Biochimica et Biophysica Acta 812 (1985) 447–452
Elsevier

BBA 72447

Effect of the concentration and the molecular structure of phosphonic acid dialkyl esters on the kinetics of Pr^{3+} permeation through egg phosphatidylcholine small unilamellar vesicles

Michael Hentschel and Gotthard Klose

Department of Physics, Karl-Marx-University, Linnestrasse 5, 7010 Leipzig (G D R)

(Received July 6th, 1984)

Key words: Model membrane, Membrane permeability; Phosphonic acid dialkyl ester; Molecular asymmetry; Pr^{3+}; NMR

The dependence of the Pr^{3+} permeation kinetics in unilamellar vesicles of egg phosphatidylcholine on the incorporation of phosphonic acid dialkyl esters in the membrane was studied by ^{31}P- and ^{1}H-NMR. The phosphonic acid dialkyl esters cause drastic changes of the permeation half-time $t_{1/2}$ of the Pr^{3+} ions into the inner vesicle compartment. The ion permeation induced by the esters was interpreted as due to the formation of metastable pores. The permeation half-time $t_{1/2}$ depends on the ester concentration in the membrane and on temperature. The threshold concentration of the ester necessary for a drastic decrease in permeation half-time $t_{1/2}$ reflects certain structural differences of the esters. The results suggest a correlation between the effective molecular shape of the phosphonic acid esters and the ion permeation. The esters are mainly incorporated in the outer vesicle monolayer. This could be understood because of the positive molecular asymmetry of the phosphonic acid dialkyl esters. The permeation half-time $t_{1/2}^{*}$ of Pr^{3+} ions out of the vesicles is much larger than the half-time $t_{1/2}$ for the opposite process.

Introduction

The transport through model membranes can be used to deduce information about the lipid dynamics and the physical state of the bilayer [1,2]. Such investigations can be accomplished on closed model membranes such as liposomes or small unilamellar vesicles. High-resolution NMR is well suited to investigate the permeation of paramagnetic ions through model membranes.

The ion permeability of phospholipid bilayer membranes can be modulated by various substances, e.g. anaesthetics [2,3], lysophosphatidylcholine [4,5], fatty acids [6], cholesterol [3], etc. A new class of substances, phosphonic acid dialkyl esters (PAE), and its influence on the ion permeability is the subject of the present paper.

PAE are used as pesticides, herbicides [7,8], insecticides, defoliants and desiccants [8]. Independently of the mechanism of their action in the cell they have to penetrate through or to be incorporated in membranes. The motional behaviour of PAE in model membranes [9,10] and its incorporation in hepatic microsomes [11] have already been studied. The investigation of their influence on the permeability properties of small unilamellar vesicles contribute to understanding the behaviour of PAE in membranes and their biological efficiency [9]. On the other hand the relation between the effective molecular asymmetry of molecules incorporated in membranes and their influence on the membrane properties poses a fundamental problem. The advantage of the PAE

Abbreviations: egg PC, egg phosphatidylcholine; PAE, phosphonic acid dialkyl ester.

in comparison with other molecules (as for example proteins) is that their molecular shape can be easily obtained, for example by the Monte-Carlo method [25].

Stable small unilamellar vesicles containing lipid and PAE can be easily prepared and controlled. They represent very asymmetric model membranes. Such systems can be used to study the nonspecific ion transport as a function of the direction. The Pr^{3+} permeation into the vesicles from egg PC and different PAE was followed by ^{31}P- and ^{1}H-NMR taking advantage of the properties of hydrophilic paramagnetic ions [13–15]. The Pr^{3+} permeation out of the vesicle can also be studied by adding EDTA which complexes with the Pr^{3+} ions.

Materials and Methods

Egg PC was isolated and purified according to the method of Singleton [16]. Only samples which show no impurities after the preparation procedure when checked by TLC ($CHCl_3/CH_3OH$/25% aqueous ammonium solution (65 : 35 : 5, v/v)) were taken for the kinetic analysis.

In Table I the PAE involved in the investigations and their notations are presented.

