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Cation permeability induced by valinomycin, gramicidin D and amphotericin B in large lipidic unilamellar vesicles studied by ^{31}P-NMR

M. Hervé[1,2,*], B. Cybulska[**], and C. M. Gary-Bobo[1]

[1] Service de Biophysique, C.E.N. Saclay, F-91191 Gif-sur-Yvette, France
[2] U.E.R. de Médecine et Biologie Humaine, Université Paris XIII, F-93012 Bobigny, France

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Abstract. Permeability induced by mobile carriers and channel-forming compounds in large unilamellar lipidic vesicles (LUV) has been studied by the proton-cation exchange method. Proton movement has been monitored by pH-stat and ^{31}P-NMR techniques. pH-stat measurements indicate that, in the presence of valinomycin, the proton efflux develops with a rate dependent upon valinomycin concentration, until equilibrium is reached. ^{31}P-NMR spectra, monitoring pH-dependent intravesicular phosphate ionization, show that after addition of valinomycin the initial pH peak (pH 5.5; $\delta = 0.25$) shifts progressively to the position corresponding to the pH at equilibrium (pH 7.4; $\delta = 2.20$).

In the presence of the channel-forming compounds, gramicidin D or amphotericin B, permeability developed in a few minutes whatever the concentration used. The percentage of total titratable proton released depends upon the antibiotic concentration. ^{31}P-NMR spectra shows two signals from internal phosphate: one signal corresponding to the initial pH and a second signal corresponding to the pH at equilibrium indicating an all-or-none mode of action; just after addition of the antibiotic, two populations of vesicles coexist in proportions that depend on ionophore concentration; after longer incubation times all vesicles are permeabilized.

The results obtained primarily reflect the differences in the mode of interaction with the membrane, of valinomycin as compared to the channel-forming reagents, gramicidin D or amphotericin B.

Key words: Ionophores, large unilamellar vesicles, ^{31}P-NMR

* To whom offprint requests should be addressed
** On leave from the: Department of Pharmaceutical Technology and Biochemistry, Technical University of Gdansk, PL-80-952 Gdansk, Poland

Introduction

The activity of an ionophore on cellular membranes depends not only upon the intrinsic permeability properties of the pathway this ionophore provides but also upon its mode of interaction and incorporation in the membrane. For this reason, the measurement of ionic fluxes induced in a cell population upon the addition of a given ionophore cannot, a priori, be interpreted in terms of permeability only. Using lipidic vesicles, which are the model system closest to a cellular population, ionic fluxes as well as interaction measurements can be made in well defined physicochemical conditions.

Clement and Gould (1981a, b) studying ion fluxes induced by gramicidin D in small unilamellar vesicles (SUV) came to the conclusion that due to the very high ion mobility in the gramicidin D channel, the ion flux rate measured did not reflect the channel permeability, but rather the kinetics of channel formation. A study of amphotericin B induced permeability using a similar SUV system (Bolard et al. 1981), led us to the same conclusion. Even at low antibiotic concentrations the permeability observed is a very fast, all-or-none process. The limiting factor is the pore formation, since, once the pore is formed, the very small intravesicular medium reaches equilibrium in a very short time. The smallness of the SUV internal medium is not the main factor, since the same phenomenon is apparently observed using large unilamellar vesicles (LUV) (Cybulska et al. 1983) in which the internal volume is at least a thousand time larger. In contrast, the situation is different with other ionophores. The results of ion flux measurements carried out on SUV with valinomycin (Clement and Gould 1981a, b) as well as on LUV with crown-ethers (Thomas et al. 1983) seem to capable of interpretation in terms of permeability.

Therefore it seems that in vesicular systems, SUV or LUV, and also perhaps in cellular systems, the interpretation which can be given of an ion flux induced by ionophores would depend on whether this ionophore is a mobile carrier or a channel-former.

In order to test this hypothesis, a study of the kinetics of ion fluxes mediated by ionophores of both types has been carried out on LUV using the method of electroneutral proton-cation exchange. Proton fluxes have been monitored concurrently by two techniques, firstly, the pH-stat method (Cybulska et al. 1983; Thomas et al. 1983) which allows determination of proton fluxes in the extravesicular medium and secondly, ^{31}P-NMR spectroscopy (Tran-Dinh et al. 1981). The latter technique, using the orthophosphate ion as a probe for measuring intravesicular pH, has the decisive advantage that it allows a direct observation of the intravesicular medium.

