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The Permeability of Liposomes to Nonelectrolytes

I. Activation Energies for Permeation

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Summary. The effect of temperature on the permeability of nonelectrolytes across liposomal membranes above and below their transition temperature has been studied by using an osmotic method. Below their transition temperature, liposomes are osmotically insensitive structures but, on addition of gramicidin A, the water permeability so increased that the permeability of solutes could be studied. The measured activation energies for permeation of a variety of nonelectrolytes has been found to increase when a) there is an increase in the capability of the solutes to form hydrogen bonds in water, b) the cholesterol concentration in the membranes increases and c) the membranes pass from a liquid-crystalline to a solid-crystalline state. The change in the activation energy for permeation per hydrogen bond is about 1.8 kcal/mole for all the different liposome systems investigated; the only solute tested that deviated from this correlation was urea, whose activation energy for permeation across a gramicidin-containing system was much lower than expected from its hydrogen-bonding capacity. This finding suggests that urea is permeating across the gramicidin pore. Although the literature contains only incomplete data relating the activation energies for permeation of nonelectrolytes across biological membranes to their hydrogen-bonding capacity, the available evidence suggests that there is a similar correlation to that found in liposomes. Thus, the average increase in the activation energy per hydrogen bond for permeation across ox red cell membranes (Jacobs, Glassman & Parpart, *J. Cell. Comp. Physiol.* **7**:197, 1935) is 2.2 ± 0.4 kcal/mole, a value that is similar to that obtained in liposomes. However, the activation energies for water and urea are—in such a system—very much lower than expected, suggesting that they, too, are permeating by some parallel route such as an aqueous pore.

The basic importance of one's understanding of the mechanism of the transport of molecules across a homogeneous or continuous lipid membrane can be considered to have originated from the first measurements of permeability in biological systems made by Overton (1895) which revealed that

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molecules penetrate cells in the same relative order of their oil-water partition coefficient. However, the actual details of the process by which a molecule passes through the cell membrane were not considered explicitly until Danielli proposed a theory to account for the rates of permeation on the basis of the existence of a continuous lipid bilayer as the basic permeability barrier of the cell membrane. The analysis of the temperature dependence of the permeabilities played an important role in this theory (Davson & Danielli, 1943).

Danielli's analysis was based on treating the bilayer as a thin layer of liquid oil in which the water-lipid interphase is the main energy barrier that a penetrating solute must overcome in order to pass the membrane. Relatively water-soluble molecules which have a low rate of permeation (as expected from Overton's rules) would have a high temperature coefficient of permeation for the number of molecules with the energy necessary to overcome the barrier are small; when the permeability of the solute is high and so its lipid solubility, all the molecules have the energy necessary to pass the water-lipid interphase and so only the relatively small activation energy involved in diffusion through a liquid oil will be of importance. Danielli applied this treatment to the Q_{10}'s determined by Jacobs, Glassman and Parpart (1935) for ox red cell membranes and concluded that these membranes behave, to a first approximation, as a homogeneous lipid bilayer (Davson & Danielli, 1943).

Zwolinski, Eyring and Reese (1949) generalized Danielli's theory by applying the absolute rate theory to the process of diffusion across barriers. They derived an expression for the permeability of a solute similar to that obtained by Danielli, but considered that there were no reasons for differentiating between rate-determining barriers on the basis of the magnitude of the permeability coefficients. According to this theory only the relative values of the rate constants associated with each of the permeation steps are important in determining the main barrier for the permeation of a solute.

The possibility of studying the permeability properties of an isolated lipid bilayer became available in 1962 when Mueller, Rudin, Tien and Wescott reported a technique for preparation of thin lipid films separating two aqueous compartments. Concerning the mechanism of solute transport across these films, there have been several investigations of the activation energies for water permeation (Price & Thompson, 1969; Redwood & Haydon, 1969) but the use of such preparation for the study of the transport of uncharged species has been limited by technical difficulties associated with unstirred layers and the presence of excess solvent (hydrocarbon).

On the other hand, the thin lipid system has been extensively used to investigate the properties of a group of natural and synthetic compounds that, in relatively small amounts, produce an increase in the conductance of the system by altering the cation permeability. A body of evidence indicates that most of these compounds, such as valinomycin, the nonactin series, and cyclic polyesters, act as ion carriers by combining with an ion to form a lipid-soluble complex which can diffuse back and forth across the membrane (for a review, *see* Haydon & Hladky, 1972; Eisenman, Szabo, Ciani, McLaughlin & Krasne, 1973). However, polyene antibiotics such as nystatin and amphotericin B and small polypeptides like gramicidin A, appear to act by forming aqueous pores in the lipid bilayers (Hladky & Haydon, 1970; Holz & Finkelstein, 1970; Krasne, Eisenman & Szabo, 1971; Urry, Goodall, Glickson & Mayers, 1971).

The system of phospholipid vesicles (liposomes) developed by Bangham (1968) has many advantages for permeability studies of uncharged species. Thus, liposomes are osmotically sensitive structures that swell and shrink under a concentration gradient of permeable solute and so relative permeabilities to nonelectrolytes can be measured conveniently by optical techniques (Cohen & Bangham, 1972).

In the present work, studies have been made of the effect of temperature on the permeability of liposomes to a variety of nonelectrolytes and the effect of the incorporation of gramicidin A into liposomes. The purpose of this investigation is to provide some insight into the basic mechanism for solute permeation in biological membranes.

Materials and Methods

Experimental Equipment

(a) Light-Scattering Apparatus. Light-scattering measurements were used to monitor the time course of the volume changes of liposomes when rapidly mixed with a hypertonic solution of nonelectrolytes. An SP 700 Unicam spectrophotometer was used. This double beam instrument automatically records the transmission or optical density of a sample relative to a reference cell. It coupled a high speed strip chart recorder (Honeywell Control Ltd.) with a time constant of 1/4 sec for full scale pen travel.

A thermostated cell housing of the circulatory type was used to keep the temperature constant. This was measured by a thermometer conveniently placed in the reference cell.

(b) Digital Voltmeter and Print-out System. The Honeywell recorder was equipped with a second slidewire and a second set of contactors for linear retransmission of input signals to a digital voltmeter and a printer drive unit (Weir Electronics Ltd.) using a stable power source of 125 d-c volts.

An electric clock was adapted to send print signals at predetermined time intervals. In this way, the time course of the transmission changes were monitored simultaneously

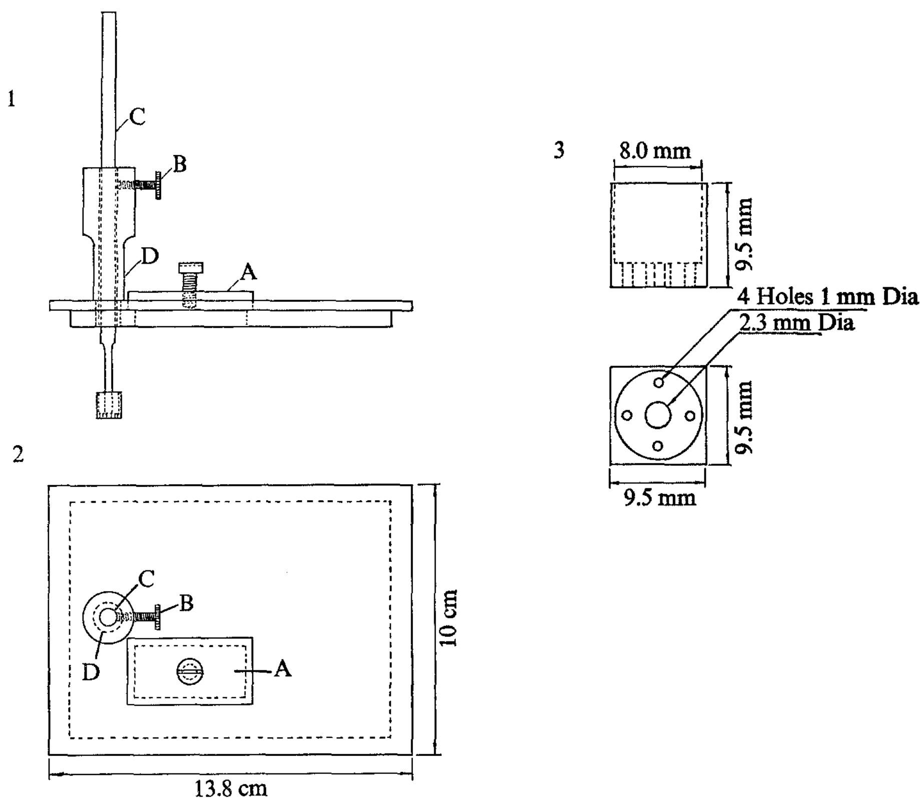

Fig. 1. Frontal (1) and top view (2) of the modified cell compartment cover of the SP 700 spectrophotometer. (*A*) Viewing window; (*B*) locking screw; (*C*) mixing device; (*D*) supporting column. An enlarged frontal and top view of the small cuvette at the tip of the mixing device is shown at (3)

on the strip recorder and by printing out of the voltage analogue at 1 sec time intervals or slower.

