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PROTON GRADIENT ACROSS MEMBRANES OF LECITHIN VESICLES AS MEASURED BY ^{31}P-NMR : ASYMMETRICAL BEHAVIOUR OF INTERNAL AND EXTERNAL LAYERS

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SUMMARY

The ionization of phosphate ions trapped inside sonicated egg yolk lecithin vesicles was determined as a function of internal pH values using a ^{31}PNMR. The chemical shift of the internal phosphate signal reflects the intravesicular pH perfectly. At 30°C, protons once inside vesicles (pH int < pH ext) cannot get across the inner layer even under a pH gradient up to five units. On the other hand, when pH (int) > pH(ext),the results suggest that protons may cross the outer but not the inner layer and interact with the internal phosphate ions.

INTRODUCTION

^{31}PNMR is one of the powerful techniques available for studying lipid-related properties of biological membranes [1]. Recently, it has been shown [2-7] that observation of the ^{31}P signal from various intracellular phosphates (P_i, ADP, ATP, etc...) can provide a convenient, non destructive technique for determining intracellular pH. It seems likely that ^{31}PNMR may lead to a better understanding, at the molecular level, of phenomena occurring inside a membrane subjected to a proton gradient ; in order to systematically investigate these phenomena, it seems wise to test the phospholipid behaviour in model systems.

In this paper, we present the result of a ^{31}PNMR study of sonicated egg yolk lecithin vesicles and demonstrate their asymmetric behaviour with respect to a proton gradient. Orthophosphate has been used as the intra and extra vesicular pH probe.

MATERIALS AND METHODS

Egg yolk lecithin (3-sn-Phosphatidyl) choline (EPC) was prepared by the Singleton method [8]. Chromatographically pure sodium phosphatidate (from egg yolk lecithin) was obtained from Koch-Light and dipalmitoyl phosphatidyl choline (DPPC) from Sigma. Cholesterol (CH) and stearic acid (SA) were supplied by Fluka. The vesicles were prepared by dispersing 80 to 120 mg of phospholipid mixture in 4 ml of phosphate buffer (400 mM NaH_2PO_4 + 1 mM EDTA) adjusted to the required pH by addition of NaOH. The dispersion was then sonicated under a nitrogen stream for

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20 mn, with a 4 x 180 mn sonotrode dissipating 20 W at 20 KHz. In order to remove large vesicles and titanium particles, the sonicated solution was centrifuged at 13000g for 1 h. It was checked by TLC on aliquots that no decomposition of the phospholipids occurred during sonication. The vesicle solution was dialysed for 20 h against a citrate isotonic buffer (+1 mM EDTA)to eliminate external orthophos phate ions. This operation facilitated the observation of the internal phosphate signal (P_i int signal) in particular when the difference between pH int and pH_{ext} was small.

The ^{31}PNMR spectra (with proton noise decoupling) were recorded at 40.5 MHz on a Varian XL 100-12 WG Spectrometer at 29-30°C. The chemical shifts were measured from 85 % H_3PO_4 as an external reference (δ positive upfield).

RESULTS AND DISCUSSION

a) Characterization of vesicles and titration curves of internal phosphate ions

Fig. 1 shows typical spectra obtained at constant pH (int) = 4.5 with pure EPC at pH (ext) = 6.5 (Fig. 1A) and with EPC + 40 mole % cholesterol at pH (ext) = 9.5 (Fig. 1B). The low field signals which depend on pH (ext) are attributed to the external phosphate ions. The peak at -0.15 ppm, the position of which depends only on pH (int) (= pH of the buffer trapped inside the vesicles), is due to the internal phosphate. The broad peak at 0.9 ppm, insensitive to pH (int) and pH (ext) is, of course, due to the phospholipid.

