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THE EFFECT OF CHOLESTEROL INCORPORATION ON THE TEMPERATURE DEPENDENCE OF WATER PERMEATION THROUGH LIPOSOMAL MEMBRANES PREPARED FROM PHOSPHATIDYLCHOLINES

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Summary

The permeation of water through liposomal membranes composed of phosphatidylcholine plus varying amounts of cholesterol was studied as a function of temperature.

1. Increasing amounts of cholesterol caused a gradual disappearance of the abrupt change in the rate of water permeation near the gel to liquid-crystalline phase transition temperature of dipalmitoylphosphatidylcholine and dimyristoylphosphatidylcholine liposomes. At cholesterol concentrations above about 30 mol % there was no longer a discontinuity in the rate of water permeation.

2. The incorporation of cholesterol produces a steep change in the activation energy of the water permeation above the transition temperature of the saturated lecithin occurring at about 15 mol % of cholesterol. Below the transition temperature there was a gradual decrease in the activation energy of the water permeation in the region of 0 to 33 mol % of cholesterol.

3. In systems containing unsaturated phosphatidylcholines cholesterol also enhanced the activation energy of the water permeation although to a lesser extent. The results indicate that the position of the *cis*-double bond in the fatty acid chain is very important in this respect.

4. In systems in which cholesterol increased the temperature dependence of the water permeation there is also an enhancement of the temperature dependence of the isotonic glycerol and erythritol swelling by the same number of kcal/mol.

Introduction

Model membrane systems have been widely used to explore the molecular consequences of the presence of cholesterol in biomembranes [1,2]. Monolayer

studies have shown that the introduction of cholesterol into liquid expanded films of e.g. phosphatidylcholine produces a reduction in the area per molecule [2,3], indicating an increased intermolecular interaction. Spectroscopic studies on liposome systems have demonstrated a reduction of the rotational freedom of motion of the fatty acid chains of phospholipids by cholesterol [1,2,4]. From calorimetric studies it appeared that increasing concentrations of cholesterol in phosphatidylcholine bilayers cause a reduction in the energy content of the gel to liquid-crystalline phase transition and support the view that each cholesterol molecule withdraws two phosphatidylcholine molecules from the cooperative lipid phase transition [5—8]. From the above studies it can be concluded that cholesterol affects the mobility of the phospholipids such that the lipid bilayer reaches an "intermediate state", in which the hydrophobic interior has a high viscosity.

A good correlation has been found between the effect of cholesterol on the packing and mobility of mixed phospholipid/cholesterol bilayers and the permeability properties of these structures.

In general it can be concluded that cholesterol reduces the rate of permeation when introduced into liquid-crystalline lipid bilayers [3,9—12], and into the membranes of *Acholeplasma laidlawii* ([13—15]. Below the transition temperature cholesterol increases the permeability of the phospholipid bilayers [16]. No significant changes were observed in the activation energy of the isotonic non-electrolyte swelling of liposomes of relatively unsaturated lecithins [17] and of *A. laidlawii* cells [13] upon cholesterol incorporation.

A recent study [18] revealed that water permeation through membranes of saturated lecithins is drastically reduced on cooling below the transition temperature, but still occurs to a measurable extent when the lipid bilayer is in the gel state. Temperature dependent studies demonstrated a change in the activation energy for the water premeation on going from one phase to the other [18].

In the present paper the effect of cholesterol incorporation on the temperature dependence of water permeation through liposomal membranes of synthetic lecithins is described. In view of the fact that cholesterol-containing membranes are brought to an intermediate state and the recent finding that the energy barrier of water permeation in the gel state and liquid-crystalline state has different values [18] special attention was paid to the activation energy of water permeation through the bilayers of cholesterol-containing phosphatidylcholine liposomes.

Materials and Methods

Lipids

Egg phosphatidylcholine was purified from egg yolk by acetone precipitation and subsequent chromatography over alumina oxide and silica gel. Egg phosphatidic acid was prepared from egg phosphatidylcholine by phospholipase D degradation. 1,2-Dimyristoyl-*sn*-glycero-3-phosphorylcholine, 1,2-dipalmitoyl-*sn*-glycero-3-phosphorylcholine, 1,2-dioleoyl-*sn*-glycero-3-phosphorylcholine, 1,2-dierucoyl-*sn*-glycero-3-phosphorylcholine and 1-stearoyl-2-oleoyl-*sn*-glycero-3-phosphorylcholine were synthesized as described previously [19]. The purity

of all lecithins was checked by thin-layer chromatography. Fatty acid impurities of the synthetic lipids were less than 1% as shown by gas chromatography. Cholesterol (99% pure) was obtained from Merck (G.F.R.) and used without further purification.