(c) Mixing Device. Rapid mixing of the liposomes with a test solution was achieved by using a mixing device (Ottewill & Sirs, 1957) that consisted of a small teflon cuvette, with a diameter that nearly matches the dimensions of an ordinary 1 cm^2 glass cuvette, held at the end of a supporting rod (Fig. 1).

The teflon cuvette has holes in the bottom, small enough to prevent drainage of 0.2 to 0.3 ml of an aqueous solution but large enough to ensure an adequate mixing when, loaded with liposomes, it is pushed downwards into the glass cuvette containing the test solution.

To hold the mixing device in position at zero time (i.e., at the top part of the glass cuvette), the sample cell compartment of the SP 700 was replaced by one as depicted in Fig. 1. (The SP 700 has two separate cell compartments, one for the reference cell and the other for the sample.) Tube *D* holds the mixer in position by the screw *B* and the window *A* allows one to locate it at the top part of the glass cuvette.

Lipids and Reagents

Egg-lecithin was extracted from egg yolks by alumina and silicic acid chromatography, according to the procedure of Papahadjopoulos and Miller (1967). Cholesterol and 1,2-dimyristoyl-L-3-lecithin were obtained commercially (Koch-Light Labs Ltd. and Sigma Chemical Co.). Phosphatidic acid was prepared from egg-lecithin by enzymatic hydrolysis (Papahadjopoulos & Miller, 1967). New batches of lecithins and phosphatidic acid were routinely examined for purity on activated silica gel plates using chloroform/methanol/7 M aqueous ammonia (230:90:15, v/v/v) (Abramson & Blecher, 1964). All lipid solutions in chloroform were stored at −20 °C under an atmosphere of nitrogen, until required. Nonelectrolytes were obtained from British Drug House Ltd. (Poole, England), Hopkin and Williams Ltd. (Essex, England) and May and Baker Ltd. (Dagenham, England). Gramicidin A was obtained from Koch-Light and dicetylphosphate from Sigma Chemical Co. All these reagents were analytical grade and were used without further purification. Water used to prepare the solutions of nonelectrolytes was twice distilled, the second time from $KMnO_4$ in borosilicate apparatus.

Experimental Procedure

The permeability of liposomes to nonelectrolytes was measured using the method developed by Hill and Cohen (1972). This method is based on the observation that when liposomes are put into a hypertonic solution of a nonelectrolyte, the measured changes in volume following the minimum volume point remain constant for a certain time depending upon the type of solute (Cohen & Bangham, 1972). The linear volume changes after the minimum volume obtained when the ratio of impermeable solute C_i to permeable solute C_s (C_i/C_s) outside the liposomes is much smaller than 1 has been shown to be proportional to the permeability coefficient of the solute (Hill & Cohen, 1972). A small C_i/C_s ratio can be easily obtained by mixing 0.18 ml of a liposome suspension with 2.5 ml of a 100 mM solution of a nonelectrolyte. Thus, in this case $C_i/C_s=0.029$ and the measured slope proportional to the permeability coefficient can be considered to be independent of the reflection coefficient of the solute.

Light scattering at 450 nm was used to measure the change in volume of liposomes. Thus, from the empirical relation between the volume of liposomes and their optical density E (Bangham, De Gier & Greville, 1967),

$$dV/dt = -1/E^2\, dE/dt.$$

The initial value of E of a liposome suspension is the same for all systems of liposomes prepared and as the range of E, over which the slope after the minimum volume is measured is small, changes in dE/dt will be proportional to dV/dt.

The procedure routinely applied when recording changes in transmission of a suspension of liposomes, unless otherwise stated, was as follows:

(1) 2.5 ml of the 100 mM nonelectrolyte test solution was pipetted into the glass cuvette and placed in the cell holder of the SP 700, equilibrated at a given temperature.

(2) A portion of 0.18 ml of a concentrated liposome suspension was pipetted carefully into the small teflon cuvette of the mixing device, and placed in the zero-time position.

(3) With the window A closed (*see* Fig. 1) and the recording system on, the mixing device was released and pushed downwards rapidly but gently until it touched the bottom of the glass cuvette. Then, it was returned back and left out of the optical path of the instrument. This whole operation took less than a second.

The volumes of the suspensions were recorded for 5 to 20 min depending on the type of solute, temperature and liposome system. The slope following the minimum volume was calculated from the sequence of voltage readings ψ corresponding to the portion where $d\psi/dt$ is a constant. For the most permeable solutes, it was found necessary to use only the readings covering an interval of 5 to 10 sec after the minimum volume. However, the slopes for slowly permeating solutes remain constant, up to 5 min. These slopes are referred to as permeability values (P) and are reported in voltage units per second.

Preparation of Liposomes for Light-Scattering Measurements. A stock solution of the lipids in chloroform (lecithin, cholesterol) was placed in a round-bottomed flask of 100 or 250 ml capacity, at the appropriate molar ratios but always with 4% of negatively charged lipid. The chloroform was evaporated under reduced pressure, using a rotary evaporator, so that the lipids were left as a smooth thin film on the surface of the flask. When bubbles were left trapped in the film, chloroform was added again and the procedure was repeated until a satisfactory film was obtained. A 20 mM KCl solution was then added in an amount to make the concentration of lipid to about 10 mM. The lipid was dispersed into this aqueous phase by gentle agitation until the thin film on the surface of the flask was removed. No further mechanical or sonication process of any sort was applied to the preparation. When saturated phospholipids were used the temperature of the aqueous solution was adjusted to be about 10 °C above the transition temperature of the lipid. Egg-lecithin liposomes were prepared at room temperature. Before starting the measurements, the optical density of the 10 mM liposome suspension was further adjusted to a constant optical density by adding an appropriate amount of 20 mM KCl solution.

Measurements were usually started 1 hr after preparing the liposomes, unless otherwise stated.

Theory

The movement of molecules across lipid bilayers under an osmotic gradient may be considered to be carried out fundamentally by a diffusion process rather than by viscous or bulk flow. This means that water and solutes cross the hydrophobic barrier independently by dissolving into the lipid phase and then diffusing through it.

In the Absolute Rate theory treatment of a process of diffusion (Glasstone, Laidler & Eyring, 1941) a diffusional flow of molecules is regarded to occur by a series of successive jumps from one equilibrium position to another. Each of these steps has associated a rate constant whose magnitude is given by:

$$k=\frac{\kappa T}{h}\exp\left(-\Delta G^{\ddagger}/RT\right)$$

where κ is the Boltzman constant, T is absolute temperature, h is Planck's constant and $\Delta G^{\ddagger}$, the free energy of activation, is the difference in free energy between the initial and an activated state.

The free energy of activation is related to the enthalpy of activation ($\Delta H^{\ddagger}$) and the entropy of activation ($\Delta S^{\ddagger}$) by the expression

$$\Delta G^{\ddagger} = \Delta H^{\ddagger} - T\Delta S^{\ddagger}.$$

To obtain a value for $\Delta H^{\ddagger}$, the Arrhenius equation is used:

$$\ln k = \ln A - \Delta E/RT$$

where A is constant and ΔE is the activation energy of the process.