The esters are oil-like liquids except PAE 16 which is a wax at room temperature. Their solubility in water is low [9,10]. The short-chain PAE 6 was obtained from Professor Dr. Großmann of the Technical University, Dresden; PAE 12 and PAE 16 from Dr. Haage of the Central Institute of Organic Chemistry, Berlin; and PAB-T, PAE-C and PAB-C from Dr. Günther of CKB, Bitterfeld. All PAE were of analytical purity and showed no impurities within their ^{1}H-, ^{13}C- and ^{31}P-NMR spectra.

Weighed amounts of PC and PAE were codissolved in a 2 : 1 chloroform/methanol solution, lyophilized and resuspended in 0.15 M deuterated sodium acetate in $^{2}H_2O$ to give a final lipid concentration of 5% (w/v). The $p^{2}H$ was adjusted by acetic acid to be 5.9 and the suspension was sonicated in a bath-type sonifier for two hours under nitrogen [17]. After sonication the sample was centrifuged for one hour at 180 000 × g to remove remaining multilamellar liposomes [18].

The diameter of the resulting vesicles was characterized by quasi-elastic light scattering and ^{1}H- and ^{31}P-NMR as being smaller than 50.0 nm. These results were supported by gel chromatography, electron microscopy and the ^{1}H-NMR pulsed field gradient technique.

TABLE I

PHOSPHONIC ACID DIALKYL ESTER R–P(=O)$(OR')_2$

R	R′	Notation
C_6H_{13}	C_2H_5	PAE 6
$C_{12}H_{25}$	C_2H_5	PAE 12
$C_{16}H_{33}$	C_2H_5	PAE 16
	C_2H_5	PAE-C
	C_4H_9	PAB-T
	C_4H_9	PAB-C

^{31}P- and ^{1}H-NMR spectra were recorded at 36.43 and 200.13 MHz using Bruker HX-90 and WP-200 Fourier-transform spectrometers. The temperature was controlled so as to be accurate within ±1 K by the Bruker temperature units.

The molar ratio PAE/egg PC within the samples was determined by planimetry of the ^{31}P-NMR intensities. Our experimental conditions guaranteed signal intensities with an accuracy of about 5% for samples with PAE/egg PC molar ratios higher than 0.1. Otherwise the error was considerably higher and was estimated to be about 50%.

Typically 1.5 ml of the freshly prepared vesicle suspension was pipetted into an NMR sample tube. At time zero 75 µl of a 0.1 M praseodymium nitrate solution in $^{2}H_2O$ was added so that the Pr^{3+} concentration reached 4.8 mM. The Pr^{3+}-induced chemical shift of the outer vesicle headgroup resonances results in two well-resolved ^{31}P (Fig. 1b) or $N(C^{1}H_3)_3$ proton signals.

At time zero the shift difference between the inner and outer egg PC signals is maximal and decreases with permeation of Pr^{3+} ions into the inner vesicular compartment, thus resulting in a time-dependent Pr^{3+}-induced chemical shift of the inner resonances. One can follow the permeation of Pr^{3+} ions by recording the time-course of the inner resonances. However, at the phospholipid concentration used (50 mg/ml) the vesicle population exhibits a total inner volume of about 1% [20].

Therefore, a time-dependent shift of the outer resonances due to the loss of Pr^{3+} concentration into the inner medium was not observed. Thus the shift difference between inner and outer signals was followed within this paper (cf. Fig. 1b–e). In the concentration range used the Pr^{3+}-induced chemical shift was found to be directly proportional to the aqueous Pr^{3+} concentration.

The rate of transport to the inner vesicle compartment obeys a first-order kinetics with correlation coefficients of at least 0.98. The first-order rate constant k is directly related to the permeation half-time $t_{1/2}$ by Eqn. 1

$$k = \ln 2/t_{1/2} \quad (1)$$

The permeation half-times $t_{1/2}$ longer than 30 min were obtained from ^{31}P-NMR measurements and those faster than 30 min were derived from ^{1}H-NMR experiments. The accuracy of the permeation half-time $t_{1/2}$ was determined mainly by the time necessary to record an NMR spectrum. The accuracy was found to be ± 30 min for ^{31}P and better than ± 3 min for ^{1}H-NMR.