Permeability studies

Multilayered liposomes, containing 4 mol % of egg phosphatidic acid, were prepared at temperatures above the gel to liquid-crystalline phase transition temperature as previously described [9]. For the osmotic shrinkage experiments the liposomes were prepared in 10 mM glucose and diluted in this medium to a final lipid concentration of 0.30 mM. 9.6 ml of this suspension was transferred into a thermostatted cuvette and vigorously stirred. After temperature equilibration an osmotic shock was given by rapidly injecting 0.40 ml of 1.0 M glucose, preincubated at the same temperature. Changes in the turbidity were determined with a spectrophotometer (Vitatron, MPS type) at 438 or 450 nm. From the recorder tracings the relative initial shrinkage velocity, $[d(1/A)/dt]\%$, was calculated as previously described [9].

For studying the swelling of liposomes in isotonic non-electrolyte solutions the liposomes were prepared in 100 mM glucose. The final lipid concentration was 4.0 mM. After temperature equilibration 0.75 ml of the liposome dispersion was rapidly pipetted into 9.25 ml of a 100 mM glycerol or erythritol solution, and the relative initial swelling rate of the liposomes was measured.

All reagents were commercial and of analytical reagent grade.

The activation energies presented in this paper were all calculated by the least squares methods from plots in which $\ln[d(1/A)/dt]\%/T$ was plotted against $1/T$, T being the absolute temperature. Since the osmotic pressure is given by $\Pi = RT\Delta c$ [20], activation energies calculated in this way are more correct than those obtained in previous studies [18] where the temperature dependence of the osmotic pressure was neglected. The values obtained by this method are about 3% lower than those obtained from the previous method.

Results

Fig. 1 shows the effect of cholesterol incorporation into dipalmitoylphosphatidylcholine liposomes on the temperature dependence of the osmotic shrinkage of these mixed liposomes. In agreement with other studies [16,21] it was found that there is a gradual disappearance of the steep change in the rate of shrinkage near the transition temperature of dipalmitoylphosphatidylcholine. That there is still a discontinuity in the temperature dependence of the osmotic shrinkage of dipalmitoylphosphatidylcholine liposomes containing 20 mol% cholesterol, can be clearly shown by plotting the data in an Arrhenius plot (Fig. 2A). If more than about 30 mol % cholesterol was incorporated such a discontinuity was no longer found (Fig. 2B). The data in Table I suggest that the activation energy for the water permeation decreases rather gradually below the transition temperature of dipalmitoylphosphatidylcholine from about 28 kcal/mol to about 21 kcal/mol. Of particular interest is the increase in the activation energy above the transition temperature. The incorporation of more than about 15 mol % cholesterol into dipalmitoylphosphatidylcholine bilayers

