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ON THE MECHANISM OF NON-ELECTROLYTE PERMEATION THROUGH LIPID BILAYERS AND THROUGH BIOMEMBRANES

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SUMMARY

The temperature effects on the permeation of polyhydroxy alcohols through the lipid bilayers of liposomes with a great variety in chemical composition were studied. Although important differences in the permeability of the various lipid bilayers were observed, Arrhenius plots demonstrated that the activation energy is independent of the degree of unsaturation or the presence of cholesterol in the paraffin barriers. The activation energies found for the penetration of a bilayer with a liquid paraffin core are 14.3 kcal for glycol, 19.4 kcal for glycerol, and 20.8 kcal for erythritol. These values are in agreement with the energies that can be expected for complete dehydration of the permeant molecules. The idea that the activation energy is determined by the number of hydrogen bonds with water is supported by the finding that a series of different diols did demonstrate practically identical activation energies. Studies on a number of biological membranes demonstrated the same activation energies for the penetration of glycerol and erythritol as found in the experiments with liposomes. These facts support the view that both the lipid bilayers and the biological membranes are penetrated by single fully dehydrated molecules.

INTRODUCTION

The permeation of non-electrolytes such as the polyhydroxy alcohols through the membranes of biological cells, in particular of erythrocytes, has been studied by various investigators[1–3]. From these studies some interesting features can be noted. It is apparent that there are considerable differences in the penetration rates of the polyhydroxy alcohols into erythrocytes of various animal species. Furthermore, the temperature coefficients of the permeation process are found to be remarkably high for the erythrocytes of a certain group of animals and much lower in another group[3]. In addition to the permeation through biological membranes, the diffusion of glycerol through lipid bilayers as can be obtained in model systems has been the subject of investigation as well. It has been shown that the permeability coefficients of nonelectrolytes for these artificial membranes are in the same range as those found for the bio-

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membranes[4]. Using the liposome system, we have demonstrated that also between various phospholipid bilayers considerable differences in permeability can exist[5]. There is a pronounced increase in the penetration rate of glycerol with an increase in unsaturation or a decrease in chain length of the fatty acid constituents. Also the diffusion through the lipid bilayers is highly dependent on the temperature. In this paper we compare and try to analyse the temperature effects on non-electrolyte permeation through various lipid bilayers and biological membranes.

EXPERIMENTAL

Materials

Egg-yolk lecithin was isolated and purified by precipitation as $CdCl_2$ adduct and chromatography on silicic acid. Phosphatidic acid was obtained from this phospholipid by degradation with phospholipase D extracted from Savoy cabbage. EPL is a highly unsaturated natural lecithin containing 70 % linoleate and was generously supplied by Dr. H. Eikermann of Natterman and Cie, Köln. The synthetic phospholipids were obtained by reacylation of glycerol–phosphorylcholine, which in turn was obtained by deacylation of the egg-yolk lecithin. ^{14}C-labelled erythritol was obtained from the Radiochemical Centre, Amersham, England. All other reagents were commercial and of Analytical Reagent Grade.

Methods

Liposomes were prepared from the dry lecithin (or lecithin *plus* cholesterol) containing 4 % of phosphatidic acid by dispersing into 50 mM KCl as described earlier[5]. We obtained osmotic sensitive particles which can be considered to consist of fully closed concentric bilayers of phospholipid molecules intercalated by aqueous layers. The permeability of the bilayers for non-electrolytes was determined by following the swelling rate of the artificial "cells" in isotonic solutions of the various permeants, which could be measured by registering $d(1/A)/dt$, the change in the reciprocal of the absorbance at 450 nm with time[5].

Erythrocytes were obtained from fresh heparinised blood and washed with saline. The glycerol permeability of pig erythrocytes was followed as the swelling rate of the cells in a 1:1 (v/v) mixture of 300 mM glycerol solution and an isotonic Krebs–Ringer phosphate buffer. The change in optical density was measured at 824 nm. The half-time of leakage into a buffered saline medium of ^{14}C-labelled erythritol trapped in rabbit erythrocytes by means of preincubation was measured at an equilibrium concentration of 50 mM in phosphate buffer of pH 7.3 using the method of MAWE AND HEMPLING[6]. A rapid separation between cells and medium without haemolysis was obtained by filtration through a combination of two millipore filters with effective pore diameters of 5 and 8 μm.

