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NON-ELECTROLYTE PERMEABILITY AS A TOOL FOR STUDYING MEMBRANE FLUIDITY

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Summary

1. The reflection coefficient for the permeation of thiourea through bilayers of phosphatidylcholine is a function of the fatty-acid composition of the lipid molecules. By means of these reflection coefficients an index for membrane fluidity has been given to each of those lipids, relative to that of egg phosphatidylcholine.

2. The maximum number of water molecules that can copermeate with each molecule of solute by means of solute-solvent interaction is a function of the packing of the lipid molecules in the bilayer. This parameter has been used in this paper for characterizing the fluidity of cholesterol-containing membranes and for membranes with their lipids in the gel state.

Introduction

According to the irreversible thermodynamic considerations of Kedem and Katchalsky [1], the simultaneous permeation of a non-electrolyte and water through a selectively permeable membrane should be described by three parameters. The filtration coefficient (L_p) describes the conductivity of the membrane for water, and the permeation coefficient (ω) the conductivity of the membrane for the solute. Interaction between the permeation of the solute and the solvent is described by the so-called reflection coefficient (σ). This solute-solvent interaction was found to play an important role during non-electrolyte permeation through lipid bilayers [2].

Liposomes have been widely used as a model system for studying the barrier properties of biological membranes [3]. The irreversible thermodynamic

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parameters describing the non-electrolyte permeation through liposomal membranes can be determined with optical techniques by following the volume change of the cell during the permeation processes [2,4]. The filtration coefficient and the solute permeation coefficient cannot be obtained in an absolute way with this technique since all permeation rates are proportional to the outer area of the liposomes, which is normally unknown [2]. Since this outer area may be a function of the membrane lipid composition, an exact comparison of the rates of permeation through liposomal membranes with different lipid compositions is not possible. The ratio of the permeation coefficient and the filtration coefficient, normally expressed in $\omega \bar{V}_s/L_p$ in which $\bar{V}_s$ is the molar volume of the solute, and also the value of σ can be determined in an absolute way, independent of the outer area and independent of optical parameters [2,4]. The use of these two parameters makes it possible to compare the permeability properties of liposomes with a different membrane composition.

In this paper the reflection coefficients for the permeation of thiourea through liposomal membranes with different chemical composition are compared. A correlation will be made between these σ-values and the packing of the lipid molecules in the membrane. In addition, the permeation of non-electrolytes through cholesterol-containing bilayers and through bilayers with their lipid molecules in the gel state will be investigated.

Materials and Methods

Egg phosphatidylcholine was purified from egg yolk by acetone precipitation and subsequent chromatography over alumina oxide and silica gel. Soya phosphatidylcholine (essential phospholipid) was a gift of Dr. H. Eikermann from Natterman and Cie., Köln, G.F.R. The fatty acid composition of these two phospholipids has been given in ref. 5. Egg phosphatidic acid was prepared from egg phosphatidylcholine according to Davidson and Long [6]. 1,2 Dimyristoyl-*sn*-glycerol-3-phosphocholine (14 : 0/14 : 0-phosphatidylcholine), 1,2 dipalmitoyl-*sn*-glycero-3-phosphocholine (16 : 0/16 : 0-phosphatidylcholine), 1,2 dioleol-*sn*-glycero-3-phosphocholine (18 : 1_c/18 : 1_c-phosphatidylcholine), 1,2 dielaidoyl-*sn*-glycero-3-phosphocholine (18 : 1_t/18 : 1_t-phosphatidylcholine) and 1,2-dierucoyl-*sn*-glycero-3-phosphocholine (22 : 1_c/22 : 1_c-phosphatidylcholine) were synthesized as described previously [7]. Cholesterol was obtained from Fluka (Buchs, Switzerland). All other reagents were of Analytical Reagent Grade and used without further purification. For the permeability studies the procedure described in the previous paper [2] has been used. Multilamellar liposomes, containing 4 mol% egg phosphatidic acid were prepared in 20 mM glucose (unless stated otherwise). Differential scanning calorimetry was performed as described in ref. 8.