The activation energy ΔE is related to the enthalpy of activation by (Glasstone *et al.*, 1941)

$$\Delta E = \Delta H^{\ddagger} + RT.$$

Now the amount of solute passing through a square centimeter of surface per second (J) under a concentration gradient (dC/dx) is given by Fick's first law as,

$$J = -D\,dC/dx$$

where D, the diffusion coefficient, is equal to

$$D = \lambda^2 k,$$

λ being the average distance between two equilibrium positions.

The kinetic analysis of membrane permeability developed by Danielli (Davson & Danielli, 1943) and Zwolinski *et al.* (1949) is based on the assumption that Fick's law can be applied to the process of permeation across a thin membrane. These authors have proposed that solutes may have three main sites of resistance for permeation across a lipid layer. They are, the transfer of the solute from the water phase to the lipid membrane, diffusion through the membrane, or the transfer of solute from the lipid membrane to the water phase.

By dividing the membrane into a series of energy barriers, each one separated by a distance λ, Zwolinski *et al.* (1949) derived the following expression for the permeability coefficient P of a solute:

$$P = \frac{k_{sm} k_m \lambda}{2k_m + m k_{ms}} \quad (1)$$

where k_{sm} and k_{ms} are the rate constants for diffusion from the water phase to the membrane and vice versa, k_m is the rate constant for diffusion through the membrane and m is the number of equilibrium positions in the membrane.

Eq. (1) can be simplified if

$$m k_{ms} \gg 2 k_m;$$

then,

$$P = \frac{k_{sm} k_m \lambda}{k_{ms} m} = \frac{K D_m}{l} \tag{2}$$

where K, the partition coefficient is defined as the ratio of the rate constants for diffusion through the solution-membrane interphase l is the thickness ($l = \lambda m$) and D_m is the diffusion coefficient of the solute in the membrane.

Eq. (2) is generally considered to be valid for the process of permeation across thin lipid membranes (where m is small). However, it must be kept in mind that there is no quantitative data to support the assumption that $m k_{ms} \gg 2 k_m$.

The process of permeation across a lipid bilayer can be separated into the following number of events, each of them contributing to the total rate of permeation:

(1) adsorption of the solute at the lipid membrane/water interphase;

(2) dehydration of the solute;

(3) diffusion through the hydrocarbon chains.

The rate of permeation will be proportional to the product of the probability of occurrence of the successive events,

$$P = \lambda^2 \frac{\kappa T}{h l} \exp(-\Delta G^{\ddagger}_{ads}/RT) \exp(-\Delta G^{\ddagger}_{deh}/RT) \exp(-\Delta G^{\ddagger}_{dif}/RT) \tag{3}$$

where $\Delta G^{\ddagger}_{ads}$ and $\Delta G^{\ddagger}_{dif}$ are the contribution to the free energies of activation of the adsorption and diffusion steps, respectively, that are not due to hydrogen bonds. $\Delta G^{\ddagger}_{deh}$ is the free energy of activation for the dehydration process. Also,

$$\Delta G^{\ddagger}_{ads} = \Delta H^{\ddagger}_{ads} - T \Delta S^{\ddagger}_{ads}$$

$$\Delta G^{\ddagger}_{dif} = \Delta H^{\ddagger}_{dif} - T \Delta S^{\ddagger}_{dif}$$

where $\Delta H^{\ddagger}_{ads}$, $\Delta H^{\ddagger}_{dif}$ and $\Delta S^{\ddagger}_{ads}$, $\Delta S^{\ddagger}_{dif}$ are the corresponding enthalpies and entropies of activation that are not due to hydrogen bonds.

A variable that can be singled out as determining the extent of dehydration that a solute will suffer in passing through a hydrophobic barrier is its capacity to form hydrogen bonds in water. This was the independent variable chosen by Stein (1967) in his analysis of membrane permeability. Thus, if N_H is the potential number of hydrogen bonds that a solute can form in water,

$$\Delta G^{\ddagger}_{deh} = N_H \Delta G^{\ddagger}_H = N_H (\Delta H^{\ddagger}_H - T\Delta S^{\ddagger}_H) \quad (4)$$

where $\Delta G^{\ddagger}_H$ is the free energy of activation for "breaking" a hydrogen bond and $\Delta H^{\ddagger}_H$ and $\Delta S^{\ddagger}_H$ are the corresponding enthalpy and entropy changes per H-bond.

Results

The effect of temperature on the permeability of liposomes to a variety of solutes differing in the number of H-bonds that they might form in water was investigated in the following two systems (the lipid concentrations are given in molar ratios): (a) 96% egg-lecithin/4% phosphatidic acid; (b) 66% egg-lecithin/30% cholesterol/4% phosphatidic acid. For every solute, the permeability was measured an average of five times at a given temperature. Measurements were made at five different temperatures between 10 and 35 °C.

The activation energies for permeation were obtained by plotting the logarithms of the permeabilities against the inverse of the absolute temperature. Typical plots are shown in Figs. 2 and 3. A straight line relationship between log P and the inverse of the absolute temperature was obtained in all cases investigated.

Two different procedures were followed in determination of their activation energies. Firstly, the measurements of permeability for a set of solutes at five different temperatures were completed using a single suspension of liposomes, the stock suspension of liposomes being kept at 4 °C under nitrogen for the five-day period of the experiment. Secondly, fresh suspensions of liposomes were prepared every day for their determination at each temperature and, in order to normalize the differences in surface areas between these preparations, the permeability to malonamide was used as a reference. The activation energy for the permeation of malonamide was carefully determined and from this value, the activation energies for the different solutes were calculated.

Activation energies for the permeability of nonelectrolytes across egg-lecithin liposomes are given in Table 1. The values listed in columns 1 and 2

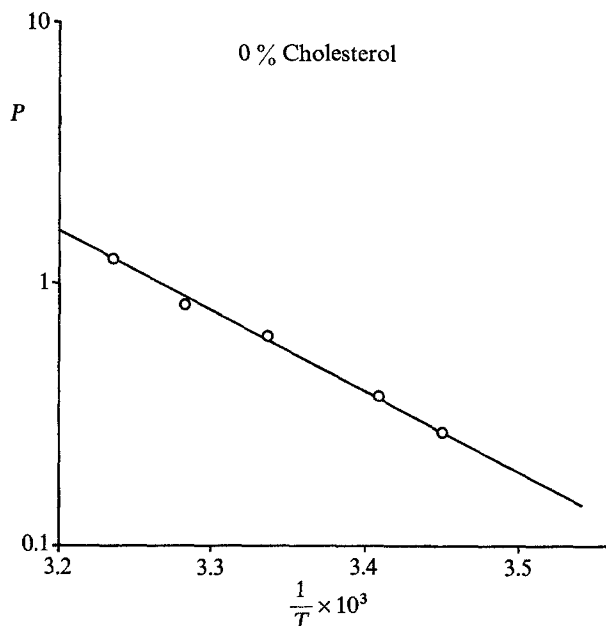

Fig. 2. Arrhenius plot for the permeability of malonamide across egg-lecithin/PA (96:4) liposomes. Ordinate: logarithms of the permeability values (in voltage units per second). Abscissa: the inverse of the absolute temperature

Table 1. Activation energy values for the permeability across egg-lecithin liposomes (kcal/mole ± SD)