Mycoplasma laidlawii B cells were grown and collected as has been described[7]. The cells were grown in lipid poor medium supplemented with equimolar amounts of palmitic and oleic acid. The cell pellet was washed with 200 mM sucrose and then resuspended in this solution at room temperature for 1 h before the swelling in isotonic glycerol or erythritol was measured as described for the liposomes.

RESULTS

In Fig. 1 the initial swelling rate in isotonic erythritol is given for a number of liposome systems prepared of various synthetic and natural phospholipids. In the given sequence there is a marked increase in unsaturation of the paraffin chains. A direct correlation between permeability for erythritol and the degree of unsaturation is apparent. Increasing unsaturation enhances the permeability for the non-electrolyte. This confirms our results on the penetration rate of glycerol[5].

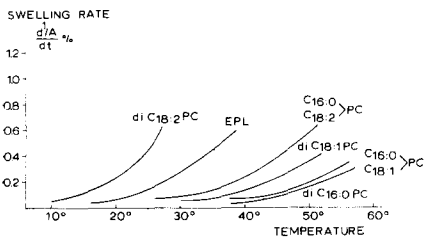

Fig. 1. Initial swelling rate in isotonic erythritol of liposomes prepared of (dilineoyl) lecithin, EPL (isolated lecithin containing 70% linoleate), (1-palmitoyl-2-lineoyl) lecithin, (dioleoyl) lecithin, (1-palmitoyl-2-oleoyl) lecithin, and (dipalmitoyl) lecithin. All systems contained about 4 mole % of phosphatidic acid derived from egg lecithin.

In Fig. 2 the permeability is demonstrated for glycol, glycerol, and erythritol as measured on liposomes prepared from dioleoyl lecithin. The diol, glycol, permeates rapidly even at low temperatures while the tetrahydroxy alcohol, erythritol, permeater only at higher temperatures, and glycerol is in between. The temperature dependence is remarkably high for a simple diffusion process. The general Arrhenius expression fos the temperature dependency of the permeability will be $P = P_0 e^{-E/RT}$ where E is the activation energy. This equation predicts that logarithmic plots of the permeability against $1/T$ will demonstrate straight lines with a slope equivalent to $-E/R$. Examples of these plots for the dioleoyl system are given in Fig. 2b.

The calculations have been made also for the other liposome systems and the results are summarized in Table I. When we consider the unsaturated systems, the mean values are 20.8, 18.4, and 14.3 kcal for erythritol, glycerol, and glycol, respectively. The variations between the systems of different composition are very slight and

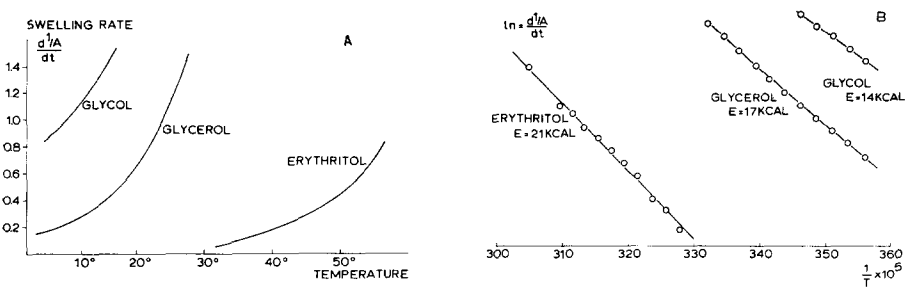

Fig. 2. A. Initial swelling rate of liposomes prepared from (dioleoyl) lecithin in isotonic solutions of glycol, glycerol, and erythritol. B. the Arrhenius plots of the data demonstrate the activation energies for the permeation process.

