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PROTON PERMEABILITY OF LIPOSOMES FROM NATURAL PHOSPHOLIPID MIXTURES

MICHEL ROSSIGNOL, PIERRE THOMAS and CLAUDE GRIGNON
Laboratoire de Biochimie, ENSA-INRA, 34060 Montpellier Cedex (France)

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A method to determine the proton permeability coefficient of phospholipid membrane with the fluorescent probe pyranine is described. Very high proton permeability coefficients of liposomes from natural extracts are measured with great accuracy. The proton permeability appears to be linearly related to the fluidity of the bilayers. This relation as well as the comparison of the activation energies of proton permeability and fluidity support the hypothesis (Nichols J.W. and Deamer D.W. (1980) Proc. Natl. Acad. Sci. USA 77, 2038–2042) of a transfer process along a network of hydrogen bonded water molecules. It is suggested that the common lipid characteristics of biological membranes (net surface charge and unsaturation) favor a high proton permeability.

Introduction

The cell pH homeostasis requires the presence of an efficient permeability barrier for H^+ and OH^-. As the phospholipid bilayer moiety of the membrane is the major permeability barrier it is particularly relevant to determine the permeability of this barrier to protons. Recently Nichols and coworkers have used the fluorescent probe 9-aminoacridine to measure proton permeability coefficients of liposomes from phosphatidylcholine and phosphatidic acid [1,2]. Suprisingly their results indicate a very high proton permeability of the phospholipid bilayer. In this paper we have studied the proton permeability barrier of complex membrane phospholipid mixtures. The proposed method involves the use of the fluorescent probe pyranine. In addition we have examined the relation between the proton permeability and fluidity of bilayers.

Abbreviation: CCCP, carbonylcyanide *m*-chlorophenyl hydrazone.

Material and Methods

Material. Lipids were extracted from roots of horse bean (*Vicia faba* L. var. *minor*). Phospholipids were separated by silicic acid column chromatography [3]. The final mixture showed a fatty acid auto-oxydation of 0.4% as judged by its absorbance at 231 nm [4]. The phospholipid and fatty acid composition of the extracts has already been published [5].

Liposome preparation. Liposomes were prepared by the vaporization method of Deamer and Bangham [6], using petroleum ether (boiling range 40–60°C) instead of diethyl ether, in 50 mM KH_2PO_4/KOH buffer at pH 5.6 containing 0.1 M KCl, 1 mM RbCl and 1 mM pyranine (8-hydroxy-1,3,6-pyrenetrisulfonate, Eastman). Extra-vesicular RbCl and pyranine were eliminated by filtration through a Sephadex G 50 column. Phospholipid concentration was measured by colorimetric determination of the lipid phosphorus. The internal volume of the vesicles was calculated from their Rb content; it was generally 15–20 litre/mol.

Internal pH determination. The internal pH was

calculated from the ratio I_{463}/I_{405} of the pyranine fluorescence intensities at 511 nm after excitation at 405 and 463 nm [7]. The use of pyranine is particularly convenient for the following reasons: (i) When entrapped in negatively charged liposomes it is entirely solubilized in the internal bulk water pool; this is ascertained by its fluorescence polarization (0.009 in this study) which corresponds to that measured for aqueous solutions of pyranine [7]. (ii) The pH determination is very sensitive; this is allowed by the 1000-fold change in the ratio I_{463}/I_{405} between pH 4 and 9. (iii) The internal pH determined by this means corresponds effectively to that measured with a glass electrode; this was controlled in experiments where the pH gradient across the phospholipid bilayer was abolished by addition of CCCP (carbonyl cyanide *m*-chlorophenyl hydrazone, Sigma).

Permeability calculations. Under the assumptions that (i) the fluxes are independent, (ii) the surface potentials at both sides of the membranes are identical, (iii) there is no diffusion potential, the observed proton flux (J), which is the result of the individual proton and hydroxyl fluxes across the phospholipid bilayer, is:

$$J = P_H \cdot \Delta_{oi}(H^+) - P_{OH} \cdot \Delta_{oi}(OH^-) \quad (1)$$

where P_H and P_{OH} are the permeability coefficients of the bilayers for protons and hydroxyls; Δ_{oi} are the proton or hydroxyl activity differences between the inside and the outside of the liposomes. The preceeding assumptions will be discussed later.