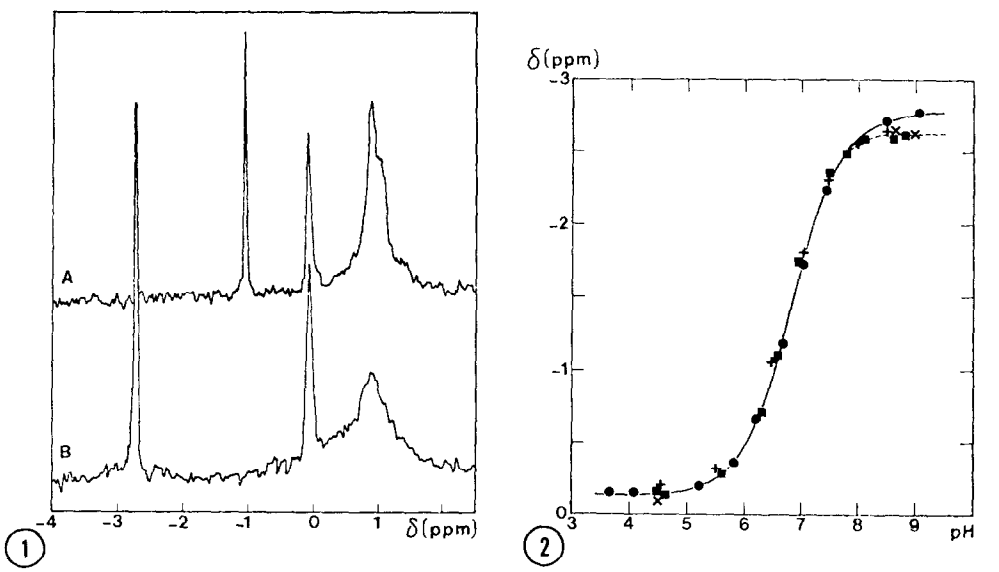

Fig. 1. ^{31}P NMR spectra of vesicle solutions:pH (int) = 4.5, internal phosphate concentration $[P_i int]$ = 400 mM. A) with pure EPC at pH (ext) = 6.5 ; B) with EPC + 40 mole % cholesterol at pH (ext) = 9.5 (see text).

Fig. 2. Titration curves of internal phosphate-ions trapped inside vesicles of various compositions. ■ = pure EPC ; + = EPC + 4 mole % PA ; x = EPC + 40 mole % cholesterol ; ● = $P_i$ext. $[P_i int]$ = 400 mM.

In order to verify that the vesicles prepared were unilamellar, Pr^{3+} and La^{3+} ions were added separately to samples dialysed against 400 mM NaCl solution : the phospholipid signal of the inner layer remained rather sharp, whereas the signal of the outer layer shifted and broadened ; the area ratio between these two signals, due to the outer and the inner phospholipid layer, is about two, characteristic of unilamellar vesicles [9].

If we took 22 nm as the mean outer diameter [10, 11] and 3.5 nm as the thickness of the bilayer [12], we obtained the following mean parameters : internal vesicle volume = $1.8\ 10^{-18}$ ml ; on the average each vesicle contains about 2160 phospholipids molecules, 59,000 water molecules, 430 phosphate ions and 0.1 free H^+ for a pH (int) = 4.5.

Fig. 2 shows the titration curves of the internal and external phosphates. They are practically the same in the 4 to 9 pH range. The same curves were obtained with various membrane compositions : pure EPC, EPC + 40 % mole CH, EPC + 4 mole % SA. In general, the pH (int) signal remains sharp (3 to 7 Hz at half height) when pH (int) varies between 4 and 9.

The results show clearly that i) internal phosphate behaves similarly to bulk phosphate, even in a small volume (15 nm inner diameter) and ii) the chemical shift of P_i int reflects the intravesicular pH perfectly.

b) Asymmetric behaviour of EPC vesicles with respect to the sign of the pH gradient (Δ pH = pH int - pH ext).

At 30°C, the NMR spectra of the suspension, in particular that of the P_i int signal, remain unchanged over a period of several days when pH (int) is more acidic than pH (ext) ($\Delta pH < 0$) even with a negative pH gradient up to five units (Fig. 1). The vesicle bilayer is therefore perfectly impermeable to proton and phosphate ions. The proton once inside the vesicle cannot get out.

On the other hand, when pH (ext) becomes more acidic than pH (int), $\Delta pH > 0$, the internal phosphate signal undergoes important changes : it shifts upfield as if the internal medium was becoming more acid and broadens progressively until it vanishes in the noise (Fig. 3 & 4). At this stage, if pH ext is adjusted to the initial pH int, i.e. the pH gradient is cancelled by NaOH addition, the P_i int signal reappears and finally returns to its starting position with the same shape and area as before (Fig. 4). During this experiment, the phospholipid resonance line remained unchanged and for fully dialysed samples no outer phosphate signal was observed (not shown). This reversible phenomenon does not depend on the nature of the anion in the external medium (citrate, chloride or sulphate). The disappearance of the P_i int signal is certainly not due to either aggregation or vesicle burst.