Material and methods

L-α-phosphatidyl choline was prepared from egg-yolk according to Patel and Sparrow (1979). Phosphatidic acid was prepared from phosphatidyl choline according to Roux et al. (1983). Cholesterol was from Fluka, and was twice recrystallized from ethanol before use. FCCP (carbonyl cyanid-p-trifluoromethoxyphenyl hydrazone) was from Boehringer. Valinomycin and Gramicidin were from Sigma and Amphotericin B was from Squibb.

Large unilamellar vesicles (LUV) were prepared according to Szoka and Papahadjopoulos (1978) using a lipid mixture of L-α-phosphatidylcholine, phosphatidic acid and cholesterol in a 70 – 10 – 20 molar ratio, unless otherwise indicated.

Proton flux determination

a) pH-stat method. LUVs were prepared using 40 μmol of lipid mixture per millilitre of a 100 m*M* sodium phosphate/100 m*M* sodium sulfate solution (pH 5.50). After vesicle formation by reverse phase evaporation under reduced pressure, the vesicle suspension was diluted three times in 194 m*M* sodium sulfate and then was sequentially filtered through polycarbonate porous membranes (Nuclepore Corporation, Pleasanton, Calif.), of 1 μm, 0.4 μm and 0.2 μm pore size.

0.5 ml of filtered vesicles (6 μmol of lipids) was diluted in 3.5 ml of 194 m*M* sodium sulfate solution in the titrating vessel of a pH-stat (Radiometer, Copenhagen). The vesicle suspension was then equilibrated at 20° under a nitrogen stream, and the pH was brought to 7.50. 10 μl of a 1 m*M* FCCP solution in ethanol was then added. Subsequently, the desired amount of ionophore was added as microlitre amounts of its solution in the appropriate organic solvent. The proton efflux was measured as the volume of a 5 m*M* NaOH solution in 194 m*M* sodium sulfate necessary to maintain pH 7.50.

b) ^{31}P-NMR method. LUVs were prepared using 70 μm of lipid mixture per millilitre of a 200 m*M* sodium phosphate + 200 m*M* sodium sulfate + 1 m*M* EDTA solution in 40% D_2O (pH 5.50). The vesicles, diluted four times in 400 m*M* sodium sulfate were filtered as described above. 2.5 ml (about 45 μmol of lipid) were brought to pH 7.50. 10 μl of a 10 m*M* FCCP solution was added and then the desired amount of ionophore. After a given incubation time at room temperature, 20 μl of a 100 m*M* $MnCl_2$ solution was added, the suspension was transferred to a 10 mm bore NMR tube and the spectrum was recorded. The accumulation time was 9 min.

^{31}P-NMR spectra of phosphate ions, with proton noise decoupling were recorded at 36.4 MHz on a Brucker WH 90. The pH variations in the internal and external media were calculated from the chemical shifts of the phosphate signal using 85% H_2PO_4 as an external reference (Prigent et al. 1980).

Results

Characterization of the experimental system

The permeability induced by antibiotic ionophores in LUVs was measured by the proton-cation exchange method. In this method, a transmembrane ΔpH of about 2 units established in the LUV suspension drives protons outward, whereas a corresponding cation gradient drives them inward. Since neither phosphate nor sulphate ions can cross the membrane, any cation movement can take place only by electroneutral Na^+/H^+ exchange. Therefore the permeability induced by a given ionophore in vesicles subjected to such a ΔpH can be adequately and conveniently measured by monitoring the proton movement through the membrane, providing that the proton efflux is not the limiting factor in this exchange. This proton movement has been measured either in the external medium, recording the time-course of the titration by the amount of NaOH necessary to maintain constant external pH (pH-stat technique) or monitoring the change with time of the position and intensity of the intravesicular phosphate ion NMR signal. Both techniques should yield the same result, when the differences in the experimental conditions are taken into account.