Solute	N_H	(1)	(2)	(3)	Average values
Erythritol	8	15.8 ± 0.6	16.4 ± 2.0		16.1
Malonamide	6	13.3 ± 0.6	12.9 ± 0.5	12.5 ± 0.6	13.0
Hydroxy-urea	6	10.7 ± 1.0	11.0 ± 0.5		10.9
Glycerol	6	11.9 ± 0.6	10.1 ± 0.5		11.0
Urea	5	9.5 ± 0.5	9.4 ± 0.3	9.3 ± 0.6	9.3
Thiourea	5	8.4 ± 0.4	7.8 ± 0.5	9.0 ± 1.0	8.4
Monactin	4		8.2 ± 0.5		8.2
Methylurea	4		7.5 ± 0.2		7.5
Ethylurea	4	6.2 ± 0.5		6.1 ± 1.0	6.2
H_2O	4	8.6 ± 0.9			8.6
Ethyleneglycol	4	6.2 ± 0.2			6.2
Acetamide	3	5.1 ± 0.3		6.1 ± 1.0	5.6
Methylacetamide	3	5.0 ± 0.3			5.0
Thioacetamide	3	5.2 ± 0.3			5.2
Propionamide	3	5.4 ± 0.3		6.1 ± 1.0	5.8
Formamide	3	4.8 ± 0.3		6.1 ± 1.0	5.5
Urethane	3	7.1 ± 0.4			7.1
Cyanoacetamide	3	5.3 ± 0.5			5.3
Dimethylformamide	2	5.3 ± 0.5			5.3

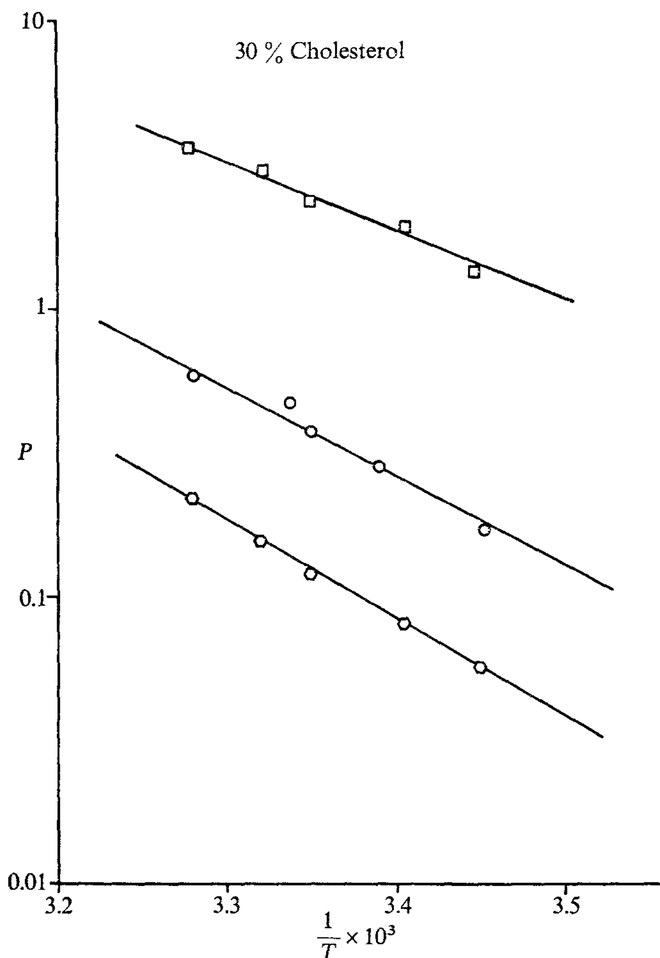

Fig. 3. Arrhenius plots for the permeability of some solutes across egg-lecithin/cholesterol/PA (66:30:4) liposomes. Ordinate: logarithms of the permeability values (in voltage units per second). Abscissa: the inverse of the absolute temperature. O malonamide; ○ urea; □ formamide

correspond to the activation energies obtained using the first procedure. The values in column 3 were obtained by the second method. It can be seen that both procedures gave quite similar results. However, the standard deviation is greater when a different suspension of liposomes is used at each temperature.

The average values of the activation energies obtained for permeation of solutes across egg-lecithin/PA liposomes (Table 1) are correlated with the number N_H of H-bonds that the molecules might form in water (open circles in Fig. 4). The number N_H of H-bonds for different functional groups

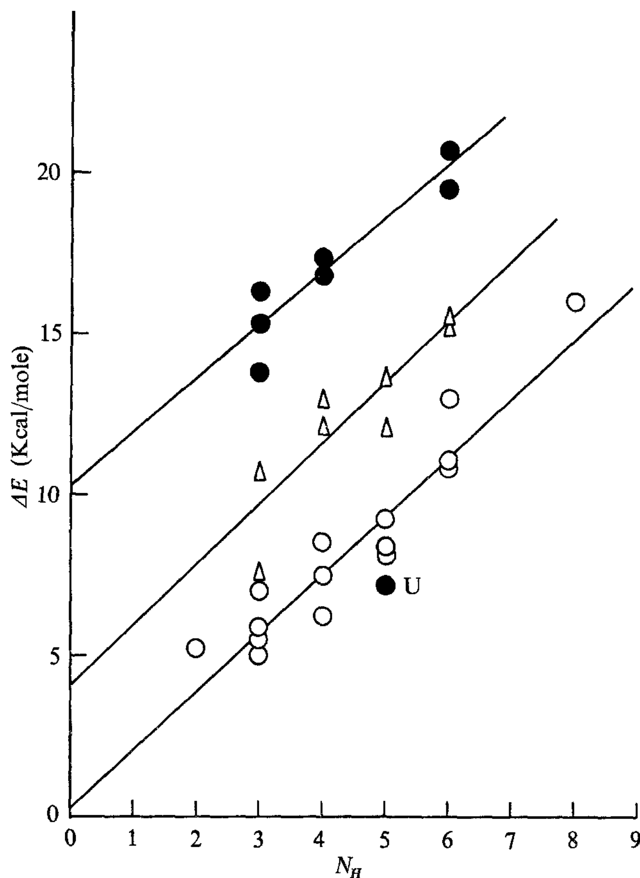

Fig. 4. The relation between the activation energies for permeation of nonelectrolytes across different liposome systems and the capacity of the solutes to form hydrogen bonds in water. Ordinate: activation energies (ΔE) in kcal/mole. Abscissa: the number of hydrogen bonds. ○ egg-lecithin/PA (96:4) liposomes; △ egg-lecithin/cholesterol/PA (66:30:4) liposomes; • dimyristoyl-lecithin/DCP (96:4) liposomes containing gramicidin A. All the activation energies were determined between 10 and 35 °C except in the gramicidin-containing system where they were between 10 and 20 °C

are assigned according to the criteria of Stein (1967). The data were fitted by the least-square method and it was found that the regression coefficient for 19 solutes is 0.93. The slope of the straight line indicates a value of 1.8 ± 0.2 kcal/mole for the activation energy (ΔE) of permeation per H-bond. Extrapolation to zero number of H-bonds gives a value of 0.26 ± 0.8 kcal/mole for the activation energy of a molecule that does not form H-bonds in water. This indicates that in these membranes, the contribution of the nonhydrogen bonding terms [Eq. (3)] to the total activation energy for permeation is very small.

Table 2. Activation energy values for the permeability across egg-lecithin liposomes containing 30% cholesterol (kcal/mole ± SD)

Solute	N_H	(1)	(2)	Average values
Malonamide	6	15.2 ± 0.8	14.1 ± 0.6	14.7
Glycerol	6	15.5 ± 0.5		15.5
Lactamide	5	14.8 ± 1.0		14.8
Urea	5	13.7 ± 0.8	13.6 ± 0.7	13.7
Thiourea	5	12.2 ± 0.8		12.2
Ethyleneglycol	4	13.1 ± 1.7		13.1
Methylurea	4	12.2 ± 0.7		12.2
Acetamide	3	7.6 ± 1.3		7.6
Formamide	3		10.8 ± 0.8	10.8

The values for the activation energies in the egg-lecithin system containing 30% cholesterol are given in Table 2. In this case, the same suspension of liposomes was used to obtain the values in each of the columns.

The presence of 30% cholesterol in egg-lecithin membranes (Table 2) led to a constant increase in the activation energies for permeation of the different solutes, compared with the noncholesterol values (triangles in Fig. 4). Thus, the corresponding correlation with the number of H-bonds gives a quite similar value for the activation energy per H-bond, 1.9 ± 0.4 kcal/mole. The contribution of the nonhydrogen bonding terms to the total activation energy for permeation through these membranes is now 4.1 ± 1.8 kcal/mole.