From the rate constant k the permeability coefficient P can be calculated using Eqn. 2

$$k = P\ A/V = 3\ P/r \quad (2)$$

where A is the surface area, V the volume and r the radius of the vesicle [21].

Results

The ^{31}P spectra of small unilamellar vesicles from egg PC and PAE consist of two lines, one at low magnetic field from the ester and the other from the lipid (Fig. 1a).

As shown in Fig. 2 the permeation half-time $t_{1/2}$ depends strongly on the PAE concentration. This dependence is quite similar for all esters studied. The permeation half-time $t_{1/2}$ decreases drastically within a narrow concentration range. By a linear least-squares fit of the permeation

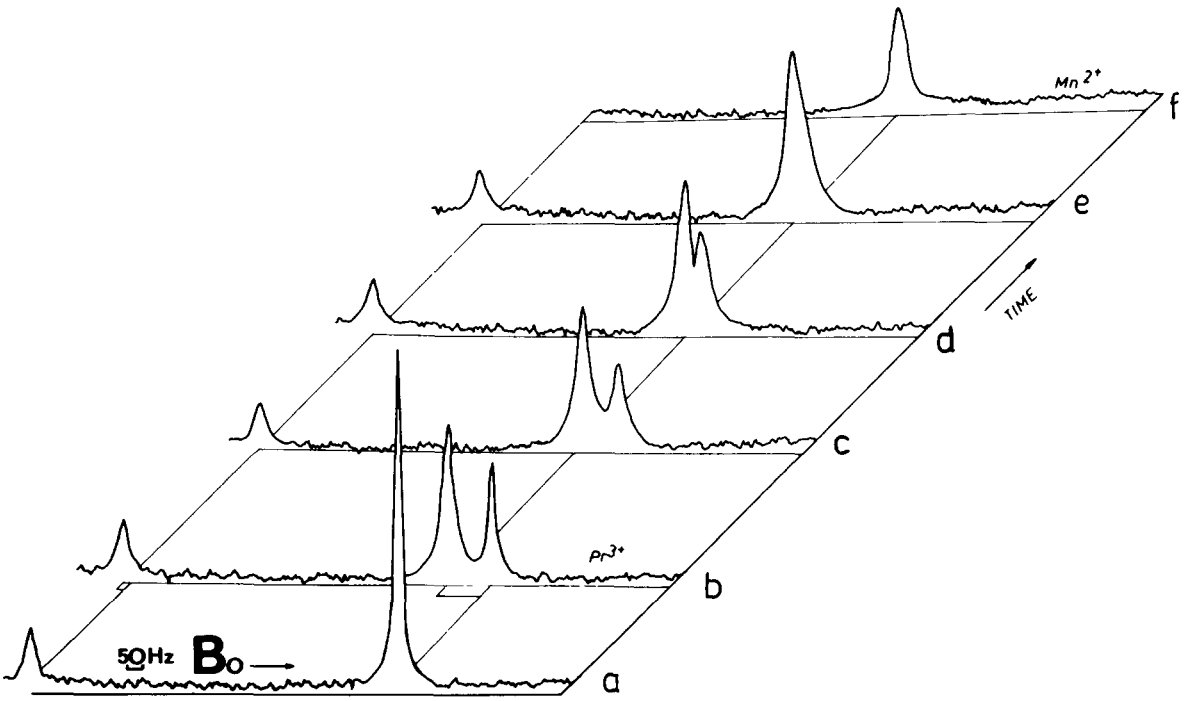

Fig 1. The time-course of the ^{31}P-NMR spectra of small unilamellar vesicles (egg PC/PAE 6, 1 0.15) at 24°C and 36.43 MHz (5 kHz spectral width, 1.1 s repetition time, 4096 data points, about 7 μs for a 90° pulse, 1024 scans, 4 Hz line broadening) a, before and b–e, after addition of 4.8 mM Pr^{3+}, f, 1 mM Mn^{2+} added