TABLE I

ACTIVATION ENERGIES CALCULATED FOR THE PENETRATION OF GLYCOL, GLYCEROL, AND ERYTHRITOL THROUGH THE LIPID BILAYERS OF VARIOUS LIPOSOMES

	Activation energy (kcal/mole)		
	Erythritol	*Glycerol*	*Glycol*
Unsaturated systems			
(1-Palmitoyl-2-oleoyl) lecithin	20	19	14
(1-Palmitoyl-2-oleoyl) lecithin with 30 % cholesterol	—	19	—
(1-Palmitoyl-2-lineoyl) lecithin	20	20	—
Egg lecithin	21	18	15
Egg lecithin with 30 % cholesterol	21	19	14
(Diolecyl) lecithin	21	17	14
(Dioleoyl) lecithin with 30 % cholesterol	—	18	—
EPL unsaturated lecithin	20	17	—
(Dilineoyl) lecithin	22	18	—
(Dilineoyl) lecithin with 30 % cholesterol	21	19	—
Mean	20.8	18.4	14.3
Standard deviation	0.8	1.0	0.5
Saturated systems			
(Distearoyl) lecithin	21	52*	61*
(Distearoyl) lecithin with 30 % cholesterol	—	21	21
(Dipalmitoyl) lecithin	20	42*	50*
(Dipalmitoyl) lecithin with 30 % cholesterol	—	20	18
(Dimyristoyl) lecithin	22	31*	65*
(Dimyristoyl) lecithin with 30 % cholesterol	25	19	14

* In these cases the Arrhenius plot did not show straight lines.

can be considered to be within the limits of experimental error. In series of experiments with different samples of the same system (egg lecithin) comparable variations were observed. In case of the saturated systems, much higher variations are apparent. The Arrhenius plots of the permeability of glycol and glycerol for the saturated system without cholesterol did not give straight lines, but curves with slopes which were much higher than those for the corresponding lines of the unsaturated systems. The values listed in Table I are the slopes of the higher temperature part of these curves (*cf.* Fig. 3). For these saturated systems, the swelling in isotonic glycerol could be measured only

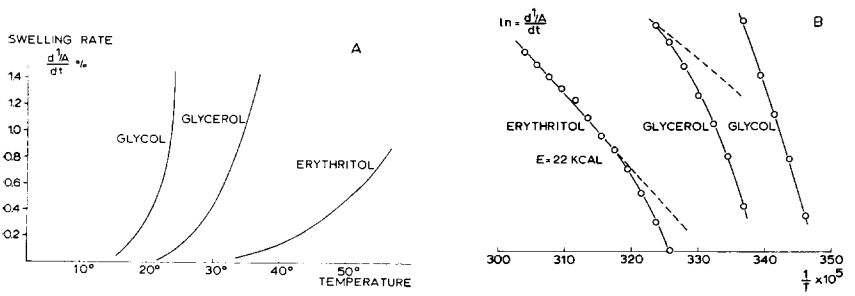

Fig. 3. A. Initial swelling rate of liposomes prepared of (dimyristoyl) lecithin in isotonic solutions of glycol, glycerol, and erythritol as a function of temperature. B. The Arrhenius plots of these data.

at temperatures relatively close to the regions of phase transitions in the paraffin core. The data on erythritol were obtained at considerably higher temperatures and, compared with the unsaturated systems, normal values were found.

The introduction of 30 mole % of cholesterol into the saturated bilayers did bring down the activation energies to values much closer to those found for the unsaturated systems. A remarkably high value was observed for erythritol penetration into the (dimyristoyl) lecithin–cholesterol system.

TABLE II

ACTIVATION ENERGIES CALCULATED FOR THE PENETRATION OF VARIOUS DIOLS THROUGH THE BILAYERS OF LIPOSOMES PREPARED FROM EGG LECITHIN CONTAINING 30 MOLE % OF CHOLESTEROL

Permeant	*Activation energy (kcal/mole)*
Ethane-1,2-diol	14
Propane-1,3-diol	13.5
Propane-1,2-diol	11
Butane-1,4-diol	13
Pentane-1,5-diol	12.5

TABLE III

COMPARISON OF ACTIVATION ENERGIES CALCULATED FOR PERMEATION PROCESSES THROUGH LIPID BILAYERS OF LIPOSOMES AND THROUGH BIOLOGICAL MEMBRANES

	Activation energy (kcal/mole)	
Permeant:	*Glycerol*	*Erythritol*
Liposomes	18.4	20.8
Pig erythrocytes	19	—
Rabbit erythrocytes	—	23
Mycoplasma laidlawii B	20	22

In Table II the activation energies are given for 5 different diols as measured on the egg lecithin–cholesterol system (70–30 mole %). The conclusion that can be drawn is that the values are very close.