Fig. 4 also shows that the disappearance rate of the P_i int signal (<1 h) is faster than its reappearance ($\simeq 15$ h). It is to be remarked that the

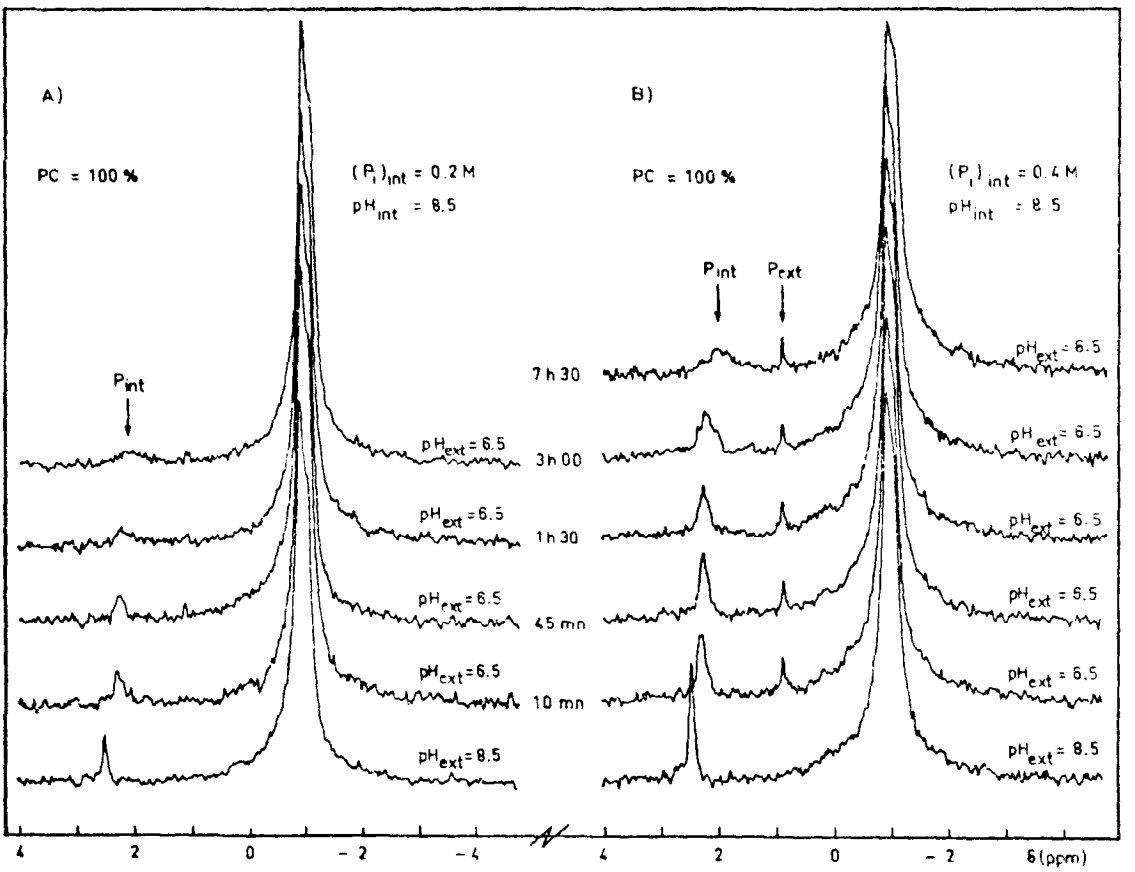

Fig. 3. Behaviour of the P int signal for various internal phosphate concentrations when a negative proton gradient is set up : pH int = 8.5, pH ext = 6.5. A) $[P_i\ int]$ = 0.2 M, B) $[P_i\ int]$ = 0.4 M. At the beginning pH ext and pH int were the same (= 8.5). Zero time corresponds to the addition of hydrochloric acid to the external medium pH ext = 8.5 ⟶ pH ext = 6.5.

disappearance rate increased with pH gradient and varied with the vesicles compositions (EPC + SA > EPC > EPC + CH).

The shift and the broadening of the P_i int signal observed (Fig. 3 & 4), when pH (int) > pH (ext), could not be due to a heterogeneity in vesicle size, resulting in a distributed influx of protons in the internal aqueous compartments ; since the protons, once inside the vesicles, cannot get out; the broadening of the P_i int signal should remain unchanged when the pH gradient is cancelled. The fact that the P_i int signal behaves in a strictly reversible way with respect to the pH gradient, indicates that pH int is undoubtedly unchanged.