Results and Discussion

The permeability properties of liposomes as a function of membrane lipid composition. Solute-solvent interaction plays an important role during the permeation of non-electrolytes through lipid bilayers [2]. This was deduced

from the observation that for these permeation processes $(1-\sigma) >> \omega\bar{V}_s/L_p$ [1]. It has been shown in ref. 2 that this solute-solvent interaction can be fully described by assuming that with each molecule of solute N molecules of water will copermeate, making

$$(1-\sigma) = \frac{\omega(\bar{V}_s + N\bar{V}_w)}{L_p} = \left(\frac{\omega\bar{V}_s}{L_p}\right)\left(\frac{\bar{V}_s + N\bar{V}_w}{\bar{V}_s}\right) \quad (1)$$

In this equation $\bar{V}_s$ is the molar volume of the solute and $\bar{V}_w$ that of water. Since the values of σ, $\omega\bar{V}_s/L_p$ and N are independent of the outer area of the liposomes and independent of optical parameters, it is possible to compare these parameters for different liposomes. Table I shows the values of these three parameters for the permeation of thiourea at 15 or 30°C through liposomal membranes prepared from various phosphatidylcholines. As can be seen, σ and $\omega\bar{V}_s/L_p$ are a function of the membrane lipid composition, while N is not. It has already been shown for this type of system that σ and $\omega\bar{V}_s/L_p$ are a function of temperature and of the nature of the permeant [2]. The value of N, however, was found to be constant under all these conditions.

In order to investigate how the reflection coefficient for the permeation of thiourea varies with the membrane lipid composition, it is necessary to know the values of σ at a certain temperature. It has been shown in ref. 2 that $(1-\sigma)$ increases exponentially with rising temperature. The temperature dependence of this parameter, expressed in the form of an activation energy, was found to be independent of the membrane lipid composition and to have a value of 3.0 kcal/mol for the permeation of thiourea [2]. In this way σ values measured at

TABLE I

IRREVERSIBLE THERMODYNAMIC PARAMETERS FOR THE PERMEATION OF THIOUREA THROUGH BILAYERS OF PHOSPHATIDYLCHOLINES WITH DIFFERENT FATTY-ACID COMPOSITION

Liposomes, containing 4 mol % egg phosphatidic acid were prepared in 20 mM glucose. The ratio of water and glucose molecules inside the liposome (R) is 2750 under these conditions. Values and standard deviations were obtained from duplicate experiments.

Membrane lipid composition	Temperature (°C)	σ	$\omega\bar{V}_s/L_p$ X10^4	N	Phase transition temp. (°C)
Soya phosphatidylcholine	15	0.36 ±0.04	7.91 ±0.82	2500 ±100	<—20
18 : 1_c/18 : 1_c-Phosphatidylcholine	15	0.50 ±0.01	7.20 ±0.20	2100 ±100	—20
Egg phosphatidylcholine	15	0.54 ±0.01	5.14 ±0.86	2700 ±400	approx. 0
Egg phosphatidylcholine	30	0.41 ±0.02	7.05 ±1.03	2500 ±300	approx. 0
22 : 1_c/22 : 1_c-Phosphatidylcholine	30	0.57 ±0.03	5.31 ±1.00	2400 ±500	11.5
18 : 1_t/18 : 1_t-Phosphatidylcholine	30	0.59 ±0.01	4.85 ±0.12	2500 ±100	9.5
14 : 0/14 : 0-Phosphatidylcholine	30	0.61 ±0.01	3.30 ±0.18	3400 ±400	23
16 : 0/16 : 0-Phosphatidylcholine	30	1.00	—	—	41.5

different temperatures can be compared. For liposomes of the very unsaturated phospholipids it was of advantage to make measurements at relatively low temperatures, since all permeation processes take place very rapidly through these bilayers. Some of the experiments, however, had to be performed at higher temperatures because only the permeability properties of bilayers in the liquid-crystalline state were to be compared in this study (temperatures for the gel to liquid-crystalline phase transition as detected with differential scanning calorimetry have been listed in Table I).

Fig. 1 illustrates how the reflection coefficient at a certain temperature can be calculated from values found at other temperatures. By plotting ln $(1 - \sigma)$ against the reciprocal of the absolute temperature for the various phosphatidylcholines, a set of parallel lines is obtained each with a slope which corresponds to an activation energy of 3.0 kcal/mol. When the values of the reflection coefficient for thiourea at 30° C are compared for the different liposomes (Fig. 1, vertical dotted line), it can be seen that a more unsaturated character of the lipid molecules results in a lower value of σ. Since the molecular area of phospholipids increases with a more unsaturated character of the lipid molecules [9], it can be stated more generally that a closer packing of the lipid molecules in the bilayer gives rise to a higher value of σ. It can indeed be imagined that an increase in the packing of the phospholipid molecules in the bilayer will result in a stronger reduction of the permeation coefficient of the large solute molecules than of the much smaller water molecules, thus leading to a lower value of $\omega \bar{V}_s/L_p$. Since N is a constant under all conditions used here, $(1 - \sigma)$ is proportional to $\omega \bar{V}_s/L_p$ (see Eqn. 1). It can, therefore, be concluded that under isothermic conditions differences in $(1 - \sigma)$ and $\omega \bar{V}_s/L_p$ can be related to differences in the packing of the lipid molecules.