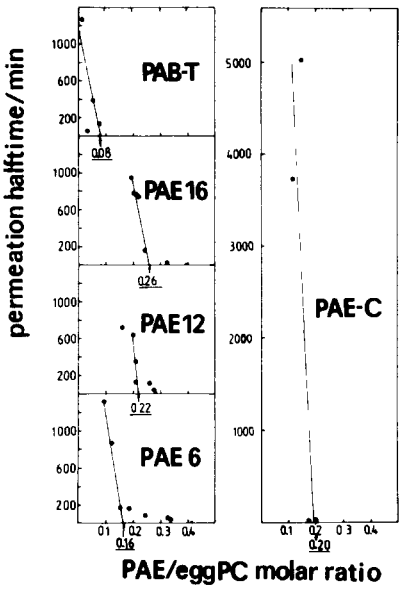

Fig 2 Dependence of the permeation half-time $t_{1/2}$ of Pr^{3+} ions on the molar ratio of the different PAE in the vesicle bilayer at 24°C

half-times $t_{1/2}$ in this region (full lines in Fig. 2) we obtained the threshold PAE concentration from the point of intersection of the slope of the permeation half-times $t_{1/2}$ with the concentration axis. The threshold concentrations are specified in Table II. PAB-C increases the Pr^{3+} permeation approximately in the same concentration range as PAB-T (data not shown). However, a distinction between these two esters by the permeation half-time $t_{1/2}$ was not possible.

The unchanged barrier properties of the egg PC/PAE vesicles after the termination of the permeation process (only one line from the outer and inner egg PC remains, Fig. 1e) were checked by adding Mn^{2+} or EDTA. Thus after the addition of 1 mM Mn^{2+} the intensity of the egg PC signal was only reduced by the portion of the outer phosphatidylcholine molecules which interact with the Mn^{2+} ions (Fig. 1f). After the addition of 10 mM EDTA which complexes the Pr^{3+} ions in the outer aqueous compartment the initially shifted outer signal appears at the position of the unshifted inner signal and vice versa.

A time dependence of the splitting of the inward and outward facing molecules was again observed. The inner signal approaches the outer one but the direction of this process is upfield now. This can be caused by the permeation of Pr^{3+} out of the vesicles and/or the permeation of EDTA into the vesicles. The latter process seems to be less important because of the relatively large size of EDTA and its slow permeation rate. In all samples investigated the half-times $t^*_{1/2}$ for the permeation of Pr^{3+} out of the vesicle (superimposed by the inward transport of EDTA) were considerably longer than the inward Pr^{3+} transport, e.g. in small unilamellar vesicles (egg PC/PAE 6, 1 : 0.41) $t_{1/2} = 6$ min and $t^*_{1/2} = 120$ min.

Further we studied the temperature dependence of the permeation half-time $t_{1/2}$ below 50°C as egg PC tends to auto-oxidate and degrade above this temperature [22]. The results are summarized in Fig. 3. For small unilamellar vesicles (egg PC/PAE

TABLE II

THRESHOLD CONCENTRATION

PAE	c_{thresh} (PAE/egg PC molar ratio)
PAE 6	0.16 ± 0.01 [a]
PAE 12	0.22 ± 0.01
PAE 16	0.26 ± 0.01
PAE-C	0.20 ± 0.01
PAB-T	$0.08 \pm 0\,04$

[a] The error results mainly from the uncertainty in the ester concentration.

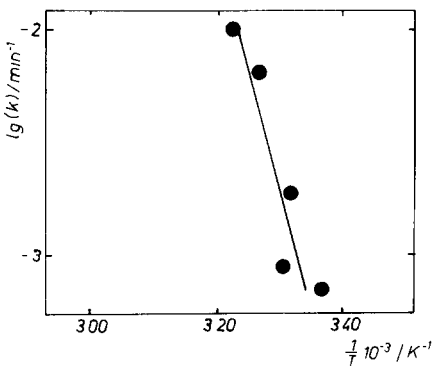

Fig. 3 Arrhenius plot of the rate constants, k, against the inverse temperature for small unilamellar vesicles (egg PC/PAE 6, 1 0 09). The full line represents a linear least-squares fit

6, 1 :0.09) an activation energy of $E_a = 181$ kJ/mol was obtained.

