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AN NMR METHOD FOR THE STUDY OF PROTON TRANSPORT ACROSS PHOSPHOLIPID VESICLES

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SUMMARY

An NMR method to study proton transport into and out of vesicles under the condition of obligatory proton-cation exchange is presented.

Below the transition temperature, T_c, the intravesicular proton concentration is found to be unchanged even when DPPC vesicles are subjected to a large pH gradient of four or five units. Above T_c, proton can get in or out of DPPC vesicles ; this depends on the sign of the pH gradient. Valinomycin can be transferred from one vesicle to another ; the intravesicular contents however do not mix.

The vesicle size distribution and the proton gradient appear to be responsible for the evolution of the P_{int} signal which broadens at the beginning and becomes narrow again at the end of the proton transport process.

INTRODUCTION

In recent years, NMR spectroscopy has proved to be a successfull method for investigating transport processes. Many studies have been devoted to water (1-4), carboxylic acid (5) and ion (6-7) transport. Unfortunately, the transport of protons has not been directly investigated so far. This is due to the fast exchange between H_2O and H^+, the small concentration of the latter and also to the fact that the cation transport is much slower than water diffusion.

In the present communication, an NMR method is proposed for the study of proton-cation exchange through vesicle bilayers subjected to a pH gradient and some preliminary results on the proton transport are presented.

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NMR method to investigate proton transport

The total internal volume of all vesicles in solution is very small, about 1 % of the external medium volume. For this reason, a large variation in the internal proton concentration observed during the transport process will cause only a small change in the external proton concentration. It is therefore of the utmost importance to know the acidity inside the vesicles at any time. This can easily be achieved with the NMR technique by introducing into the intravesicular medium a probe fulfilling the following conditions :

a) the chemical shift of the probe must be pH-dependent.

b) the nucleus observed should have a large gyromagnetic ratio (^{1}H, ^{19}F, ^{31}P) to facilitate signal detection.

c) the pK of the probe should not be very different from the physiological pH ($\simeq$ 7.0).

d) the vesicle bilayer must be perfectly impermeable and inert (chemically and structurally) to this probe.

In previous papers, pH measurements in biological media have been carried out using ^{31}P-NMR spectroscopy of phosphorylated compounds (8-11). Orthophosphate ions appear to be a good probe for measuring pH_{int} since the internal and external phosphate ions, P_{int} and P_{ext}, behave similarly and the P_{int} chemical shift reflects the intravesicular pH perfectly (12).

Vesicles were prepared from L-α-dipalmitoyl phosphatidyl choline (DPPC). Below the transition temperature (T_c = 42°C) of the membrane, the internal proton and phosphate ion concentrations are found to be unchanged for days even when DPPC vesicles are subjected to a large pH gradient of 4 or 5 units ; we took advantage of this property to keep constant the composition of the internal vesicular medium during dialysis and NMR measurements.

In the system studied here, due to the impermeability of the DPPC membranes to anions, proton fluxes can occur only through electroneutral exchange with another cation : an easy pathway for the exchanging cation may be provided by adding valinomycin to the membrane to increase the proton flux.

Experimental procedure

Valinomycin and L-α-dipalmytoyl lecithin (DPPC) were purchased from SIGMA. DPPC was further purified by preparative HPLC according to (13). All other reagents were analytical grade. Vesicles were prepared by sonicating at 60°C, 90µmol DPPC in 3 ml of 400 mM phosphate buffer + 1 mM EDTA as described previously (12).

In some preparations, valinomycin was added to DPPC prior to sonication at the ratio of 1 valinomycin for 2200 DPPC, that is about 1 valinomycin per vesicle. The vesicle suspension was then dialysed overnight against an isotonic citrate buffer (+ 1 mM EDTA) in order to remove the great part of external phosphate ions. pH gradients were established across the vesicle membrane by addition of small amounts of NaOH or HCl ; the vesicle suspension was then introduced into a 10 mm NMR tube which was capped and sealed with fast epoxy glue.