The Effect of Gramicidin A on the Permeability to Water and Nonelectrolytes of Liposomes Below Their Transition Temperature

Liposomes made with saturated lecithins, i.e. dimyristoyl, dipalmitoyl of distearoyl-lecithin, are osmotically sensitive structures that swell or shrink under a concentration gradient of impermeable solute provided that the system is examined at a temperature above their corresponding transition temperature (De Gier, Mandersloot & Van Deenen, 1968). Thus, no changes in light scattering are observed when dimyristoyl-lecithin liposomes are mixed with hypertonic solutions of KCl at 10 °C (top curves in Fig. 5), for the transition temperature of this system is at 23 °C. Above this temperature, the system is osmotically active.

It was found, however, that if a small amount of gramicidin A, 50 μg/20 μmoles lipid, dissoved in methanol, is added to the lipid solution before

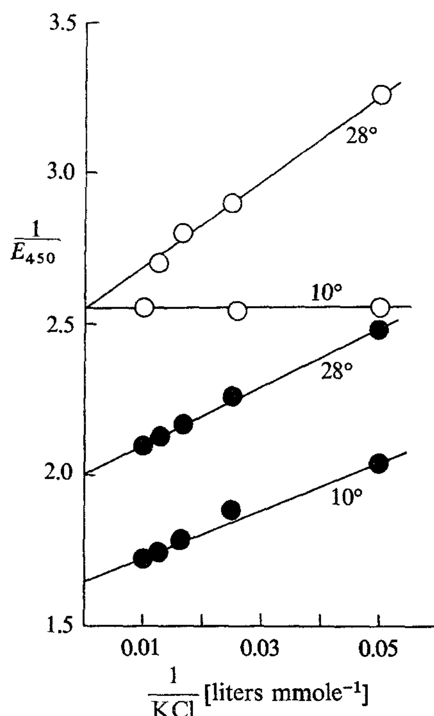

Fig. 5. The effect of gramicidin A on the osmotic behavior of dimyristoyl-lecithin/DCP (96:4) liposomes below their transition temperature. Ordinate: the inverse of the equilibrium optical densities of liposomes prepared in a 20 mM KCl solution when put into a hypertonic KCl solution. Abscissa: the inverse of the KCl concentration outside the liposomes. ○ liposomes prepared without gramicidin A; • liposomes containing gramicidin A. The transition temperature of dimyristoyl-lecithin is 23 °C

the liposomes are formed, the liposomes now show changes in light scattering below as well as above their transition temperature (bottom curves in Fig. 5). Equilibrium optical densities of liposomes mixed with various hypertonic KCl solutions are achieved very rapidly indicating that the water permeability of the system has substantially increased. It can be seen in Fig. 5 that in the gramicidin-containing liposomes, the final liposome volume (proportional to $1/E_{450}$) is directly related to the external KCl concentration at both temperatures.

It was also observed that if the gramicidin A liposomes containing 20 mM KCl are mixed with pure water at a temperature well below the transition, a decrease in optical density, indicative of a swelling, occurs but that the final equilibrium optical densities appear to depend on the temperature, decreasing as the system approaches the transition temperature (Fig. 6). It can be seen in this Figure that the OD of the suspension in water (curve *b*)

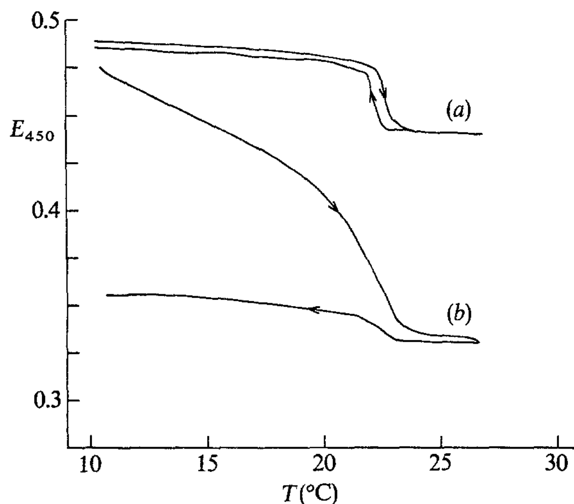

Fig. 6. The effect of temperature on the equilibrium optical density of dimyristoyl-lecithin/DCP (96:4) liposomes containing gramicidin A. Liposomes were prepared in a 20 mM KCl solution at 30 °C and a small aliquot transferred to water at 10 °C. When this system was in equilibrium, the temperature was slowly increased (curve *b*). In the control experiment (curve *a*) the liposomes were put into the same solution in which they were prepared. The temperature was continuously recorded by a thermocouple placed at the reference cell

decreases up to the transition temperature, in contrast to the suspension in isotonic 20 mM KCl (control) where no change occurs (curve *a*). At the transition, both suspensions show a relatively large decrease in optical density, which is reversible. Gramicidin A does not perceptively change the transition temperature of the lipid.

The optical density changes at the transition temperature have been observed before by Abramson (1971) and have been adequately accounted for by a change in refractive index resultant upon a decrease in density of the bilayer and by a concomitant decrease in the anisotropy of the bilayer (Yi & McDonald, 1973).

Gramicidin A has no effect on the osmotic behavior of liposomes if it is added only to the external aqueous solution.

Activation Energies for Permeation of Nonelectrolytes Across the Gramicidin-Containing System

As was described in the previous section, liposomes prepared with saturated phospholipids treated with gramicidin A are osmotically sensitive structures below their transition temperature in contrast to such liposomes

Table 3. Activation energies for permeation across dimyristoyl-lecithin/DCP (96:4) liposomes with gramicidin A below their transition temperature

Solute	N_H	ΔE (kcal/mole ± SD)
Malonamide	6	19.5 ± 2.0
Glycerol	6	20.7 ± 2.0
Urea	5	7.2 ± 0.5
Methylurea	4	16.8 ± 1.0
Ethylurea	4	17.3 ± 1.0
1,1 diethylurea	3	16.3 ± 1.0
Acetamide	3	13.8 ± 1.0
Valeramide	3	15.3 ± 1.0

free of gramicidin A. In consequence, the present osmotic method could be used to measure the permeability to different solutes. However, assessment of the activation energies for permeation of nonelectrolytes across such a system was complicated because the "equilibrium volumes" of the structures as determined from final OD vary with temperature when, for example, they are mixed with water (*see* Fig. 6). In order to avoid this problem, these liposomes were prepared in pure water instead of the standard 20 mM KCl solution.

Liposomes prepared in pure water form structures whose volume-to-surface ratio is maximal and no further swelling is possible below or above the transition. They can only be shrunk and the extent of shrinkage is not affected very much by the temperature (bottom curves in Fig. 5).

Measurements of relative permeabilities in such a system were carried out at 10 and 20 °C. For this purpose, liposomes were prepared from a mixture of 96% dimyristoyl-lecithin and 4% dicetylphosphate. Gramicidin A, dissolved in methanol, was added to the lipid phase before the liposomes are formed, in a concentration of 100 µg per 20 µmoles of lipid. The activation energies were calculated from the average Q_{10} of two separate experiments and are presented in Table 3.

The measured activation energies for permeation across dimyristoyl-lecithin liposomes treated with gramicidin A below their transition temperature (Table 3) are plotted against the number of H-bonds per molecules (filled circles in Fig. 4). It can be seen in Fig. 4 that solutes permeating across such a system have a much higher ΔE than the previous two systems but there is also a correlation with the number of hydrogen bonds, with the exception of urea (U). Thus, the activation energy for permeation of urea is 7.2 kcal/mole, a value that is even smaller than the ΔE obtained for this solute in the egg-lecithin membranes.

The increase in the activation energy per H-bond calculated for the solutes permeating across the gramicidin-containing system (excluding urea) is 1.7 ± 0.3 kcal/mole. The intercept at $N_H = 0$ is 10.3 ± 1.2 kcal/mole.

Discussion

The Activation Energies for Permeation Across Liposomes

The linear correlation that has been found between the activation energies for permeation across continuous lipid bilayers and the capability of the solutes to form hydrogen bonds in water shows clearly that dehydration of the solutes plays an important role in the process. This result is even more meaningful in the light of the gross simplifications made in the estimation of the number of potential hydrogen bonds where no account was taken of their strength or steric disposition.

