges. The alignment of the phospholipid permanent dipoles with the positive poles towards the hydrocarbon interior (suggested by the surface potential (ΔV) values of oriented monolayers) would be expected to be unfavorable for cation diffusion, but perhaps favorable for anions. Uncharged molecules will be less affected by the form of the potential. Although very little is known about the details of such potential profile across the bilayer, it would be expected that temperature-dependent structural changes will modify it, with concomitant effects on cation permeability. The presence of cholesterol will again produce its effect on cation permeability by inhibiting the temperature-dependent structural changes. Accordingly, Cl^- and uncharged permeants will not be affected since their diffusion is not restricted by an unfavorable dipole orientation. The above possibilities are still rather speculative and further experimentation is needed before a more definitive statement can be made on the subject.

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