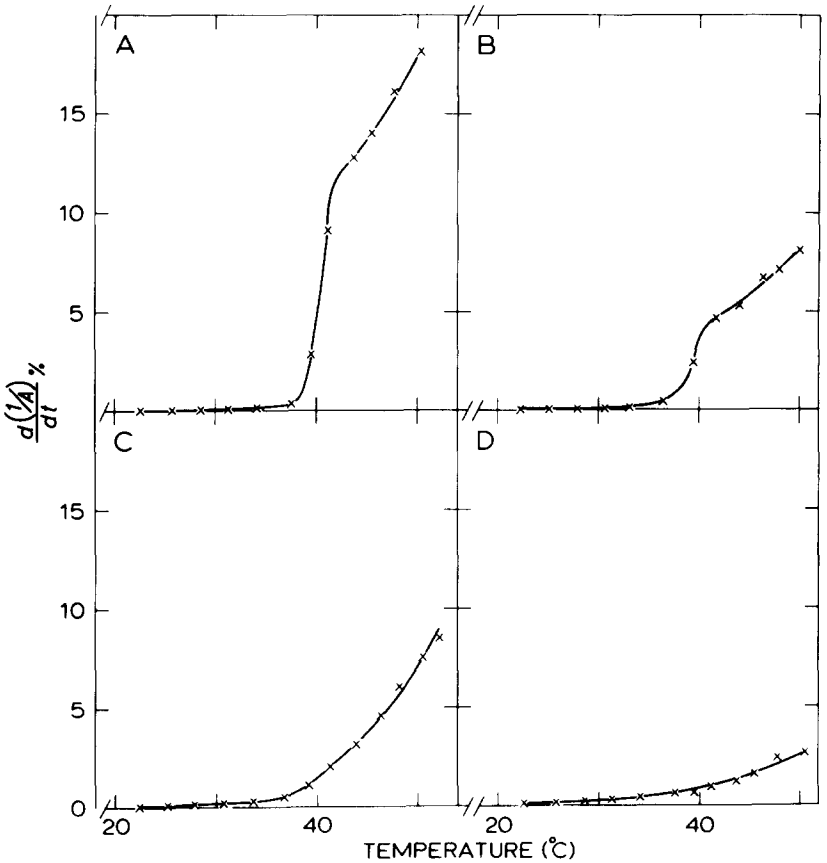

Fig. 1. Temperature dependence of the osmotic shrinkage of dipalmitoylphosphatidylcholine liposomes following the addition of hypertonic glucose. The experiment was carried out as described in Materials and Methods with liposomes containing 0 (A), 10 (B), 20 (C) and 33 (D) mol % cholesterol.

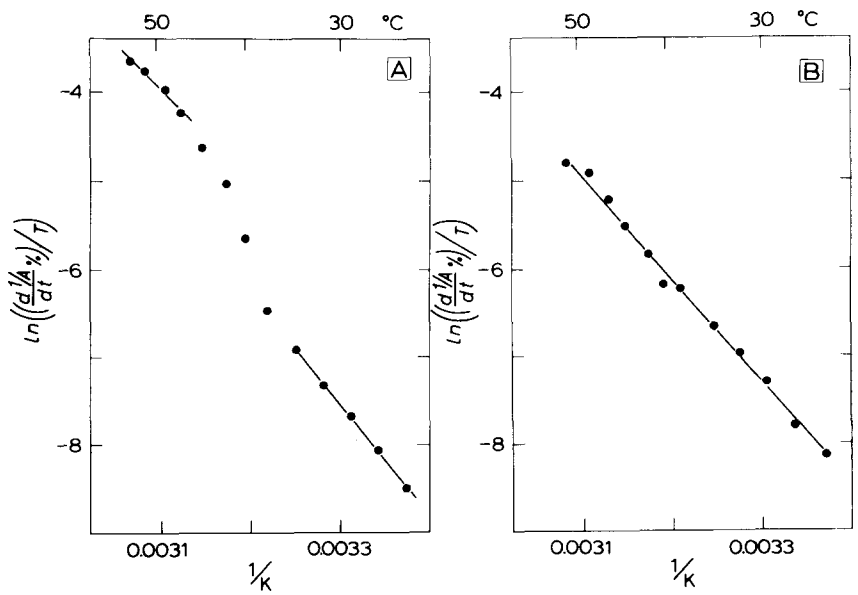

Fig. 2. Arrhenius plot of the osmotic shrinkage of dipalmitoylphosphatidylcholine liposomes, containing 20 (A) and 33 mol % cholesterol (B). The data of these plots are the same as in Fig. 1 C and D.

TABLE I

ACTIVATION ENERGY FOR THE PERMEATION OF WATER THROUGH DIPALMITOYLPHOSPHATIDYLCHOLINE MEMBRANES, CONTAINING VARYING AMOUNTS OF CHOLESTEROL

Mol % cholesterol	$T \leqslant °C$	Activation energy (kcal/mol)	$T \geqslant °C$	Activation energy * (kcal/mol)
0	34.1	28.3 ± 1.0	43.8	12.5 ± 0.7 10.2 ± 2.7
10	33.1	27.2 ± 0.7	46.3	12.7 ± 2.2 15.1 ± 1.2
15	33.1	27.2 ± 0.5	46.3	21.5 ± 4.2 22.8 ± 2.2
20	30.7	25.2 ± 0.8	46.5	20.8 ± 2.6 22.0 ± 7.7
33	34.1	24.2 ± 1.1	43.8	21.8 ± 4.2 23.0 ± 1.7
50	30.7	21.9 ± 0.4	44.0	21.4 ± 4.3 20.9 ± 0.8

* The first value is calculated from measurements in which the initial glucose gradient was 40 mM; the second one from measurements with 20 mM glucose.

caused a steep change in the activation energy for the water permeation from about 11 to about 21 kcal/mol (Table I). Very similar shifts in the activation energies of water permeation were observed in mixed dimyristoylphosphatidylcholine/cholesterol liposomes (Fig. 3).