On the other hand, the magnitude of the activation energies has been found to be related to the physical state of the hydrocarbon chains in the bilayer. Liposomes prepared from egg-lecithin are, at room temperature, in the liquid crystalline state (Chapman, Williams & Ladbrooke, 1967). Since it can be considered to be very unlikely that hydrogen bonds play an important role in the process of diffusion through a hydrophobic barrier, the results obtained in such a system suggest that the energy necessary to form a "hole" in the bilayer is small. Thus, the value that can be calculated for the ΔE of this process is a mere 0.26 ± 0.8 kcal/mole. This value should be compared with the activation energy for diffusion of water in hydrocarbons given as 4.5 kcal/mole for squalene and 2.6 to 3.4 kcal/mole for saturated hydrocarbons of 10 to 16 carbon atoms (Schatzberg, 1965). Such a comparison suggests that the hydrocarbon chains in a liquid crystalline state are in a rather disordered conformation (Luzzati, 1968; Levine & Wilkins, 1971).

When 30% cholesterol is incorporated in egg-lecithin membranes the contribution of the nonhydrogen bonding terms to the activation energy increases in about 4 kcal/mole. If now it is assumed that the incorporation of cholesterol does not significantly change $\Delta H^{\ddagger}_{ads}$ [*see* Eq. (3)] this value can be assigned to an increase in the restriction for diffusion (or in the enthalpy for the formation of a "hole") across the bilayer. This effect would be consistent with the calorimetric data which suggest that cholesterol modifies the hydrocarbon chains to an "intermediate fluid condition" between a solid and a liquid crystalline state (Ladbrooke, Williams & Chapman, 1968). Indeed, cholesterol reduces the area per molecule from

about 61 Å in an egg-lecithin membrane (at 35% weight water content) to 48 Å in an egg-lecithin/cholesterol bilayer (1:1 molar ratio) (Lecuyer & Dervichian, 1969). This is a very tight packing considering that a phospholipid molecule in a maximally condensed state occupies 40 Å (Van Deenen, Houtsmuller, de Haas & Mulder, 1962) that leads to a reduction in the mobility of the hydrocarbon chains and a decrease in the probability of trans to gauche conformations (Hubbell & McConnell, 1971; Levine & Wilkins, 1971).

The contribution of a solid-crystalline membrane to the total activation energy for permeation cannot be evaluated directly by using the present osmotic method since the system is rather impermeable to water. On the other hand, even though in the presence of gramicidin A the permeability to water increases and relative permeabilities can be measured, it is not known if the number of gramicidin channels is independent of temperature. However, it can be noted that the calculated contribution of the nonhydrogen bonding terms to the total activation energy for permeation across the dimyristoyl-lecithin/gramicidin system below the transition temperature (10.3 kcal/mole) is larger than the corresponding term for membranes in a liquid-crystalline state but it is not so large as would have been expected for a membrane system that is in a solid-crystalline condition. Thus, it has been estimated that about nine phospholipid molecules are involved in the passage of a molecule across liposome membranes (Hill, 1974). Since the enthalpy change of the transition of dimyristoyl-lecithin from the solid to the liquid-crystalline state is 6.7 kcal/mole of phospholipid (Phillips, Williams & Chapman, 1969), it is to be expected that the activation energy for diffusion across the bilayer is about 60 kcal/mole. The discrepancy between this predicted value and the value obtained might be explained on the basis that the conformation of the majority of the hydrocarbon chains in such a system remains solid except around the gramicidin molecule. The solutes that are unable to penetrate the pore will be crossing the lipid bilayer via such a region.

The temperature dependence of the permeability of some nonelectrolytes across liposomes have been studied before by de Gier, Mandersloot, Hupkes, McElhaney and van Beek (1971). These authors calculated values of 21, 18 and 15 kcal/mole for the activation energies of permeation of erythritol, glycerol and ethyleneglycol, respectively, but found no variations whether or not cholesterol was incorporated or upon the degree of saturation of the lipids. However, since the permeability measurements were made at temperature ranges different for the various solutes and lipid systems used, no conclusions can be drawn from their data about the contribution of diffusion through

the membranes to the activation energy for permeation. Also, the fact that the calculated values for the various activation energies are higher than those obtained in the present work for similar systems may be ascribed to the rather uncertain nature of the extrapolation procedure used by de Gier *et al.* (1971) to estimate the permeabilities.

Relation Between the Enthalpy Changes and the Entropy Changes for Permeation

The activation energies for permeation of solutes have been calculated in the present work by using the Arrhenius equation,

$$\ln P = \ln P_o \exp - \Delta E/RT$$

according to the theory of the absolute rates,

$$\Delta E = \Delta \overset{+}{H^{+}} + RT$$

and

$$\ln P_o = \text{constant} + \Delta \overset{+}{S^{+}}/R.$$

It follows that information about the changes in entropy associated with the enthalpy changes can be obtained by comparing values of $\ln P_o$ and ΔE for different solutes (Fig. 7).

The values in Fig. 7 correspond to measurements carried out using the same suspension of liposomes (Tables 1 and 2). The linear relationship obtained is described by an equation of the form,

$$\Delta E = RT_c \log P_o + \text{constant} \tag{5}$$

where T_c is the so-called "compensation temperature".

The value of T_c calculated from the data in Fig. 7 is 786 ± 24 °K. This temperature is the value at which the permeabilities for all solutes are the same. Thus, at this temperature,

$$\Delta(\Delta \overset{+}{H^{+}}) = T_c \Delta(\Delta \overset{+}{S^{+}}).$$

Since $\Delta(\Delta \overset{+}{H^{+}})$ per H-bond is 1.8 kcal/mole, the corresponding $\Delta(\Delta \overset{+}{S^{+}})$ is 2.3 cal/mole °K.

A number of equilibrium processes in aqueous solutions have been found to exhibit a linear relationship between the enthalpy and the entropy

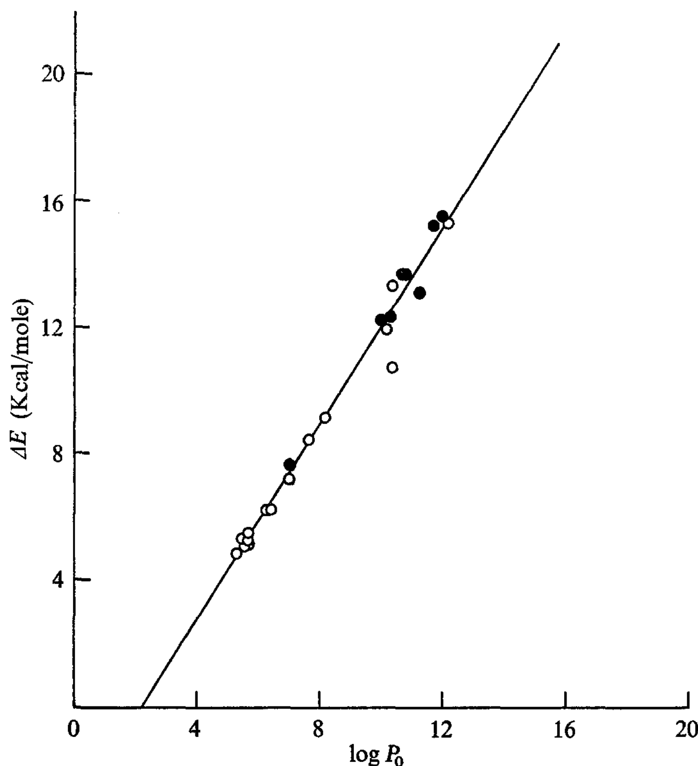

Fig. 7. The relation between the activation energies for permeation across liposomes and the corresponding entropy changes. ○ egg-lecithin/PA (96:4) liposomes.
• egg-lecithin/cholesterol/PA (66:30:4) liposomes

changes (Ives & Marsden, 1965; Lumry & Rajender, 1970). It has also been observed for the process of permeation across biological membranes (Good, 1967).