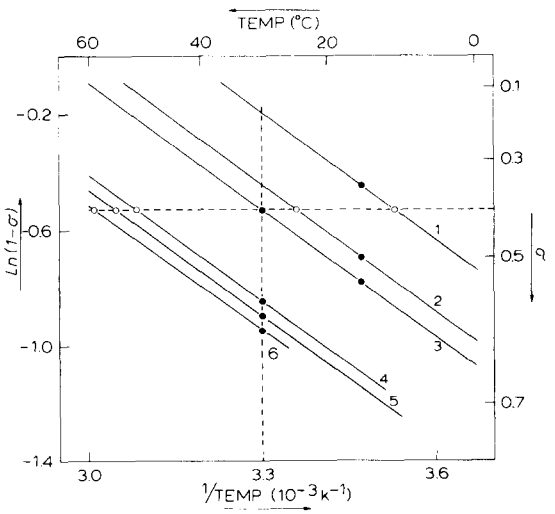


Fig. 1. Temperature dependence of the reflection coefficient for the permeation of thiourea through liposomes with different membrane lipid composition. 1, soya phosphatidylcholine; 2, 18 : 1_c/18 : 1_c-phosphatidylcholine; 3, egg phosphatidylcholine; 4, 22 : 1_c/22 : 1_c-phosphatidylcholine; 5, 18 : 1_t/18 : 1_t-phosphatidylcholine; 6, 14 : 0/14 : 0-phosphatidylcholine. Closed circles denote the measured σ values (taken from Table I), the open circles denote the temperature at which $\sigma = 0.41$.

Taking the value of the reflection coefficient for permeation of thiourea through bilayers of egg phosphatidylcholine at 30°C as a reference, these differences in molecular packing can be quantified by dividing the value of $(1-\sigma)$ for a certain membrane by the value of $(1-\sigma)$ for the reference. The values of Z_{THU}, obtained in this way, are listed in Table II. Z is not only the ratio of the $(1-\sigma)$ values, but also of the $\omega\bar{V}_s/L_p$ values. Since the temperature dependence of $(1-\sigma)$ is similar for all these membranes its value holds for every temperature and is, therefore, a characteristic for a certain membrane. It is important to note that although the values of Z are a function of the permeant used, the sequence of lipids with respect to their Z values will not depend on the choice of the non-electrolyte.

Knowing the temperature dependence of σ, it is also possible to calculate at which temperature a given liposome will have a certain σ value towards permeation of thiourea. Taking the σ value for the permeation of this solute through bilayers of egg phosphatidylcholine at 30°C again as a reference ($\sigma = 0.41$), it is shown in Fig. 1 (horizontal dotted line) and also in Table II at which temperature (T_f) each of the tested membranes will have this σ value. In addition, the difference between this calculated temperature and the reference temperature (30°C) has been listed (ΔT_f). Since N is a constant under all conditions used here, it can be concluded that there is a certain temperature (T_f) for each of the phospholipid bilayers at which they will have the same values for all three parameters σ, $\omega\bar{V}_s/L_p$ and N, as egg phosphatidylcholine at 30°C. From kinetic considerations the rate of penetration of a non-electrolyte molecule into the lipid bilayer will be proportional firstly to the concentration of solute molecules in the medium, secondly to the probability that such a solute molecule can leave the water phase by breaking down the hydrogen bonds with its environment and thirdly to the concentration of "cavities" in the bilayer which have the proper size for the penetrating molecule to fit in. The proportionality constant will reflect the lipid affinity of the permeant. From the high values of the activation energy for non-electrolyte permeation and from the correlation between these values and the polarity of the permeant, as was observed for a number of poly-alcohols, it has been concluded that the energy required for the dehydration of the non-electrolytes gives the largest contribution to the total activation energy of the permeation pro-

TABLE II

COMPARISON OF THE PERMEABILITY PROPERTIES OF BILAYERS OF PHOSPHATIDYLCHOLINES WITH DIFFERENT FATTY-ACID COMPOSITION TOWARDS THIOUREA AT 30°C, USING EGG PHOSPHATIDYLCHOLINE ($\sigma = 0.41$) AS A REFERENCE

For interpretation of the symbols see text. (THU is an abbreviation for thiourea.)