In similar experiments with liposomes prepared from mixtures of cholesterol and unsaturated lecithins, it was found that cholesterol did not significantly elevate the activation energy of the water permeation through mixed dioleoylphosphatidylcholine/cholesterol membranes. In contrast to this finding, the

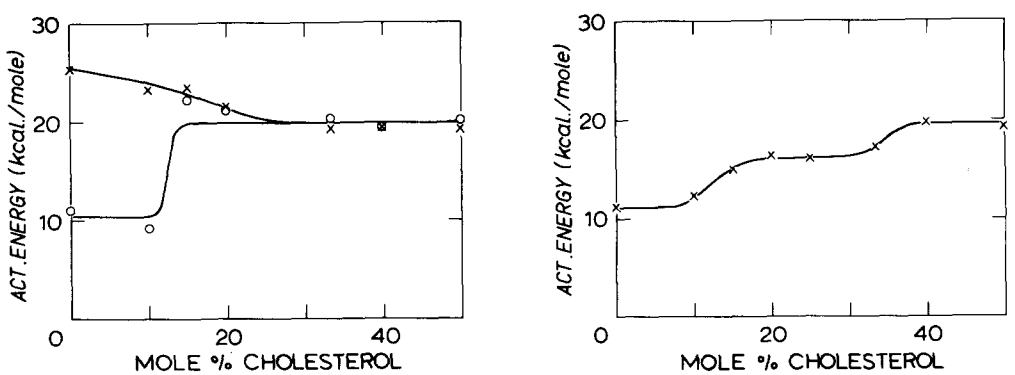

Fig. 3. The effect of cholesterol on the activation energy of the osmotic shrinkage of dimyristoylphosphatidylcholine liposomes (○———○), above and (X———X), below the lipid phase transition temperature of the lecithin. The experiment was carried out according to the procedure described in Materials and Methods.

Fig. 4. The effect of cholesterol on the activation energy of the water permeation process of dierucoylphosphatidylcholine liposomes. Activation energies were calculated from measurements done in the temperature region of 15 to 30° C. Experimental details are given in Materials and Methods.

incorporation of cholesterol into dierucoylphosphatidylcholine liposomes * resulted in a significant increase in the activation energy for the water permeation above the transition temperature (Fig. 4). The shift from about 11 to 20 kcal/mol, however, is much less sharp than was found with the disaturated lecithins above their transition temperatures (compare Figs. 4 and 3). Experiments with stearoyl/oleoyl-phosphatidylcholine liposomes in the liquid-crystalline state showed that the activation energy for water permeation was increased from 10.6 to 16.7 kcal/mol by the incorporation of 50 mol % cholesterol.

Table II shows that higher concentrations of cholesterol in egg phosphatidylcholine membranes also cause a slight but significant increase in the activation energy of water permeation. From experiments, in which the molar ratio of dimyristoylphosphatidylcholine/dioleoylphosphatidylcholine and the amount of cholesterol was varied, we could conclude that the activation energy for water permeation can be varied by changing either the fatty acid composition of the lecithins or the cholesterol content of the lipid membrane. This result is illustrated in Fig. 5, showing the data for three liposome preparations, all containing 50 mol % cholesterol. The data also indicate that, even in the presence of high cholesterol contents, the introduction of unsaturated phospholipid species produces an increase in the rate of water permeation. This result agrees very well with previous findings [2,9,10].

In view of the present results it was of interest to reconsider earlier experiments concerning the swelling of liposomes in isotonic non-electrolyte solutions as a function of temperature. From these studies it was concluded that the activation energy is independent of the presence of cholesterol in the paraffin barriers [17]. That conclusion, however, was based primarily on experiments in which rather unsaturated phosphatidylcholines were used, with a maximum of 30 mol % cholesterol being incorporated [17].