Otherwise the flux can be directly calculated from the relation:

$$J = \frac{\Delta pH_i}{\Delta_t} \cdot \frac{B_i \cdot V_i}{A} \quad (2)$$

where ΔpH_i is the change in internal pH during Δt; V_i the internal volume and B_i the buffer capacity to the internal volume determined from a separate acid-base titration of the buffer. A is the total membrane area estimated assuming a mean packing area per molecule of 75 Å^2 in the case of our charged and highly unsaturated mixtures [5,8].

Therefore J can be calculated for each time interval and subsequently introduced in Eqn. 1. This procedure gives a set of n equations solvable by pairs for P_H and P_{OH}. However, except near pH 7, the activities and osmotic gradients of the two ions are exceedingly different. As seen from Eqn. 1 this generally leads to a poorly accurate estimation of one of the two permeability coefficients. It is convenient to identify the OH^- flux formally with an opposite flux of H^+ equivalents and to define P_{net} as:

$$P_{net} = P_H + P_{Hequiv.} = P_H + P_{OH} \quad (3)$$

P_{net} has no simple meaning but just measures the efficiency of the bilayer in maintaining a pH gradient.

Fluidity determination. The fluidity of the phospholipid bilayers was calculated from fluorescence polarization measurements using the probe perylene embedded in the bilayers [9,10].

Results

The results of two typical experiments with opposite pH gradients are shown in Fig. 1. As seen on the curves the sensitivity of the pyranine method allows one to measure pH differences as small as 0.01 pH unit.

In such experiments the main source of error due to the experimental design is a possible pH equilibration via vesicle rupture or carbon dioxyde fluxes. The linear shapes of log J versus time (Fig. 1, bottom) indicate that there is no significant vesicle rupture. In several experiments (Table I) the buffers were made from boiled decarbonated water and then degassed by a vigorous bubbling of argon before use; in addition during these experiments the spectrofluorometer cell was continuously flushed with argon. No significantly different results were obtained in these conditions, indicating that the (CO_2-H_2CO_3-HCO_3^-) equilibria are not responsible for proton transport.

As pointed out above, Eqn. 1 involves the assumption that the surface potentials at both sides of the membrane are identical. This is very likely a priori since the ionic strength is high in the two compartments (approx. 0.1 M) and since our phospholipid mixtures do not have titratable groups in the pH range used [9].

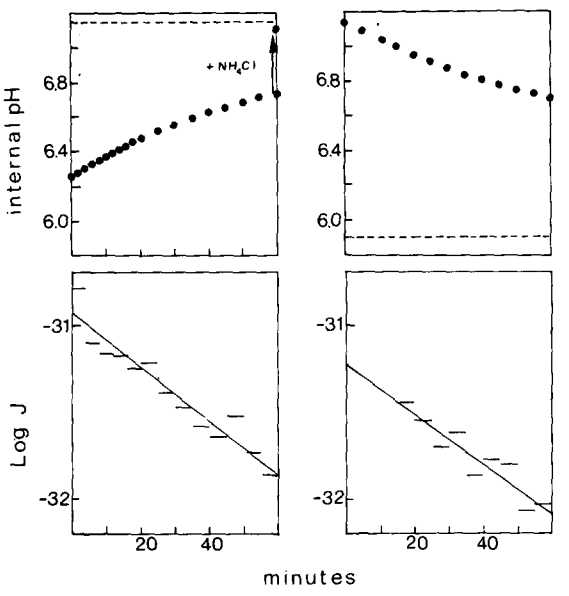

Fig. 1. Time courses of the internal pH and of the logarithm of proton flux J during alkalinization (left) or acidification (right) of the vesicle internal medium. For the acidification experiment the initial internal pH was 7.4 and the external one 5.6; these values were respectively 5.6 and 7.5 for the alkalinization experiment. $+NH_4Cl$ indicates that 0.1 M NH_4Cl was added to abolish the pH determined with pyranine; dotted line, external pH measured with a glass electrode.