Membrane lipid composition	σ (30°C)	Z_{THU}	T_f (°C)	ΔT_f (°C)
Soya phosphatidylcholine	0.18	1.39	10.6	−19.4
18 : 1_c/18 : 1_c-Phosphatidylcholine	0.36	1.09	24.5	−5.5
Egg phosphatidylcholine	0.41	1.00	30.0	0
22 : 1_c/22 : 1_c-Phosphatidylcholine	0.57	0.73	50.6	+20.6
18 : 1_t/18 : 1_t-Phosphatidylcholine	0.59	0.69	54.0	+24.0
14 : 0/14 : 0-Phosphatidylcholine	0.61	0.66	57.6	+27.6

cess [10,11]. This implies that the increase of permeation rate with rising temperature is mainly due to a larger probability for a dehydration to occur. The observation that the permeability properties of membranes can be identical when they are compared at different temperatures does not, therefore, imply that the packing properties of the lipids are the same at those temperatures. A comparison of different membranes with respect to their permeability properties is therefore only allowed under isothermic conditions. The Z_{THU} values which have been obtained for the different membranes can now be interpreted in terms of the above kinetics. The value of $\omega \bar{V}_s/L_p$ will be proportional to the ratio of the number of "cavities" in the bilayer with the size of at least a thiourea molecule and that of the size of at least a water molecule. Under isothermic conditions the proportionality between $\omega \bar{V}_s/L_p$ and this ratio of "cavities" will be the same for all membranes. From this it follows that the fraction of large "cavities" is higher for the more unsaturated phospholipids. In this way the parameter Z gives a relative value for the fluidity of the membrane. Absolute values for membrane fluidity as measured with fluorescence techniques [12] cannot be obtained in this way. Also changes in fluidity with variation of temperature cannot be measured with this technique.

The effect of surface charge on the permeation of non-electrolytes. It is shown in Fig. 2 that small variations of the percentage of egg phosphatidic acid in liposomes of egg phosphatidylcholine have hardly any influence on the reflection coefficient for the permeation of thiourea at 15°C. Since also N was found to be constant, this implies that none of the three parameters σ, $\omega \bar{V}_s/L_p$ and N, is influenced by the surface charge. Since, in the previous part of this study, phospholipids with different surface areas have been used, the introduction of a fixed percentage of phosphatidic acid will not always result in the same surface potential. The above result demonstrates, however, that the differences in σ values found for the various phospholipids cannot partly be caused by a difference in surface potential. These data disagree with those obtained by Lelievre and Rich [13], who observed large effects of the surface charge on the values for the reflection coefficient. It is not understood what may have caused this discrepancy.

The effect of the incorporation of cholesterol on membrane permeability. It

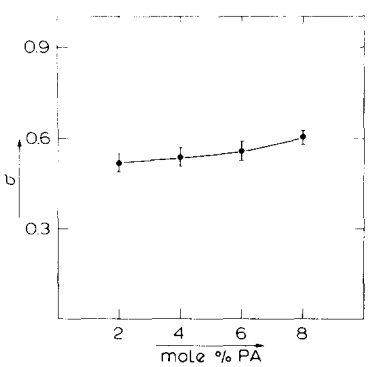


Fig. 2. Reflection coefficient for the permeation of thiourea through bilayers of egg phosphatidylcholine at 15°C as a function of egg phosphatidic acid (PA) content.

is known that cholesterol strongly influences the packing of phospholipid molecules in the bilayer [14]. Monolayer [9,14] and NMR [15] studies have shown that the interaction between cholesterol and phosphatidylcholine is dependent on the fatty acid composition of the phospholipid molecule. Phosphatidylcholine species in which both fatty acid chains are unsaturated give only a poor interaction with the sterol molecule, while those species in which only one of the chains contains a single double bond and the disaturated species give the strongest interaction. According to the observations of Blok et al. [16], the activation energy for the permeation of water and non-electrolytes through bilayers of 18 : 1_c/18 : 1_c-phosphatidylcholine is independent of the cholesterol content of the membrane, but enhanced activation energies were observed for bilayers of egg phosphatidylcholine and 14 : 0/14 : 0-phosphatidylcholine upon incorporation of cholesterol.

Table III shows the effect of incorporation of 40 mol% of cholesterol on the

TABLE III

PERMEATION OF THIOUREA THROUGH BILAYERS OF PHOSPHATIDYLCHOLINE WITH AND WITHOUT 40 MOL % CHOLESTEROL *

Standard deviations have been taken from duplicate experiments.