To see whether the temperature dependence of the isotonic non-electrolyte swelling of the more saturated liposomes is also influenced by cholesterol some systems were tested by measuring the isotonic glycerol and erythritol swelling as a function of temperature. Since the rate of permeation of these non-electrolytes is much less than that of water these experiments were done at higher

TABLE II

ACTIVATION ENERGY FOR THE PERMEATION OF WATER THROUGH EGG PHOSPHATIDYLCHOLINE MEMBRANES, CONTAINING VARYING AMOUNTS OF CHOLESTEROL

Mol % cholesterol	Activation energy [a] (kcal/mol)
0	10.6 ± 0.4
15	10.1 ± 1.2
33	13.5 ± 0.5
50	15.0 ± 0.5

[a] The data were calculated from measurements performed in the temperature region ranging from 8 to 25° C.

* Transition temperature of dierucoylphosphatidylcholine is at 10° C as shown by calorimetry (van Dijck, P.W.M., personal communication).

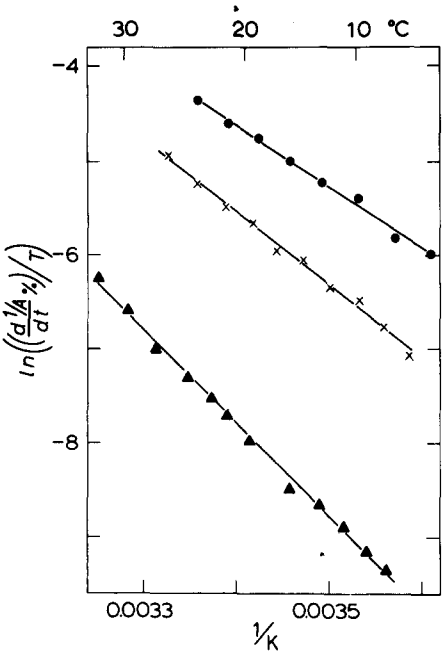

Fig. 5. Arrhenius plot of the osmotic shrinkage of liposomes, studied as described in Materials and Methods. The liposomes had the following lipid compositions: (▲———▲), dimyristoylphosphatidylcholine/egg phosphatidic acid/cholesterol, (46 : 4 : 50); (X———X), dimyristoylphosphatidylcholine/dioleoylphosphatidylcholine/egg phosphatidic acid/cholesterol, (23 : 23 : 4 : 50); (●———●), dioleoylphosphatidylcholine/egg phosphatidic acid/cholesterol, (46 : 4 : 50). The activation energies for the water permeation process through these membranes were 20.1, 15.9 and 12.7 kcal/mol respectively.

temperatures. The introduction of cholesterol into lecithin bilayers was found to produce an increase in the temperature dependence of the isotonic non-electrolyte swelling (Table III) as was found for the water permeation.

TABLE III

COMPARISON OF THE ACTIVATION ENERGIES FOR THE WATER PERMEATION PROCESS AND FOR THE ISOTONIC GLYCEROL AND ERYTHRITOL SWELLING AS A FUNCTION OF THE MEMBRANE LIPID COMPOSITION

Membrane lipid composition *		Activation energy (kcal/mol)		
Phosphatidylcholine present	Cholesterol concentration	Water permeation	Glycerol swelling	Erythritol swelling
Dioleoylphosphatidylcholine	0	11.4 ± 0.5	17 **	21 **
	50	12.7 ± 0.4	21.3 ± 0.4	25.2 ± 0.4
Egg phosphatidylcholine	0	10.6 ± 0.4	18 **	21 **
	50	15.0 ± 0.5	22.1 ± 0.8	26.5 ± 0.9
Dimyristoylphosphatidylcholine	0	11.1 ± 0.6	—	—
	50	20.1 ± 0.3	27.2 ± 0.7	30.6 ± 0.3
Dipalmitoylphosphatidylcholine	0	11.3 ± 0.9	—	—
	50	19.3 ± 0.5	25.8 ± 1.3	—

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

** Data taken from ref. 19.

Although the activation energies calculated for the water permeation and the swelling rates in isotonic glycerol and erythritol are quite different, the data of Table III indicate that the differences in the activation energies for the three permeation processes are nearly constant for each liposome preparation.