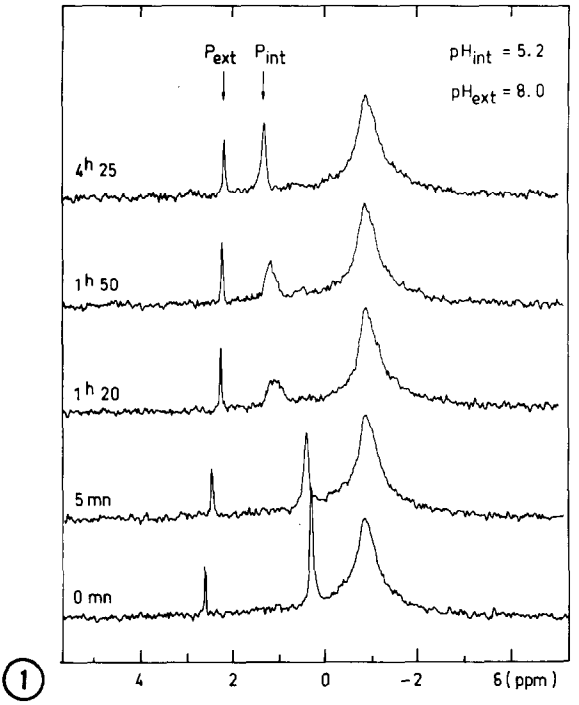
Proton diffusion experiments were carried out by incubating the NMR tube in a thermostat at various temperatures between 45 and 75°C, during given periods of time between which the tube was rapidly cooled at 27°C for NMR measurements. ^{31}P-NMR spectra (with proton noise decoupling) were recorded either at 36.4 MHz on a BRUCKER WH 90 or at 40.5 MHz on a VARIAN XL 100 spectrometer. Chemical shifts were measured from 85 % H_3PO_4 as an external reference. The pH variations in the internal and external media were calculated from the chemical shift of the corresponding phosphate signals.

RESULTS AND DISCUSSION

When DPPC vesicles are subjected to a negative or positive pH gradient ($\Delta pH = pH_{int} - pH_{ext}$) and the vesicular solution is heated above the transition temperature, the P_{int} signal shifts progressively toward the P_{ext} signal. Simultaneously its shape undergoes important changes : the linewidth increases at the beginning and then decreases to the initial linewidth as the P_{int} signal approaches the P_{ext} signal.

Fig.1 and 2 display typical series of ^{31}P-NMR spectra obtained from DPPC vesicles (containing valinomycin) subjected to a negative and positive pH gradient respectively.

In the absence of valinomycin, the same behaviour was observed but on a much longer time scale. In all cases, the area of the P_{int} signal remains the same, indicating that the P_{int} concentration remains unchanged during the transport process. These results clearly demonstrate that the pH_{int} has been modified and protons can move from the inside to the outside of vesicles or vice versa, depending on the sign of the pH gradient. Thus the proton-cation exchange (hours



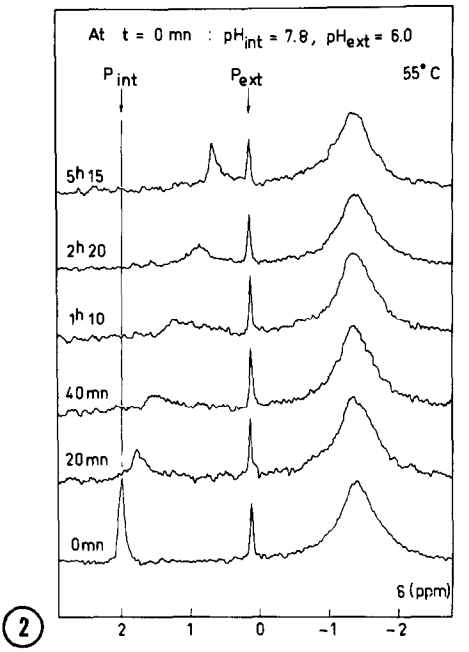


Fig.1 - ^{31}P-NMR spectra obtained from DPPC vesicles + 1 valinomycin per vesicle. P_{int} = 400 mM. At the beginning, pH_{int} = 5.2, pH_{ext} = 8.0 and KCl was added in the external medium [KCl] = 25 mM ; the vesicle solution was then heated at 55°C for various times. The broad peak at -0.9 ppm is the ^{31}P signal of DPPC.

Fig.2 - ^{31}P-NMR spectra obtained from DPPC vesicles + 1 valinomycin per vesicle. $[P_{int}]$ = 400 mM, $[K^+]_{int}$ = 3 mM ; the external K^+ ions were completely eliminated by dialysis. After a transmembrane pH gradient was established by lowering the extravesicular pH from 7.8 to 6.0, the vesicle solution was heated at 55°C for various times.

or days) is much slower than water diffusion (in the millisecond range 1-4) as expected.