In order to check that the permeability results obtained by the two methods are comparable, in spite of some differences in the mode of vesicle preparation, LUV suspensions prepared for NMR experiments were tested by the pH-stat method. It was found that: *1)* for a given antibiotic/lipid ratio the concentration of the vesicles (which is about 8 to 10 times greater for NMR than for pH-stat experiments) had no significant influence upon the proton efflux rate. *2)* presence of 1 m*M* EDTA also had no influence. *3)* The 40% D_2O used in NMR suspension medium, resulted in a general shift of the pH scale. Besides this well known effect, and within the limit of accuracy of the pH-stat method, the proton (or rather proton + deuteron) transport rate was not significantly different from that observed in pure H_2O.

Permeability to Cl^- is not negligible (Louni et al. 1983) in LUV. In the presence of $MnCl_2$, due to chloride permeability, proton-cation exchange may be not ideally stoichiometric. However this salt was present only during recording time and at very low concentration. Therefore the error introduced can be neglected. On the other hand, the proton permeability induced by valinomycin in the absence of FCCP is much lower than in the presence of FCCP (Louni et al. 1983). Therefore, even in the case of an ionophore able to perform proton-cation exchange by itself, a specific proton carrier, FCCP, was used to ensure independent proton permeability large enough not to be the limiting factor in the exchange process.

The basic permeability to monovalent cations was not systematically measured. However the following observations were made: in the presence of FCCP alone, the proton efflux from the vesicles can be considered as a measure of the cationic permeability of the lipidic bilayer. In the medium containing Na^+ as the only cation, the proton efflux elicited by FCCP was very small, and amounted to 1–2% of the total titratable intravesicular protons per hour, a rate which is at the limit of significance of the pH-stat method. In these conditions there was no significant NMR chemical shift of the internal phosphate, even after 24 h. However a slight decrease in intensity of the signal was observed. This indicates that the proton efflux measured by the pH-stat method is due to a slight vesicle destruction with time rather than to a true Na^+ permeability. In a K^+-containing medium the proton efflux becomes significant, being about 10 times greater than in a Na^+ medium. For this reason, all measurements even with valinomycin, were carried out in a Na^+ containing medium since, under these conditions, it can be assumed that all cation fluxes observed are carried by the ionophore present in the membrane.

The proportion of multilayered vesicles in a population of LUV can be measured by the difference in the maximal proton release at high Amphotericin B concentration (Teerlink et al. 1980) and the total titratable protons after Triton X-100 addition. The proportion of internal volumes enclosed in multilayered vesicle strongly depends upon filtration of the vesicles. This proportion was found to decrease from 25–30% to 8–10% and to less than 3–4% on vesicles filtered using 0.8 μm, 0.4 μm and 0.2 μm pore sizes respectively.

Permeability induced by valinomycin

Figure 1 a shows a typical time course of the proton efflux elicited by valinomycin in presence of FCCP in vesicles filtered at 0.2 μm, obtained by the pH-stat method. The valinomycin/lipid molar ratio, *R*, varying from $5 \cdot 10^{-6}$ to 10^{-4}, corresponds to about 0.5 to 10 valinomycin molecules per vesicle. The