Discussion

A large number of studies have contributed to our knowledge about cholesterol-phospholipid interactions in membranes [1,2]. In the present study the effect of cholesterol on the temperature dependence of water permeation through membranes of a number of lecithins is described. The data of Figs. 1 and 2 show that the discontinuity in the rate of water permeation near the transition temperature of saturated lecithins gradually disappears with increasing cholesterol concentration and is completely removed above ± 30 mol % cholesterol. This result agrees with other permeability studies [16,21] and also correlates very well with calorimetric data, which show that each cholesterol molecule withdraws two lecithin molecules from the cooperative gel to liquid-crystalline phase transition [5—8]. It disagrees with certain H-NMR studies [22] and fluorescence studies [23], in which a 1 : 1 stoichiometry of the lecithin — cholesterol complex is supposed. However, the interpretation of the NMR data already has been critisized in several ways [2,24,25]. Further, neither is the conclusion from fluorescence experiments clear-cut, since it has been shown that the fluorescent probe perturbs the packing of the lipids [26].

In our experiments with liposomes of saturated lecithins a steep change in the activation energy for the water permeation was observed at about 15 mol % of cholesterol (Table I; Fig. 3). In addition to results from freeze-fracture electron microscopy [27] this finding is an argument against the idea of a lateral segregation of 1 : 1 lecithin • cholesterol complexes [22,23]. If it is assumed that such a lateral segregation exists, the finding that cholesterol reduces the over-all permeability would imply that in liposomes with 15 mol % cholesterol most of the permeation occurs via the assumed regions of free lecithin (being about 80% of the total amount), without changes in the energy barrier. Since this is in contrast to the observed effect (Table I, Fig. 3) we conclude that this study does not support the idea of a cluster formation of lecithin · cholesterol complexes.

The different results of the experiments with dioleoylphosphatidylcholine and dierucoylphosphatidylcholine likely can be related to differences in the position of the *cis*-double bond. In dioleoylphosphatidylcholine liposomes, where cholesterol has only a small effect on the activation energy of the permeation processes (Table III), the *cis*-double bond is at the Δ9-position of the fatty acid, which is in that part of the paraffin chain that is involved in the interaction with the rigid sterol nucleus [4,28,29]. In erucic acid the *cis*-double bond is at the Δ13-position, so in the part of the paraffin chain that does not interact with the sterol ring, which explains the much more pronounced effect of cholesterol on the activation energy of water permeation through dierucoylphosphatidylcholine bilayers (Fig. 4).

To explain the increase in the energy barrier of water permeation by cholesterol it is necessary to know more about the mechanism of water permeation.

In the molecular theory developed by Träuble [30] kinks, arising from conformational isomerization of the paraffin chains, are considered to be intrinsic carriers in lipid membranes. The number of rotational isomers can be reduced by decreasing the temperature, by introduction of cholesterol into liquid-crystalline membranes or by a transition to the gel state [31]. The resulting decrease in the absolute number of intrinsic carriers explains the effect of these parameters on the permeability.

In earlier studies on the permeability of small non-electrolytes, which may pass the membrane in a way very similar to that of water, it was assumed that the activation energy could be almost entirely ascribed to the breaking of hydrogen bonds of the permeant molecule with water [17]. The permeation process was visualized as the penetration of the bilayer by single fully dehydrated molecules. This was based on the values of the activation energies, which turned out to be characteristic for the permeant and independent of the membrane composition [17]. The present observation that the activation energy for water permeation is elevated in the gel state (ref. 18 and Table I and Fig. 3) and by the incorporation of cholesterol (ref. 32 and present study) implies that the above assumption certainly is not correct under all conditions. In this respect comparative experiments on the temperature dependence of water permeation and isotonic glycerol and erythritol swelling processes are relevant. The data of these experiments are summarized in Table III, where it is shown that the absolute value for the activation energy of all three permeation processes is independent of the fatty acid composition in the absence of cholesterol. In the presence of 50 mol % cholesterol the absolute values also depend on the lecithin present. However, for each liposome preparation there is a nearly constant difference in the activation energy for the three permeation processes. Assuming that the entry of a fully dehydrated permeant molecule into a kink is the rate limiting step for the permeation process, part of the activation energy results from the dehydration of the permeant and part results from the kink formation process. The energy needed for the dehydration is characteristic for each type of permeant, whereas the energy needed for kink formation depends on the physical state and the chemical composition of the lipid bilayer.

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