One important question to be considered is why the shape of the P_{int} signal undergoes such modifications during proton transport. The broadening of the P_{int} signal at the beginning is due to a pH_{int} distribution which probably results from the inhomogeneity of the vesicle size and hence from a distribution in proton transport rates. On the other hand the decrease in the linewidth of the P_{int} signal at the end of the proton transport process can be explained by the fact that the proton flux depends mainly on the driving force $\Delta\tilde{\mu}H^+$. This parameter includes not only the difference in the proton

concentration $\Delta[H^+]$, but also the electrical potential difference across the bilayer which depends on the relative mobilities of proton and exchanging cation in the membrane. At the end of the transport process, as the profon flux and hence the dispersion of the internal pH decrease continously with the driving force, all the pH_{int} values tend to the same limit (which is generally different from the external pH) and the P_{int} signal becomes narrow again as before.

Therefore the greater the driving force, the faster the chemical shift variation of the P_{int} signal and the larger the broadening of its linewidth.

In the following experiment we use the present NMR method to check i) whether valinomycin can be transferred from one vesicle to another and ii) whether the two intravesicular contents mix when valinomycin transfer takes place. Initially, two vesicle populations were mixed : for the first population, the bilayer did not contain any valinomycin and pH_{int} was 5.2 ; for the second one, the membrane was loaded with valinomycin (pH_{int} = 7.3) ; the common external medium was at pH = 8.4, and the diffusion was carried out at 55°C. It can be seen in Fig.3 that the two P_{int} signals are well separated ; the signal at higher field corresponding to the first vesicle population, which did not contain valinomycin at the beginning, shifts faster and broadens more than the signal of the second population. These results clearly show that a) valinomycin is transferred from the second to the first vesicle population since the proton efflux at the same temperature is much smaller in the absence of valinomycin. b) Intravesicular contents do not mix (by vesicle fusion or other wise) during the valinomycin transfer process . c) The unidirectional proton transport rate depends on the proton gradient as expected. d) The broadening of the P_{int} signal which reflects the inhomogeneity of the vesicle size is clearly observed when the proton gradient is large and the variation of the internal proton concentration is still negligible at the beginning of the transport process. These results are in very good agreement with the above mentioned interpretation.

In conclusion, the NMR method presented here permits the proton concentration variation inside the vesicles to be observed directly and the vesicle stability controlled, and

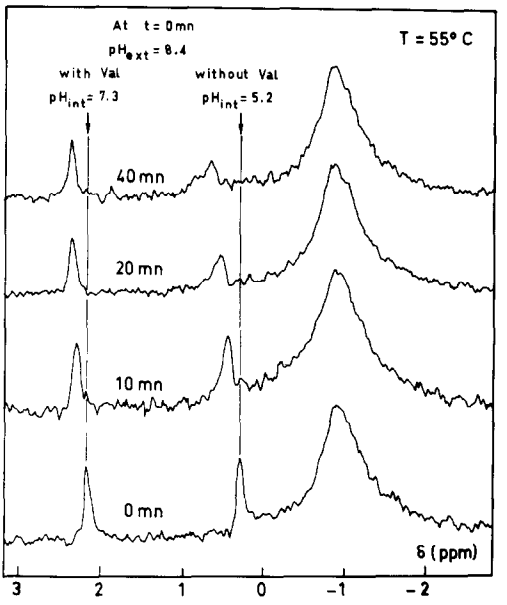


Fig.3 - ^{31}P-NMR spectra obtained from a mixing of two populations of DPPC vesicles entrapping a 400 mM phosphate buffer : peak at 0.3 ppm, vesicles without valinomycin, pH_{int} = 5.2 ; peak at 2.2 ppm, vesicles + 1 valinomycin per vesicle, pH_{int} = 7.3. Both vesicles batches were separately dialysed against a common phosphate free isotonic buffer. At the beginning of the experiment, the two batches were mixed (1 vol/1 vol), the pH of the external medium was raised to 8.4, and the NMR tube heated at 55°C for various times.

provides information useful for the understanding of the proton-cation exchange mechanism. This method can be used, with or without slight modifications, to investigate the transport process of other molecules and ions. In particular, dialysis of the vesicle solution after sonication is very useful as a way to establish at will a gradient of the molecules or ions studied, and at the same time facilitates the observation of the NMR signals coming from the intravesicular medium.

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