The other main limitation in using our flux equation is that one must ensure that there is no significant diffusion potential across the membrane. For this purpose experiments were done in the presence of valinomycin. The values of P_{net} determined in these conditions are higher than those previously obtained (Fig. 2). This may correspond either to direct acceleration of proton flux by the valinomycin which affinity for H^+ has been claimed to be greater than for K^+ [11] or to abolition of a diffusion potential. In order to investigate the former hypothesis, we have compared the valinomycin effect on the proton permeability in two cases: in the first one the medium was the usual one containing 0.1 M KCl; in the second one, K^+ was totally replaced by choline. No difference in proton permeability was observed between the two treatments (Fig. 2) indicating that the proton fluxes are not limited by a diffusion potential. On the other hand if the increase of proton permeability upon addition of valinomycin corresponds to the abolition of a diffusion potential, the question arises of the validity of permeability calculations which neglect the electrical forces. Although one can not argue that there is no diffusion potential across the membranes its effect is likely weak. First no significative difference was observed between alkalinization and acidification experiments (Table I) although the potentials are expected to be opposite. Theoretical calculations using the Goldman and Hodgkin-Katz relations in order to include electrical forces in the flux equation indicate that, providing that the pH gradient across the membrane is maintained lower than 1.5, the calculated values of P_{net} differ by less than one order of magnitude from that determined as de-

TABLE I

NET PROTON PERMEABILITY COEFFICIENT VALUES OBTAINED UNDER VARIOUS CONDITIONS

Expt. No.	P_{net} at 25°C ($cm \cdot s^{-1}$)($\times 10^3$)			
	Non decarbonated		Decarbonated	
	Liposome interior		Liposome interior	
	Acidified	Alkalinized	Acidified	Alkalinized
1	0.08	0.10	0.25	0.16
2	0.27	0.09	0.23	0.13
3		0.16		0.25
4				0.12
5				0.21
6				0.15

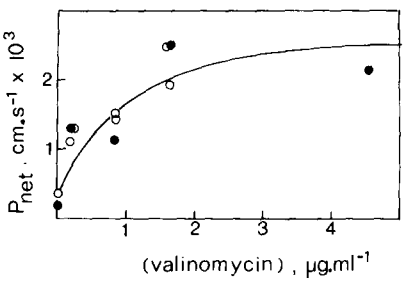

Fig. 2. Effect of valinomycin on the proton permeability coefficient. The salts used were 0.1 M KCl (●) or 0.1 M choline chloride (○).

scribed in Material and Methods. For the experiments described in this paper these calculations show that in the worst case the values of P_{net} are underestimated by a factor 3. Finally the effect of the diffusion potential should decrease as the pH gradient progressively collapses, thus leading to a parallel increase in the apparent P_{net}. Since this was not observed, it may be concluded that the results are not significantly affected by a diffusion potential.

The mean value of P_{net} at 25°C is very high:

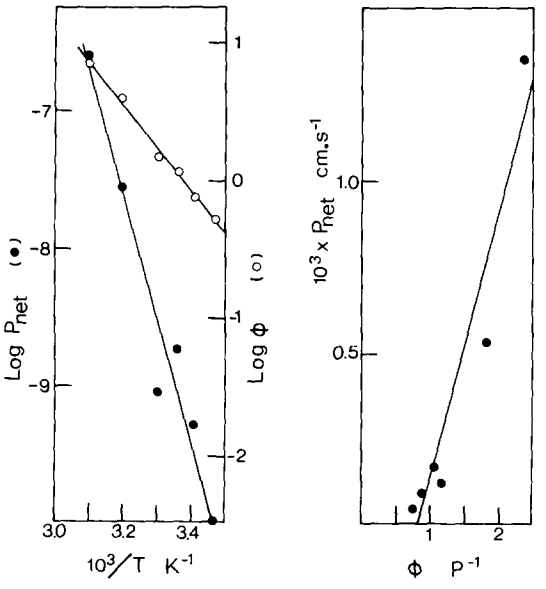

Fig. 3. Effect of temperature. (Left) Arrhenius plots of the net proton permeability coefficient (●) and of fluidity of bilayers (○). (Right) Relationship between fluidity of the bilayer interior and the net proton permeability coefficient.