Membrane lipid composition **	Temp. (°C)	σ	$\omega\bar{V}_s/L_p$ $\times 10^4$	N
Soya phosphatidylcholine				
—Cholesterol	15	0.36	7.91	2500
		±0.04	±0.82	±100
+Cholesterol	15	0.47	5.60	2800
		±0.02	±0.12	±100
Egg phosphatidylcholine				
—Cholesterol	15	0.54	5.14	2700
		±0.01	±0.86	±400
+Cholesterol	15	0.77	4.04	1700
		±0.02	±0.31	±100
Egg phosphatidylcholine				
—Cholesterol	30	0.41	7.05	2500
	30	±0.02	±1.03	±300
+Cholesterol	30	0.60	3.91	3000
		±0.04	±0.80	±200
18 : 1_c/18 : 1_c-Phosphatidylcholine				
—Cholesterol	15	0.50	7.20	2100
		±0.01	±0.20	±100
+Cholesterol	15	0.58	5.99	2100
		±0.04	±0.99	±250
18 : 1_c/18 : 1_c-Phosphatidylcholine				
—Cholesterol	30	0.35	8.47	2300
		±0.03	±1.70	±300
+Cholesterol	30	0.50	6.01	2500
		±0.01	±1.20	±300
14 : 0/14 : 0-Phosphatidylcholine				
—Cholesterol	30	0.61	3.30	3400
		±0.01	±0.18	±400
+Cholesterol	30	0.90	3.15	950
		±0.02	±0.85	±100

* Data with respect to the membranes without cholesterol have been taken from Table I of this paper.

** In addition to phosphatidylcholine and cholesterol the membranes always contained 4 mol % egg phosphatidic acid.

values of σ, $\omega\bar{V}_s/L_p$ and N for the permeation of thiourea through bilayers of various phosphatidylcholines. For all lipids tested, the introduction of this amount of cholesterol resulted in an increased σ value, illustrating a closer packing of the phospholipid molecules in the bilayer. In addition, however, variations in the value of N were observed for some of the phosphatidylcholines tested. Significantly decreased values of N were measured for permeation of thiourea through bilayers of egg phosphatidylcholine at 15°C and of 14 : 0/14 : 0-phosphatidylcholine at 30°C. No significant change in N was observed for the very unsaturated species. This suggests that a reduction in the value of N is only observed for those species of phosphatidylcholine, which also give an enhancement of the activation energies upon incorporation of cholesterol. This is illustrated in Table IV, in which the temperature dependence of the different irreversible thermodynamic parameters for the permeation of glycerol and thiourea is shown through bilayers of various phosphatidylcholines containing 40 mol% of cholesterol. (The values for the membranes without cholesterol have been given in Tables III and IV of ref. 2.) The activation energies for the various parameters were not changed for bilayers of 18 : 1_c/18 : 1_c-phosphatidylcholine upon introduction of cholesterol, and N was found to be independent of temperature. A small but significant increase was observed for the activation energy of the isotonic swelling rate and L_p for egg phosphatidylcholine (in agreement with ref. 16), but the variation of N with temperature was within experimental error. For the permeation of both glycerol and thiourea through bilayers of 14 : 0/14 : 0-phosphatidylcholine containing 40 mol% of cholesterol, however, the high activation energy for the isotonic swelling rate and L_p was accompanied by a strong temperature dependence of

TABLE IV

TEMPERATURE DEPENDENCE OF NON-ELECTROLYTE PERMEATION THROUGH CHOLESTEROL-CONTAINING MEMBRANES

Activation energies are given in kcal/mol.

Membrane lipid composition **	Solute	Isotonic swelling	L_p	ω	$1-\sigma$	$\omega\bar{V}_s/L_p$	N
18 : 1_c/18 : 1_c-Phosphatidylcholine + cholesterol (40 mol %)	Glycerol	17.2 ±1.5 (20) **	10.2 ±1.2 (12)	17.7 ±1.0	6.6 ±2.7	7.5 ±0.3	−1.0 ±2.5
Egg phosphatidylcholine + cholesterol (40 mol %)	Glycerol	20.2 ±1.0 (21)	12.4 ±2.0 (14)	18.8 ±1.7	7.4 ±1.0	6.4 ±1.0	+1.0 ±1.3
14 : 0/14 : 0-Phosphatidylcholine + cholesterol (40 mol %) ***	Glycerol	25.9 ±2.1 (25)	16.9 ±2.0 (19)	21.9 ±1.6	8.6 ±2.4	5.0 ±2.0	+3.6 ±2.0
14 : 0/14 : 0-Phosphatidylcholine + cholesterol (40 mol %) ***	Thiourea	20.5 ±0.4	16.4 ±0.5 (19)	16.4 ±1.0	3.4 ±0.5	+0.1 ±0.5	+3.8 ±1.2

* Besides phosphatidylcholine and cholesterol the liposomes always contained 4 mol % egg phosphatidic acid.

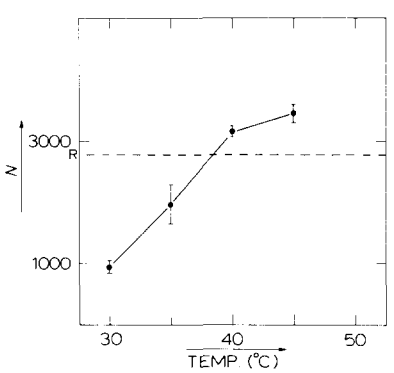
** Values taken from Blok et al. [16].