Therefore the interaction between internal phosphate ions and external protons is certainly not due to the penetration of the proton in the inner layer. On the other hand, the lack of any modification in the P int signal when pH int

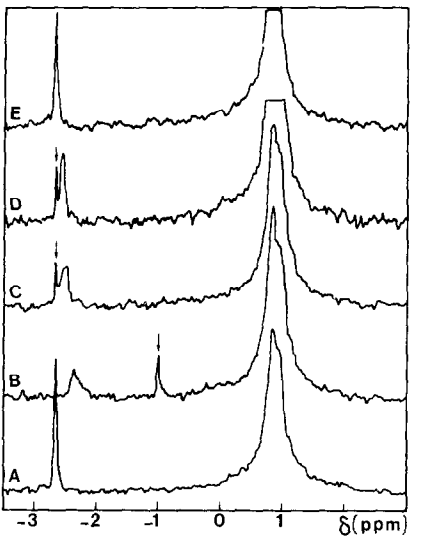

Fig. 4. Reversibility of the chemical shift and broadening of the P_i int. signal when the vesicles are subjected to a negative proton gradient. pH (int) = 8.6, $[P_i \text{ int}]$ = 400 mM. At zero time, the negative proton gradient (set up at -40 mn) was cancelled. A) no gradient ; B) 10 mn, 1 h, 15 h after the gradient was cancelled. The arrow indicates the external phosphate signal.

< pH ext, the slow broadening of this signal when pH int > pH ext and the highly dielectric medium such as the intravesicular permit us to eliminate the hypothesis of a long range interaction between the internal phosphate ions and the protons, when the latter are on the outside of the vesicle. For these reasons, we suggest that, owing to the difference between the surface tension of internal and external layers, the external layer of lecithin vesicles probably gives access to protons but not the internal layer, and that only protons passing through the external layer can interact with internal phosphate ions. The broadening of the P int signal is thus due to the exchange between free and interacted phosphate ions, whose signal is very large.

A priori, the temperature effect can be used to verify this hypothesis. However, if this effect exerces a great influence on the exchange time between two kinds of phosphate ions, it can also modify the structural parameters and the permeability of vesicles. When the temperature increases from 30° to 45°C, we have stated that the disappearence of the P_i int signal is accelerated. This could be due to a slow exchange between free and interacted phosphate ions at 30°C, but also to the fact that all the free phosphate ions have quickly been transformed into interacted ions, since the proton influx also increases with temperature.

In the lack of a different frequency ^{31}P-NMR spectrometer, we performed the same experiments with various concentrations of internal phosphate

ions and observed the broadening of the $P_i$int signal as a function of time. As it has been shown above (Fig. 3A and Fig. 3B), the broadening of the $P_i$int, at a given time after the negative pH gradient was set up, is greater at lower $P_i$int concentrations. This means that at a given time, as the number of protons trapped inside the vesicle bilayer, and hence the interacted phosphate ions, can be assumed to be the same, the proportion of free phosphate ions increases (the linewidth of the $P_i$int signal decreases) with the internal phosphate concentration in accordance with the hypothesis of an exchange between free and interacted phosphate ions. A detailed kinetic study of these phenomena exceeds the scope of the present paper. More extensive studies about the asymmetrical behaviour and the transport of ions through vesicles are in progress in our Laboratory ; the results obtained will be reported elsewhere.

In conclusion, from the data in the present paper, it seems that the inner layer represents a limiting factor for the transport of ions through the vesicle bilayer. Similarly the fluidity of the membrane is of utmost importance in this transport phenomenon.

REFERENCES

1. Cullis, P.R., McLaughlin, A.C. (1977) Trend. Biochem. Sci. 2, 196-199.
2. Casey, R.P., Njus, D., Radda, G.K., Sehr, P.A. (1977) Biochemistry 16, 972-977.
3. Moon, R.B., Richards, J.H. (1973) J. Biol. Chem. 248, 7276-7278.
4. Navon, G., Ogawa, S., Shulman, R.G., Yamane, T. (1977) Proc. Natl. Acad. Sci. USA 74, 888-891.
5. Navon, G., Ogawa, S., Shulman, R.G., Yamane, T. (1977) Proc. Natl. Acad. Sci. USA 74, 87-91.
6. Ogawa, S., Shulman, R.G., Glynn, P., Yamane, T., Navon, R.G. (1978) Biochim. Biophys. Acta 502, 45-50.
7. Pollard, H.B., Shindo, H., Creutz, C.E., Pazoles, C.J., Cohen, J.S. (1979) J. Biol. Chem. 254, 1170-1177.
8. Singleton, W.S., Gray, M.S., Brown, M.L., White, J.L. (1965) J. Am. Oil Chemist's Soc. 42, 53-56.
9. Hutton, W.C., Yeagle, P.L., Martin, R.B. (1977) Chem. Phys. Lipids 19, 255-165.
10. Barenholz, Y., Gibbes, D., Litman, B.J., Goll, J., Thompson, T.E., Carlson, F.D (1977) Biochemistry 16, 2806-2810.
11. Huang, C.H., Lee, L.P. (1973) J. Am. Chem. Soc. 95, 234-239.
12. Reiss-Husson, F. (1967) J. Mol. Biol. 25, 363-382.