In Table III the mean values of the data given in Table I are compared with activation energies calculated for permeation processes through biological membranes. The value for glycerol penetration into pig red cells was obtained from the temperature effect on the swelling of these erythrocytes in an isotonic solution. About the same value can be derived from haemolysis times given in the classical work of JACOBS *et al.*[1]. The activation energy for the erythritol permeation through the membrane of rabbit red cells was obtained by measuring the exchange of ^{14}C-labelled erythritol (*cf.* Fig. 4). This erythritol exchange over the membrane of rabbit red cells can be considered as a non-carrier transport. Extreme variations in the erythritol concentration (0.043 M up to 1.000 M) did not influence the exchange time. Nor were any differences found between the half-times of netto flux and equilibrium flux experiments. The data on the Mycoplasma cells have been obtained again from the swelling rates of the cells

in isotonic solutions. These experiments have been described elsewhere in more detail[9]. The apparent conclusion that can be drawn from this last table is that the activation energies for penetration of the lipid bilayers and of the biological membranes under investigation here are very close.

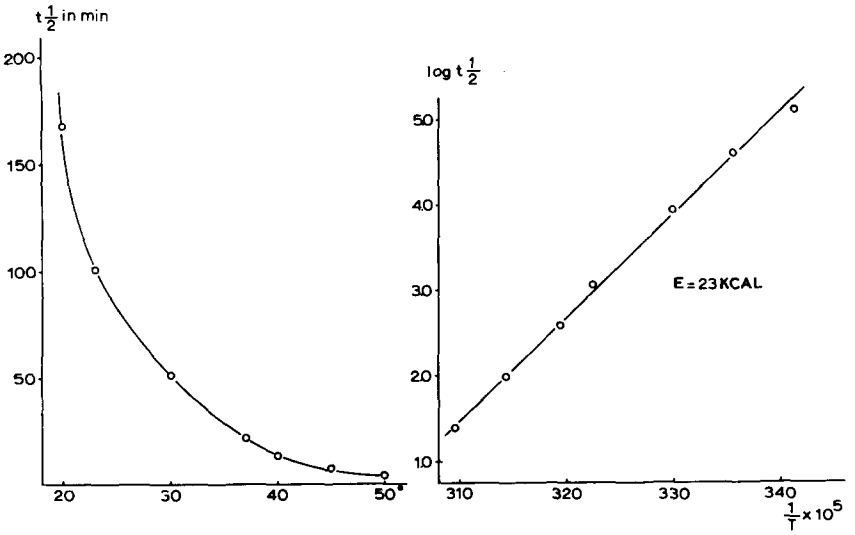

Fig. 4. Half-time of the flux of ^{14}C-labelled erythritol loaded in rabbit erythrocytes by preincubation into a medium of saline buffered at pH 7.3.

DISCUSSION

In a recent paper JOHNSON AND BANGHAM[10] have dealt with the application of the absolute rate theory on the membrane penetration process as outlined by ZWOLINSKI *et al.*[11]. The diffusion is considered as a series of successive jumps of the permeating molecules from one position with a potential free energy minimum to another (*cf.* Fig. 5). Passing the membrane, the permeants are involved in the following processes: diffusion through the water phase, penetration of the interface, diffusion through the hydrocarbon layer, and penetration of the opposite interface. On the basis of the high activation energy of the non-electrolyte penetration, it is likely that the penetration

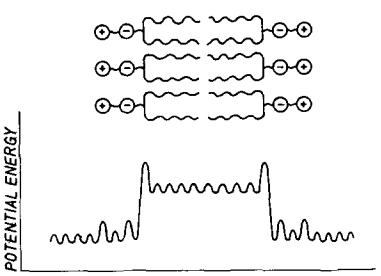

Fig. 5. Schematic presentation of the free energy ($\Delta G^* = \Delta H^* - T\Delta S^*$) profile for a penetrating non-electrolyte molecule.