*** Measured over a temperature range from 25 to 45°C.

N. As a result of this temperature dependence of N, the activation energy for the isotonic swelling rate no longer coincides with that of the permeation coefficient. The activation energy of the permeation coefficient through bilayers of 14 : 0/14 : 0-phosphatidylcholine is only slightly enhanced upon incorporation of cholesterol.

The value of N as a function of temperature for the permeation of thiourea through bilayers of 14 : 0/14 : 0-phosphatidylcholine containing 40 mol% of cholesterol is shown in Fig. 3. The increase of N with rising temperature levels off at temperatures above 40°C. The values of N which are found at these temperatures are significantly higher than 2750, which is the ratio of glucose and water molecules inside the liposomes (R) under these conditions. It should be noted, however, that a relatively high value for N has also been found for this lipid in the absence of cholesterol (see Table I). On the other hand, for bilayers of egg phosphatidylcholine containing 40 mol% of cholesterol values of N have been measured at temperatures above 30°C, which were also 10—20% higher than R (data not shown).

Fig. 4 shows the effect of the incorporation of varying percentages of cholesterol into bilayers of 14 : 0/14 : 0-phosphatidylcholine on the permeability properties of these bilayers towards thiourea at 30°C. A jump in the value of σ is observed on going from 10 to 15 mol% cholesterol, which is due to a difference in the value of N, since $\omega \bar{V}_s/L_p$ is hardly affected by the incorporation of cholesterol. It is worth mentioning in this respect that according to the observations of Blok et al. [16], there is also a sudden increase in the activation energy for water permeation for this phospholipid on going from 10 to 15



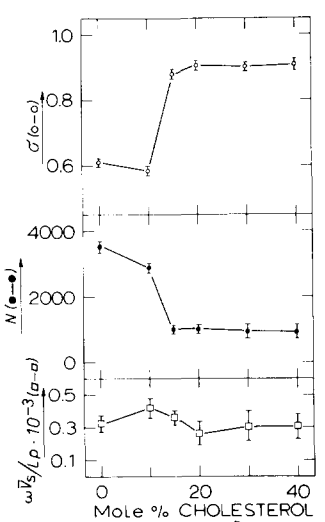


Fig. 3. The number of water molecules (N) copermeating per molecule of thiourea through bilayers of 14 : 0/14 : 0-phosphatidylcholine containing 40 mol% of cholesterol as a function of temperature. R is the ratio of water and glucose molecules inside the liposomes (=2750).

Fig. 4. The irreversible thermodynamic paramaters σ, $\omega \bar{V}_s/L_p$ and N for the permeation of thiourea at 30°C through bilayers of 14 : 0/14 : 0-phosphatidylcholine with varying percentage of cholesterol. The liposomes, prepared in 20 mM glucose, always contained 4 mol% egg phosphatidic acid.

mol% cholesterol. This suggests that after reaching a cholesterol content of 15% a critical packing of lipid molecules has been reached which does not allow a high copermeation of water molecules to take place.

The data concerning the effects of cholesterol on the permeability properties of lipid bilayers can be understood in the following way. By means of an interaction between the permeation of the solute and water, a number of water molecules can copermeate with each molecule of solute. In order to understand the measured values of copermeating molecules it was assumed [2] that there is also a compensating flow of water in the opposite direction such that the value of N is kept close to the ratio of glucose and water molecules inside the liposomes (R). The gain of entropy of the system will be optimal during isotonic swelling when $N = R$, since under those conditions no osmotic differences are induced during solute permeation. It can be speculated that there is a maximum number of water molecules that can copermeate with each solute molecule (N_{max}), depending on the packing properties of the lipids in the bilayers. Fig. 5 shows the value of N for the permeation of thiourea through bilayers of egg phosphatidylcholine at 15°C as a function of the glucose concentration in which the liposomes have been made. (These data have been taken from Table II of ref. 2.) The maximum value of N under these conditions will be higher than 5500. For bilayers of egg phosphatidylcholine containing 40 mol% cholesterol, however, N cannot exceed a maximum value of 1700 under these conditions (see Fig. 5). This indicates that a closer packing of the lipid molecules in the bilayers gives rise to a lower value of N_{max}. A closer packing of the lipid molecules will result in a reduction of the concentration of "cavities" in the bilayer. Therefore, the effect of the penetration of a solute molecule on the formation of other "cavities", which is thought to be the origin of solute-solvent interaction (see ref. 2), will also be less pronounced in a more closely packed bilayer, although the compressibility of the two monolayers of the membrane will also be smaller. It can be understood in this way