$1.7 \cdot 10^{-4} \pm 0.4 \cdot 10^{-4}$ cm · s^{-1}, but proton permeability appears to be much sensitive to temperature than the fluidity (Fig. 3): the activation energies are 17 kcal · mol^{-1} for proton permeability and 6 kcal · mol^{-1} for fluidity.

Discussion

The high sensitivity of the pyranine method allows one to obtain a good reproducibility in proton permeability determinations: the standard deviation corresponds to 39% of the mean value; for the 9-amino-acridine determinations this ratio is 114% (see Ref. 1).

Our results were obtained with very complex phospholipid mixtures containing seven polar heads and five major fatty acids [5], whereas the vesicles used by Nichols and coworkers contained a mixture of two phospholipids [1,2]. However the mean values obtained by both methods are very close. This seems to indicate a relative insensitivity of proton permeability to the peculiar composition of the phospholipid bilayer.

As pointed out [1] such values are of the same magnitude as the scarce available data for biological membranes; mitochondria [12], erythrocytes [13]. In the plant field a somewhat lower value has been published for barley roots ($0.06 \cdot 10^{-4}$ cm · s^{-1} (Ref. 14)). Nevertheless all these values are very high as compared to those obtained for other ions with similar ether-injected liposomes: $1.0 \cdot 10^{-11}$ cm · s^{-1} for sodium and $4.6 \cdot 10^{-11}$ cm · s^{-1} for pyrophosphate (from Ref. 1); $1.2 \cdot 10^{-10}$ cm · s^{-1} for nitrate and $1.5 \cdot 10^{-10}$ cm · s^{-1} for calcium (Thomas, P., unpublished data). This suggests that the proton permeability mechanism is radically different from that of the other ions.

It has been suggested that this high proton conductance is due to an H^+ exchange process along a network of hydrogen bonded water molecules present in the hydrophobic region of the bilayer, in a way similar to that of protons in ice [1]. The presence of water in the bilayer interior is supported by the relatively high water permeability across phospholipid bilayers [15–17]. The fact that water permeability coefficients are of the same magnitude as those of proton permeability also supports this interpretation.

Furthermore the high value of the activation

energy of P_{net} we have determined is reminiscent of the breaking of numerous hydrogen bonds [18] as would be the case with a transport along a network of associated water molecules. Moreover our value of the activation energy for proton permeability is very close to that measured for water permeability [19]. Also the fact that the activation energy of proton permeability is 3-fold higher than that of fluidity indicates that the transport of protons across the membrane is not directly controlled by the frictionnal forces between H^+ and the fatty acids.

In principle a permeability coefficient is directly proportionnal to the fluidity of the membrane. But the usually measured permeability coefficients correspond in fact to two events: solubilization in the membrane and diffusion across the membrane. However we observe a good proportionality (Fig. 3, right) between fluidity and proton permeability: the correlation coefficient is 0.95. This agrees well with the proposed transport mechanism: if protons are exchanged along a preexisting network of associated water molecules, the relative weight of the solubilization process in the value of the permeability coefficient should be low. Finally this should lead to an approximately linear relation between the fluidity and the permeability coefficient.

In conclusion, our results support the jumping transfer hypothesis. The two main consequences of this process are: (i) this mechanism is specific, (ii) it is related to water permeability which is directly controlled by the packing of hydrocarbon chains [16]; this may explain the high values of P_{net} observed with biological membranes and their phospholipidic extracts which are negatively charged and highly unsaturated.

Acknowledgments

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