The compensation effect is most frequently observed in a set of reactions that differ from each other mainly in the extent in which solvational changes are involved. This observation appears to agree with the present results which indicate that the enthalpy and the entropy changes may be linearly related to a simple parameter such as the number of hydrogen bonds. Thus, since the activation energies are found to be related to the number of hydrogen bonds,

$$\Delta E = N_H \Delta H_H^{\ddagger} + \text{constant} \quad (6)$$

the compensation law [Eq. (5)] can be predicted if it is assumed that,

$$\Delta S^{\ddagger} = N_H \Delta S_H^{\ddagger} + \text{constant}.$$

For in this case,

$$\log P_o = \Delta S^{\ddagger}/R + \text{constant} = N_{\text{H}}\, \Delta S^{\ddagger}_{\text{H}}/R + \text{constant}. \tag{7}$$

Substituting N_{H} from Eq. (7) into Eq. (6), we have

$$\Delta E = R \log P_o\, \Delta H^{\ddagger}_{\text{H}}/\Delta S^{\ddagger}_{\text{H}} + \text{constant} = R T_c \log P_o + \text{constant}$$

where $T_c = \Delta H^{\ddagger}_{\text{H}}/\Delta S^{\ddagger}_{\text{H}}$.

The Energy of a Hydrogen Bond

The enthalpy changes involved in the breaking of a hydrogen bond can be calculated in principle from the corresponding equilibrium values for partition of solutes in a saturated hydrocarbon. However, even in such systems considerable association of the solute can take place. Thus, it appears that only at high dilutions alcohol molecules exist as monomers in the hydrocarbon phase (Fletcher & Heller, 1967; Aveyard & Mitchell, 1969).

Aveyard and Mitchell (1969) have measured the heat of solutions of various alcohols at very low concentrations in alkanes, and from these data they have calculated that the heat of breaking 1 mole of OH bonds is about 5.2 kcal/mole. This is the same value that was obtained for the association of alcohols in alkanes using infrared absorption results (Fletcher & Heller, 1967). These values give a ΔH per potential hydrogen bond of 2.6 kcal/mole by assuming that an alcohol group forms two hydrogen bonds in water, a figure that should be compared with the 1.8 kcal/mole obtained for the activation energy per H-bond for the permeation across liposomes.

It is interesting to note that the present value for the energy of one hydrogen bond obtained from permeation data is close to some estimations of the energy of a hydrogen bond in liquid water made by various authors (Table 4). The wide range of values reported reflects the various procedures that have been used to estimate the energy of a hydrogen bond in liquid water, thus, each of the values in Table 4 corresponds to a possible model for the structure of water (Eisenberg & Kauzmann, 1969). In the same way, estimation of the fraction of the broken hydrogen bonds in liquid water varies widely in the different models proposed; e.g., 18 estimates give values between 0.02 and 0.72% at 0 °C (Eisenberg & Kauzmann, 1969). This illustrates the inherent difficulties in applying the concept of "breaking" of hydrogen bonds to any process.

Table 4. Estimated values for the energy of a hydrogen bond in liquid water (as collected by Eisenberg and Kauzmann, 1969)

Authors	Energy per H-bond (kcal/mole)
Nemethy & Scheraga (1962)	1.3
Grjotheim & Krogh-Moe (1954)	1.3 to 2.6
Worley & Klotz (1966)	2.4
Walrafen (1967)	2.5
Scatchard, Kavanagh & Ticknor (1952)	3.4
Haggis, Hasted & Buchanan (1952)	4.5
Pauling (1960)	5.2

The Dimensions of the Aqueous Pore Created by Gramicidin A in Liposomes Below Their Transition Temperature

The body of evidence that supports the idea that gramicidin A molecules form a transmembrane channel across lipid bilayers (Hladky & Haydon, 1970; Krasne *et al.*, 1971; Urry *et al.*, 1972) is reinforced by the finding that it increases the water permeability of liposomes prepared with saturated phospholipids below their transition temperature.

The osmotic insensitivity of liposomes below their transition temperature appears to be a combination of low water permeability and the solid crystal nature of the system that mechanically restricts any shape alteration. Thus, it is known from X-ray diffraction data and other physical techniques that the hydrocarbon chains of liposomes are, below their transition temperature, in a gel-like condition (Chapman *et al.*, 1967) with a rather restricted motion. The mobility of a solute across this phase is expected to be very slow compared with hydrocarbon chains in a liquid-crystalline state. In addition, the tight chain packing would probably reduce the partition coefficients of all solutes as has been found to be the case when cholesterol is present in egg-lecithin membranes (Cohen, 1975). When gramicidin A is added to such a system, the water permeability increases but the liposomes do not appear able to reach their maximum volume until they reach the transition temperature; it is evident that the "rigidity factor" restricting the swelling, only disappears at the transition temperature.

The presence of gramicidin A in a solid bilayer would also be expected to increase the permeability for those solutes with a diameter small enough to fit into the pore. Actually, the values of the activation energies for permeation across dimyristoyl-lecithin liposomes containing gramicidin A, have shown that urea does not substantially pass through the lipid part of these

Table 5. Physical size parameters for some solutes

Solute	Cylindrical radius[a] (Å)	Molar volume[b] (cm^3 $mole^{-1}$)
Formamide	2.07	40.0
Acetamide	2.38	59.2
Urea	2.41	45.0
Methylurea	2.60	61.5
Malonamide	2.78	69.3
Glycerol	3.07	73.0
Thiourea	2.86	54.2

[a] Taken from Soll (1967) and Sha'afi *et al.* (1971).
[b] Calculated by dividing the molecular weight of the solute by the density of the pure compound.

membranes. Thus the activation energy obtained for this solute was 7.2 kcal/mole instead of the 18.6 kcal/mole that corresponds in this system to a molecule with the capacity of forming five H-bonds in water. This unique behavior of urea can be understood on the basis that this solute is the only one that combines a small size with a high hydrogen-bonding capacity (Table 5).

Another solute which, according to its size (Table 5), may also pass through the gramicidin pore is formamide. The very large difference that has been observed between the permeabilities of formamide and acetamide across the gramicidin-containing system seems to indicate that this is the case even though such difference can be explained by the greater mass selectivity of the hydrocarbon chains of the lipid below their transition temperature (Cohen, 1975). However, formamide is the only member of the amides series which has a calculated diffusion coefficient in the membrane, larger than expected from its molar volume, suggesting that part of the increased permeability is due to permeation through the pore (Cohen, 1975). A measurement of the activation energy for permeation of this solute might reveal the relative contribution of the two permeation routes. Unfortunately, this parameter could not be measured due to experimental difficulties.

Acetamide has a cylindrical radius slightly smaller than urea (Table 5) and in consequence it would be expected to permeate via the gramicidin pore. However, the fact that the activation energy for the permeation of this solute does not differ substantially from the value expected in this system for molecules with three H-bonds, indicates that the largest proportion of this solute is permeating across the lipid part of the membrane.

It is interesting to note that the sequence in which the different solutes seem to be permeating across the gramicidin pore (water > formamide > urea) follows their molar volumes rather than their cylindrical radius (Table 5). Since the molar volume of a solute is a mixed parameter which includes a geometrical factor but also takes into account the hydrogen-bonding ability of a solute (Sha'afi, Gary-Bobo & Solomon, 1971), such a correlation suggests that solutes are crossing the pore with a certain degree of dehydration.

Information about the dimensions of the gramicidin pore has been obtained before from studies of the conductance of single channels as a function of membrane thickness which have indicated that the length of the pore is less than 35 Å (Haydon & Hladky, 1972). These authors have also measured the selectivity of the pore to univalent cations and the corresponding activation energies for the transport of sodium and potassium. They have found that the univalent cation selectivity is not very great and that the activation energies are very similar to those found for diffusion of cations in aqueous solutions. On the other hand, the present value for the activation energy of permeation of urea across the gramicidin system is somewhat greater than the corresponding value for diffusion of urea in water; that is 4.5 kcal/mole (Horowitz & Fenichel, 1964). This suggests that either this solute suffers some degree of dehydration in crossing the pore – as the correlation with the molar volumes has already indicated – or that there is an effect of temperature on the number of gramicidin channels in the bilayer.