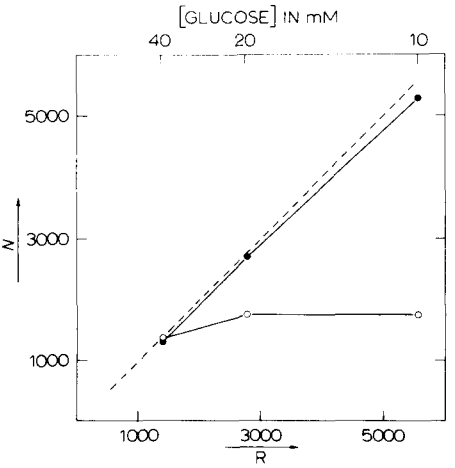


Fig. 5. The number of water molecules (N), copermeating per molecule of thiourea at 15°C through liposomes of egg phosphatidylcholine (●———●) and egg phosphatidylcholine containing 40 mol% of cholesterol (○———○) for liposomes prepared in different glucose concentrations. R is the ratio of water and glucose molecules inside the liposomes.

that an increase in temperature, which will make the membrane more fluid, leads to a higher value of N_{max} (see Fig. 3).

Under the conditions when N_{max} is larger than R (for example during permeation of thiourea at 15°C through bilayers of egg phosphatidylcholine containing 40 mol% cholesterol prepared in 40 mM glucose) the measured value of N will be equal to R, and will, therefore, be independent of temperature. When N_{max} is smaller than R (for example for these bilayers when prepared in 20 mM glucose or less), the measured value of N will be equal to N_{max}, and will thus increase with increasing temperature. As soon as N_{max} has become larger than R with increasing temperature, N will follow R again, although values of N slightly higher than R have been observed. A sharp distinction between a region in which N varies with temperature and a region in which N is independent of temperature has never been observed, although the temperature dependence of N expressed in an activation energy is certainly not constant over the whole temperature range.

Since both the values of $\omega\bar{V}_s/L_p$ and N may vary upon incorporation of cholesterol in the membrane, the fluidity of the membrane cannot directly be related to the values of σ as it has been for the systems without cholesterol. Although such a correlation could be made with the values of $\omega\bar{V}_s/L_p$, this is difficult since the error in both the value of this parameter and in its activation energy is rather large. In addition is was observed that for the permeation of thiourea $\omega\bar{V}_s/L_p$ is insensitive to the incorporation of cholesterol into membranes of, for example, 14 : 0/14 : 0-phosphatidylcholine and also for these cholesterol-containing membranes with respect to temperature.

A better characterization of the fluidity of these cholesterol-containing membranes is given by the parameter N_{max}. Although this parameter can be used more generally, it is difficult to measure its exact value for the membranes that were used in experiments shown in Table I. Since it is difficult to quantify differences in N_{max} values, this method only allows us to state that a higher value of N_{max} reflects a more fluid character of the bilayer. An advantage of the use of this parameter above the use of the reflection coefficient is that the fluidities of membranes can be compared at different temperatures.

The permeability properties of lipid bilayers in the gel state. The rate of water permeation through lipid bilayers is strongly reduced below the gel to liquid-crystalline phase transition tenperature [17]. In addition it has been observed that the activation energy for water permeation is higher in the gel state than in the liquid-crystalline state [17]. Only a limited number of solutes are able to permeate through those solid bilayers. The data of Table I show that bilayers of 16 : 0/16 : 0-phosphatidylcholine at 30°C, which is below the phase transition temperature (41.5°C), are impermeable for thiourea ($\sigma = 1.0$). Solutes which were found to permeate through these bilayers are formamide, glycol and 1,3 propanediol. In this study the permeability of solid bilayers of 16 : 0/16 : 0-phosphatidylcholine towards glycol is investigated over the temperature range from 24 to 33°C. In order to increase the rates of solute and water permeation, the liposomes were prepared (above the phase transition temperature) in 100 mM glucose which made it possible to apply steeper gradients over the membrane. Table V shows the values of the activation energy of the irreversible thermodynamic parameters for the above mentioned per-

TABLE V

TEMPERATURE DEPENDENCE OF THE PERMEATION OF GLYCOL THROUGH BILAYERS OF 16 : 0/16 : 0-PHOSPHATIDYLCHOLINE AT TEMPERATURES BELOW THE GEL TO LIQUID-CRYSTALLINE PHASE TRANSITION (24—33°C)

Liposomes containing 4 mol % egg phosphatidic acid were prepared in 100 mM glucose. Activation energies are given in kcal/mol. Values and standard deviations have been taken from 4 individual experiments.