into the hydrophobic barrier is rate limiting. The permeability can then be described by the following equation:

$$P = \frac{\lambda}{2} \cdot \frac{kT}{h} \cdot e^{\Delta S^*/R} \cdot e^{-\Delta H^*/RT}$$

in which λ is the lattice parameter, the distance between the energy minimum before and in the membrane, k is the Boltzmann constant, and h is Plank's constant. ΔS^* and ΔH^* are the molar entropy and enthalpy of activation, respectively. Comparison of this expression with the experimental Arrhenius equation demonstrates that the enthalpy of activation ΔH^* and the activation energy E are closely related. The exact relationship is $\Delta H = E - RT$ (*cf.* GLASSTONE *et al.*[16]); therefore, ΔH^* and the activation energy E differ by only about 0.6 kcal.

When we substitute in the permeability equation the various ΔH^* values for the penetration of glycol, glycerol, and erythritol, for example in case of the (dioleoyl) lecithin liposomes, and we assume for the moment that ΔS^* is independent of the permeant, we find permeability ratios for glycol:glycerol:erythritol given by $e^0 : e^5 : e^{11.7} = 1 : 148 : 120000$. However, the data in Fig. 2 show that this ratio certainly for glycol:glycerol is much less, and consequently this suggests that differences in ΔS^* for the different permeants exist as well.

Considering the ΔH^* values for the various unsaturated liposome systems (Table I) we can conclude that there is no significant variation with the degree of unsaturation at all. The standard deviation of the data allows a variation in the permeability of a factor $e^{2/RT} = 27$ at the most. Although the exact ratio at one temperature could not be measured, the data in Fig. 1 show that this variation between the (dilineoyl) lecithins and the (1-palmitoyl-2-oleoyl) lecithin systems is considerably higher. Consequently, the differences in penetration rate of the non-electrolytes into liposomes with various degrees of unsaturation cannot be explained by changes in ΔH^*. On the other hand, it is well known that introduction of double bonds in the paraffin chains will loosen the packing of the chains[12]. In the less saturated systems, there are more wagling and rotating possibilities[13]. Therefore, important differences in the entropy of activation, explaining the fact that the less saturated system is more permeable, can easily be visualized.

The paraffin core of the liposomes prepared from the unsaturated lecithins will remain in a liquid condition up to temperatures far below 0°. On the other hand, the saturated liposomes do demonstrate liquid–solid transitions of the paraffin chains in the temperature area where the permeation of glycol and glycerol is measured[15] and this fact can explain the abnormal temperature effects noticed in the experiments with these saturated systems. When cholesterol is introduced into the bilayers of the saturated liposomes, this hampers the crystallisation of the proper chains[8], and compared with the unsaturated systems normal temperature effects on the glycerol penetration are found. The also reduced but still unusually high values for the glycol penetration in these cholesterol containing systems reflect that the glycol permeation, which can be measured only at relatively low temperatures is still influenced by the to a lower temperature shifted and reduced phase transition[15]. The surprising fact that can be learned from the measurements on the liposomes prepared from 96 % of the saturated lecithin and 4 % phosphatidic acid derived from egg lecithin is that the

liquid–solid transition is demonstrating its effect over a temperature range of about 20° (*cf.* Fig. 3).