Moreover, information about the distribution of the ester between the outer and inner monolayer was obtained. After the addition of Pr^{3+} the ^{31}P signal resulting from the ester is also shifted (cf. Fig. 1b). The magnitude of the Pr^{3+} induced shift of the ester is smaller by about one order of magnitude than that of the egg PC in the outer layer. In contrast to the signals arising from the lipid the ester yields only one signal. After the addition of Mn^{2+} the signals originating from the ester (and the lipids in the outer layer) disappear (Fig. 1f). This was proved to be true for all esters. From these facts it must be concluded that within the limits of the accuracy of the experiments the esters are incorporated only in the outer monolayer of the vesicles.

The Pr^{3+}-induced shift of the ^{31}P signal of the esters is independent of the aliphatic chain length. For simplicity we assume that this shift is of pseudocontact origin only. Then the approximate position of the phosphonate group within the vesicle monolayer should be the same for all esters.

Discussion

According to Eqn. 2 the permeability coefficients P were calculated from the rate constants k for vesicles with $r = 25.0$ nm and yield about $9 \cdot 10^{-15}$ m/s for pure egg PC vesicles [12,21]. The permeability coefficients P of small unilamellar vesicles from egg PC and PAE vary in the range from $1.9 \cdot 10^{-14}$ m/s ($t_{1/2} = 5026$ min) to $1.6 \cdot 10^{-11}$ m/s ($t_{1/2} = 6$ min). Thus the NMR method presented is well suited to follow the ion permeation in small unilamellar vesicles from egg PC and PAE over four orders of magnitude.

It is well established that NMR can distinguish between different modes of transport [14]. From the Fig. 1 it can be concluded that the observed transport takes place in a continuous manner by a 'one ion at a time' type. Thus stable pores or membrane rupture are not responsible for the ion transport. Because the vesicle radii obtained by quasi-elastic light scattering did not increase within a time large compared to the permeation half-times $t_{1/2}$, fusion can also be excluded.

Until recently the molecular mechanism of non-specific ion transport in general was not well understood. Particularly in small unilamellar vesicles from egg PC and PAE we propose the idea of local and temporal fluctuations in the bilayer induced by the esters. These fluctuations disturb the membrane and allow the ion to permeate through metastable pores [24]. Computer simulations of two-dimensional systems showed that fluctuations (lateral compressibility) increase with increasing concentration of guest molecules [19].

The threshold concentration (cf. Table II) increases with increasing aliphatic chain length R of the ester and is considerably lower for esters with butyl side chains R'. This demonstrates that the threhsold concentration reflects certain structural differences of the esters. Investigations of the Pr^{3+} permeation through small unilamellar vesicles from lysophosphatidylcholine analogs by Barsukov et al. [5] found also a dependence of the Pr^{3+} permeability on the molecular structure of the lysophosphatidylcholine.

For a more quantitative discussion we use the concept of molecular shape [23,24,26]. The molecular shape of the PAE within a hydrophobic environment was calculated as described elsewhere [25]. We chose the molecular asymmetry defined by the ratio of the cross-sectional area of the head-group H to that of the chain C [25] as a representative value of the molecular shape. All aliphatic PAE exhibit a positive molecular asymmetry, vis. $H^{PAE} > C^{PAE}$ [25]. The molecular asymmetry varies in the following manner: an increase of the aliphatic chain length decreases the molecular asymmetry and an increase of the length of the ester side chains increases the molecular asymmetry. It should be stated that because of their positive molecular asymmetry the PAE are incorporated in the outer vesicle monolayer because of the packing restrictions they would experience in the inner monolayer.