Parameter	Activation energy
L_p	17.8 ± 1.1
ω	22.1 ± 1.1
$1-\sigma$	20.6 ± 1.0
N	16.6 ± 2.5
Isotonic swelling rate	35.9 ± 1.9

meation process. A high activation energy for water permeation (L_p) was observed when compared with bilayers in the liquid-crystalline state [2,17]. The value presented here, however, is much lower than the average value of 26.4 kcal/mol given by Blok et al. [17] for the activation energy of water permeation through bilayers in the gel state, using liposomes prepared in 20 mM glucose. It could be demonstrated that the activation energy for water permeation through bilayers in the gel state depends on the glucose concentration in which the liposomes have been prepared; such a phenomena has never been observed for bilayers in the liquid-crystalline state. The activation energy for osmotic shrinkage and osmotic swelling were found to be identical for all preparations tested. These activation energies for water permeation through gel state bilayers are subject of further investigations.

The initial swelling rate of these liposomes in isotonic solutions of glycol is very strongly temperature dependent. A large contribution to the activation energy of this parameter is given by the temperature dependence of N. The values of N and σ for this process as a function of temperature are given in Fig.

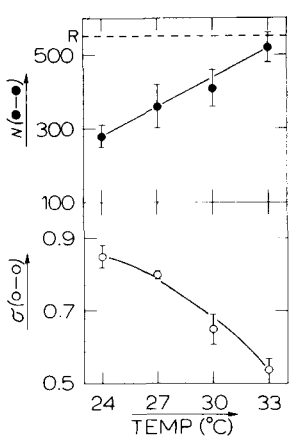


Fig. 6. Temperature dependence of N and σ for the permeation of glycol through bilayers of 16 : 0/16 : 0-phosphatidylcholine below the gel to liquid-crystalline phase transition temperature of this phospholipid. Liposomes have been prepared in 100 mM glucose (R = 550).

6. For all temperatures tested the value of N was smaller than R (= 550), which indicates that the number of copermeating water molecules is limited by the packing of the lipid molecules in the bilayer ($N = N_{max}$). The low values of N are an indication of the close packing of the lipid molecules in the gel state.

It can be stated more generally that for those situations in which $N = R$ the permeability properties of different liposomes can be best compared by means of the reflection coefficient, and in the situation that $N = N_{max} < R$ by means of the parameter N_{max}.

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References

1 Kedem, O. and Katchalsky, A. (1958) Biochim. Biophys. Acta 27, 229—246
2 Van Zoelen, E.J.J., Blok, M.C., Stafleu, G.P., Lancée-Hermkens, A.M.W., de Jesus, C. Henriques and de Gier, J. (1978) Biochim. Biophys. Acta 511, 320—334
3 Bangham, A.D., Hill, M.W. and Miller, N.G.A. (1974) Methods in Membrane Biology (Korn, E.D., ed.), 1, 1—68, Plenum Press, New York
4 Van Zoelen, E.J.J., Blok, M.C. and de Gier, J. (1976) Biochim. Biophys. Acta 436, 301—306
5 Blok, M.C., de Gier, J. and van Deenen, L.L.M. (1974) Biochim. Biophys. Acta 367, 210—224
6 Davidson, F.M. and Long, C. (1958) Biochem. J. 69, 458—466
7 Van Deenen, L.L.M. and de Haas, G.H. (1964) Adv. Lipid Res. 2, 167—234
8 De Kruijff, B., van Dijck, P.W.M., Demel, R.A., Schuyff, A., Brants, F. and van Deenen, L.L.M. (1974) Biochim. Biophys. Acta 356, 1—7
9 Demel, R.A., Geurts van Kessel, W.S.M. and van Deenen, L.L.M. (1972) Biochim. Biophys. Acta 266, 26—40
10 De Gier, J., Mandersloot, J.G., Hupkes, J.V., McElhaney, R.N. and van Beek, W.P. (1971) Biochim. Biophys. Acta 233, 610—618
11 Stein, W.D. (1967) The Movement of Molecules Across Cell Membranes, Academic Press, New York
12 Shinitzky, M. and Inbar, M. (1976) Biochim. Biophys. Acta 433, 133—150
13 Lelievre, J. and Rich, G.T. (1973) Biochim. Biophys. Acta 298, 15—26
14 Demel, R.A. and de Kruijff, B. (1976) Biochim. Biophys. Acta 457, 109—132
15 Lancée-Hermkens, A.M.W. and de Kruijff, B. (1977) Biochim. Biophys. Acta 470, 141—151
16 Blok, M.C., van Deenen, L.L.M. and de Gier, J. (1977) Biochim. Biophys. Acta 464, 509—518
17 Blok, M.C., van Deenen, L.L.M. and de Gier, J. (1976) Biochim. Biophys. Acta 433, 1—12