STEIN[14], discussing the classical permeability data of JACOBS *et al.*[1], has suggested that the activation energy is related to the number of hydrogen bonds formed between the permeant and the surrounding water molecules. The independence of the value E (or ΔH^*) of the composition of the membrane supports the view that for penetration of glycol, glycerol, or erythritol, through any of these liquid membranes, the same break of the hydrogen bonds with the water is necessary before the molecule can enter into the hydrophobic core of the lipid bilayer. When we assume that per hydroxyl group, two hydrogen bonds (an acceptor and a donor) with the water are possible, four, six, and eight bonds are to be broken for complete dehydration of glycol, glycerol, and erythritol, respectively. However, in the isolated molecule we have to consider the possibility of one intramolecular hydrogen bond for glycol, two for glycerol, and (at least) three for erythritol. Therefore, the enthalpy of dehydration for glycol, glycerol, and erythritol will be equivalent to the energy of breaking three, four, or five (or less) hydrogen bonds, respectively. As the energy needed to break one hydrogen bond is about 5 kcal/mole, this corresponds to 15 kcal for glycol, 20 kcal for glycerol, and 25 kcal (or less) for erythritol. These figures are so close to the experimental data in Table I that this is an argument in favor of the idea that the non-electrolytes penetrate into the hydrophobic core of the lipid bilayer as single, fully dehydrated molecules. The view that the activation energy, in the case of liquid bilayers is mainly determined by the dehydration energy is also supported by the close values found or the penetration of the various diols (Table II). The somewhat lower value for the penetration of propane-1,2-diol may be explained by an interaction of the unscreened CH_3 with the hydrophobic core, and has been found for the permeation of biological membranes as well (*cf.* STEIN[14]).

The agreement between the data on the synthetic and natural membranes as shown in Table III can be explained by the assumption that also in the natural membrane there is a hydrophobic core and that also these barriers are penetrated by single, fully dehydrated glycerol and erythritol molecules.

Earlier studies[3] on the haemolysis of red cells in isotonic glycerol have demonstrated that the penetration of glycerol into, for example, human erythrocytes is much less dependent on the temperature. In this case, the permeability of glycerol is strongly inhibited by traces of copper or by a drop in pH suggesting that here a facilitated diffusion process is operating. The lower activation energy in this case indicates that the presence of polar pores or a suitable carrier enables the glycerol to pass the membrane without complete dehydration.

REFERENCES

1 M. H. JACOBS, H. N. GLASSMAN AND A. K. PARPART, *J. Cellular Comp. Physiol.*, 7 (1935) 197.
2 W. D. STEIN AND J. F. DANIELLI, *Discussions Faraday Soc.*, 21 (1956) 238.
3 J. DE GIER, L. L. M. VAN DEENEN AND K. G. VAN SENDEN, *Experientia*, 22 (1966) 20.
4 H. J. VREEMAN, Thesis, Utrecht, 1966.
5 J. DE GIER, J. G. MANDERSLOOT AND L. L. M. VAN DEENEN, *Biochim. Biophys. Acta*, 150 (1968) 666.
6 R. C. MAWE AND H. G. HEMPLING, *J. Cellular Comp. Physiol.*, 66 (1965) 95.
7 R. N. MCELHANEY AND M. E. TOURTELLOTTE, *Biochim. Biophys. Acta*, 202 (1970) 120.
8 J. DE GIER, J. G. MANDERSLOOT AND L. L. M. VAN DEENEN, *Biochim. Biophys. Acta*, 173 (1969) 143.

9 R. N. McELHANEY, J. DE GIER AND L. L. M. VAN DEENEN, *Biochim. Biophys. Acta*, 219 (1970) 245.
10 S. M. JOHNSON AND A. D. BANGHAM, *Biochim. Biophys. Acta*, 193 (1969) 92.
11 B. J. ZWOLINSKI, H. EYRING, AND C. E. REESE, *J. Phys. Colloid Chem.*, 53 (1949) 1426.
12 L. L. M. VAN DEENEN, in R. HOLMAN, *Progress in the Chemistry of Fats and Related Lipids*, Vol. 8, Part 1, Pergamon Press, Oxford, 1965.
13 D. CHAPMAN, V. B. KAMAT, J. DE GIER AND S. A. PENKETT, *J. Mol. Biol.*, 31 (1968) 101.
14 W. D. STEIN, *The Movement of Molecules Across Cell Membranes*, Academic Press, New York, 1967.
15 B. B. LADBROOKE, R. M. WILLIAMS AND D. CHAPMAN, *Biochim. Biophys. Acta*, 150 (1968) 333.
16 S. GLASSTONE, K. J. LAIDLER AND H. EYRING, *The Theory of Rate Processes*, McGraw-Hill, New York and London, 1941.