These results lead to the following conclusions: First, the asymmetric distribution of PAE between the two monolayers of the small unilamellar vesicles was a result of the positive molecular asymmetry of the PAE. Second, a correlation between the magnitude of the PAE-induced perturbations, the threshold concentration and the molecular asymmetry can be suggested. Third, at present the origin of the differences in the half-

times of permeation into and out of the vesicle is not fully understood. Probably a prerequisite for effective ion transport is that the perturbing molecules are incorporated in the monolayer from which the ion permeation starts.

Acknowledgments

We want to thank Mr. S. Bilke for very helpful discussions about the results of the molecular shape calculations. We thank also the referees for helpful comments.

References

1 Marćelja, S. and Wolfe, J. (1979) Biochim Biophys Acta 557, 24–31
2 Singer, M A. (1983) in Liposome Letters (Bangham, A.D , ed.), pp. 106–112, Academic Press, London
3 Bangham, A D. (1981) in Liposomes: From physical structure to therapeutic applications (Knight, C.G., ed.), pp. 1–18, Elsevier, Amsterdam
4 Lee, Y. and Chan, S.I. (1977) Biochemistry 16, 1303–1309
5 Barsukov, L.J , Parpheniева, A.M., Victorov, A.W., Shapiro, J E , Bystrov, W.F. and Bergelson, L.D. (1974) Biofisika 19, 456–460 (in Russ)
6 Muramushi, N., Takagi, N., Muranishi, S and Sezaki, H (1981) Chem. Phys. Lipids 28, 269–279
7 Kochmann, W., Günther, E., Lottge, W. and Kramer, W (1973) Chem Techn. 25, 434
8 Kramer, W., Gunther, E , Lottge, W., Beck, R and Kochmann, W. (1975) Congress of Pesticide Chemistry, Proceedings, pp. 686–689, Helsinki
9 Klose, G., Hentschel, M. and Bayerl, T (1985) in Proceedings of the 7th school on biophysics of membrane transport, May 1984, Zakopane (Poland), Wroclaw University
10 Reference deleted
11 Bayerl, T., Klose, G., Ruckpaul, K. and Schwarze, W (1985) Biochim. Biophys Acta 812, 437–446
12 Cushley, R.J , Forrest, B.J , Gillis, A and Tribe, J (1979) Can. J Chem 57, 458–465
13 Bergelson, L D (1979) in Methods in Membrane Biology (Korn, ed), Vol 9, pp. 275–335
14 Ting, D.Z., Hagan, P S., Chan, S I , Doll, J D and Springer, C.S. (1981) Biophys J 34, 189–216
15 Pike, M M , Simon, S.R , Balschi, J A and Springer, C S (1982) Proc. Natl Acad. Sci USA 79, 810–814
16 Singleton, W S , Gray, M.S., Brown, M L and White, J L (1965) J Am Oil Chemist's Soc 42, 53
17 Frischleder, H , Grupe, R , Grundel, M and Winkelmann, H. (1978) Studia Biophys 68, 195–205
18 Szoka, F and Papahadjopoulos, D (1980) Annu Rev Biophys Bioeng 9, 467–508
19 Peinel, G , Binder, H., Bilke, S and Fritsche, S (1984) Wiss Z Karl-Marx-Universitat, Leipzig, Math Naturwiss. R 33, 349–365
20 Cornell, B A , Middlehurst, J and Separovic, F (1980) Biochim Biophys Acta 598, 405–410
21 Elamrani, K. and Blume, A. (1982) Biochim. Biophys Acta 727, 22–30
22 Lawaczeck, R., Blackmann, R and Kainosko, M (1977) Biochim Biophys. Acta 468, 411–422
23 Petrov, A G., Seleznev, A and Derzhanski, A (1979) Acta Phys. Pol A55, 385–405
24 Petrov, A.G (1981) in Proceedings of the 6th school on biophysics of membrane transport, Poland, pp 116–145, Wroclaw University
25 Bilke, S., Peinel, G. and Klose, G (1983) Studia Biophys 93, 215
26 Helfrich, W. (1973) Z Naturforsch. 28c, 693–703