Haydon and Hladky (1972) have argued that one of the dimeric helical structures proposed by Urry as a model for the gramicidin pore, the $\Pi^{6}_{L,D}$ helix, seems to be consistent with their data. The $\Pi^{6}_{L,D}$ helix has a length between 25 and 30 Å and a pore radius of 2 Å (Urry *et al.*, 1971).

The present information from the nonelectrolyte selectivity agrees with this model, even though it suggests slightly larger dimensions for the pore radius in order to accommodate a molecule of urea of 2.41 Å.

Comparison Between the Activation Energies for Permeation of Nonelectrolytes Across Some Biological Membranes and Liposomes

The linear relationship that has been found between the activation energies for permeation of nonelectrolytes across liposomes and their hydrogen-bonding ability in water suggests a method to investigate the extent in which a continuous lipid bilayer is present in biological membranes. Thus, it is to be expected that if aqueous pores are present in these membranes, a number of hydrophilic solutes would have activation energies for

permeation that deviate from the main correlation being lower than that corresponding to other solutes with a similar hydrogen-bonding capacity.

The effect of temperature on the permeability of biological membranes was investigated long ago by Jacobs, Glassman and Parpart (1935) using a hemolysis method. In ox red cell membranes these authors determined Q_{10}'s for the permeabilities of nine solutes. This is the same data that was used by Danielli (Davson & Danielli, 1943) in his analysis of membrane permeability. The corresponding activation energies are plotted in Fig. 8 against the number N_H of H-bonds.

One can see in Fig. 8 (open circles) that six of these solutes show a correlation between their ΔE's and the number of H-bonds. The average increase in the activation energy per H-bond for these solutes is 2.2 ± 0.4 kcal/mole, a value that is similar to that obtained in liposomes. However, the activation energies for urea, thiourea and water are very much lower than expected from their hydrogen-bonding capacity, suggesting that they are permeating by some parallel route.

In Fig. 8 are also plotted the activation energies measured by Hempling (1959) in ascites tumor cells (triangles) and in eggs of *Arbacia* (filled circles) by Stewart and Jacobs (1936). Unfortunately, very few values are available in these systems to justify a calculation of the increase of activation energy per H-bond.

An estimation of the contribution of the biological membranes to the total activation energy of permeation can be obtained by extrapolation of the values of the activation energies to zero number of H-bonds. It can be seen in Fig. 8 that this contribution may vary from a relatively low value for the Ehrlich ascites tumor cells to 8.8 kcal/mole for the red cell membranes but it might be even higher for the egg of *Arbacia.* These values are comparable to those obtained for liposomes suggesting that the fluidity of biological membranes may cover the whole range between a liquid-crystalline state and a more solid-like condition.

Variations in the state of the hydrocarbon chains in different biological membranes may provide an explanation for the wide range found for the activation energy of water permeation (Table 6). Thus, one can see in this Table that there is a group of systems which has an activation energy for water permeation that falls between the predicted value for water permeation across the lipid part of the dimyristoyl-lecithin/gramicidin liposomes and the value measured in egg-lecithin liposomes.

When water has an activation energy for water permeation much lower than for egg-lecithin liposomes, it would be crossing the membranes by an aqueous pore as has been suggested (Vieira, Sha'afi & Solomon, 1970).

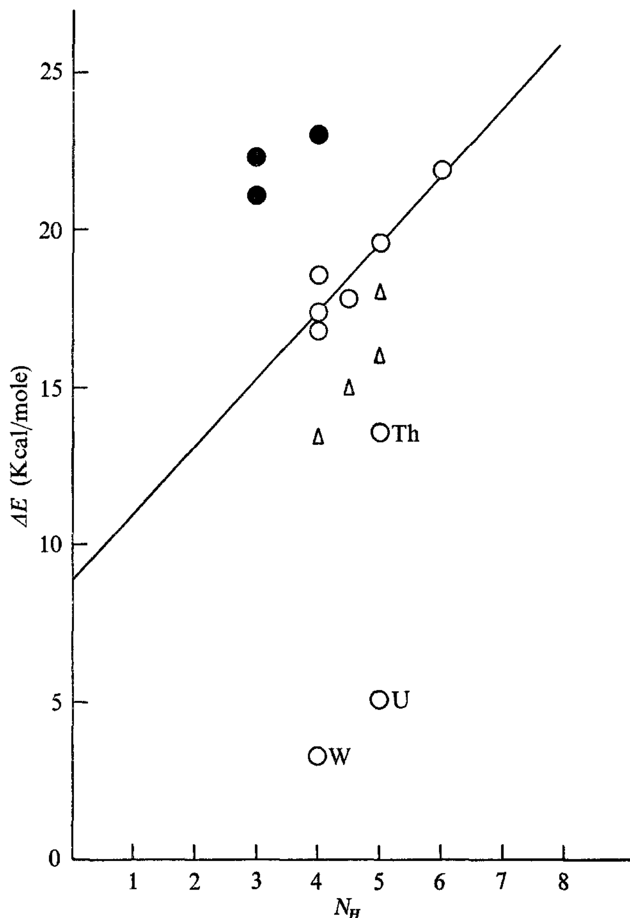

Fig. 8. The relation between the activation energies for permeation of nonelectrolytes across different biological systems and the capacity of the solutes to form hydrogen bonds in water. In ox red cells (○) the solutes are $N_H = 6$, glycerol; $N_H = 5$, urea, thiourea, triethyleneglycol; $N_H = 4.5$, diethyleneglycol; $N_H = 4$, ethyleneglycol, 1,2-propanediol, 1,3-propanediol, water. In eggs of *Arbacia* (•) the solutes are $N_H = 4$, ethyleneglycol; $N_H = 3$, propionamide, butyramide. In ascites tumor cell (△) the solutes are $N_H = 5$, urea, triethyleneglycol; $N_H = 4.5$, diethyleneglycol; $N_H = 4$, ethyleneglycol. All the activation energies for ox red cell membranes were calculated from the data given by Jacobs *et al.* (1935); the rest of the values were taken from Stein (1967)

Conclusions

Measurements of the activation energies for permeation of nonelectrolytes across liposomes has shown that for a continuous lipid bilayer the activation energies are a simple function of the number of H-bonds that a solute may form in water. However, when the bilayers contain an alternative

Table 6. Activation energies for water permeability across different membranes determined by an osmotic method

System	ΔE (kcal/mole)	Reference
Dimyristoyl-lecithin liposomes with gramicidin (predicted value)	17.0[a]	The present work
Sea urchin eggs	13–17	McCutcheon & Lucke (1932)
Thin lipid films containing cholesterol	12.7–14.6	Price & Thompson (1969); Redwood & Haydon (1969)
Chicken red cells	11.4	Farmer & Macey (1970)
Ehrlich ascites tumor cells	9.6	Hempling (1960)
Egg-lecithin liposomes	8.6	The present work
Nitella translucens	8.5	Dainty & Ginzburg (1964)
Egg-lecithin vesicles	8.25	Reeves & Dowben (1970)
Sheep red cells	7.6	Widdas (1951)
Cow red cells	4.0	Farmer & Macey (1970)
Dog red cells	3.7	Vieira *et al.* (1970)

[a] This value is the predicted activation energy for a molecule which makes four H-bonds in water and permeates across the lipid part of this system.

route of permeation such as an aqueous pore, this correlation is no longer valid for all solutes, because the solutes permeating through such a route have an activation energy for permeation that is lower than for permeation across the lipid part of the membrane. In this way, it has been demonstrated that urea is permeating via the gramicidin pore.

No systematic measurements of the effect of the number of H-bonds on the activation energies for permeation across biological membranes are available in the literature but there is some evidence suggesting that a similar correlation may be valid in these systems. The identification of solutes that permeate across "pores" in biological membranes by their departure from this correlation may be found to be easier, especially when the activation energies for permeation across the pore are lower than for permeation across the lipid part of the membrane. This appears to be the case for permeation of water and urea across some red cell membranes. However, for solutes such as urea, the presence of a more complex transport system also seems likely.

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