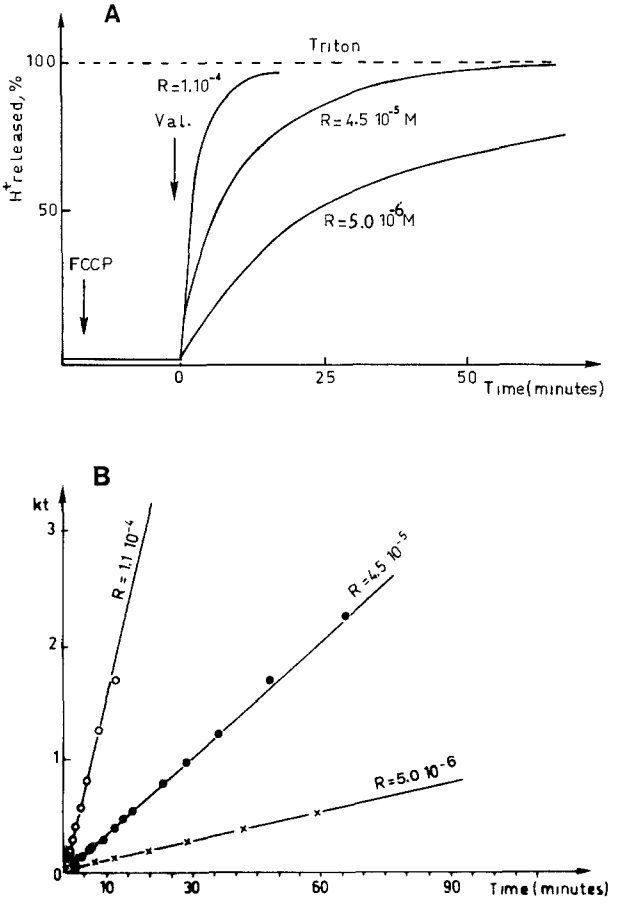

Fig. 1 A and B. Proton permeability of LUV's in the presence of valinomycin. **A.** typical time-course of proton efflux measured by pH-stat method for various valinomycin/lipid ratios (*R*); 100% proton release after addition of Triton X. **B.** Fits to the data of Fig. 1 a with the equation for k_t as a function of time. For explanation, see text

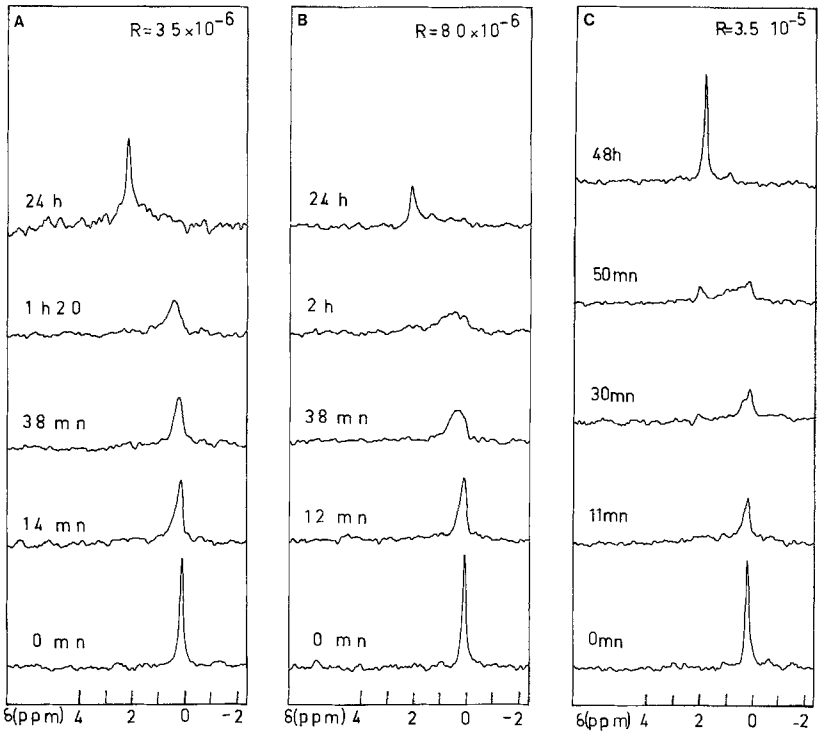

Fig. 2A–C. Proton permeability of LUV's in the presence of valinomycin measured by ^{31}P-NMR. Vesicles were filtered at: **A:** 0.2 μm, **B:** 0.4 μm, **C:** 1 μm. Indicated times are the midpoints of accumulation periods

proton efflux develops with a rate which depends upon valinomycin concentration but equilibrium (100% proton release) is always reached in the system (at this point vesicles destroyed by Triton X-100 do not release any more protons).

Experimental curves were analysed as a first-order kinetic permeability process, assuming that a mobile carrier works at its maximal rate under electroneutral exchange conditions in which no electrical parameter is involved. As shown previously (Thomas et al. 1983) the Na^+/H^+ exchange rate is measured by the net proton efflux, $J_{H^+} = K(H_i^+ - H_0^+)$ where H_i^+ and H_0^+ are the concentrations of free protons in the vesicular and external medium respectively. Taking into account the acid–base equilibrium of the phosphate buffer, noting that the external pH is kept constant, and letting α_0, α_t^0 and α_i represent the degree of ionization of the phosphate anions outside and inside at time $t = 0$ and at time t, by integrating the above equation for net proton efflux we obtain:

$$k_t = -\alpha_0(\alpha_0 - \alpha_t^0)\, x - \alpha_0^2 \ln(1-x)$$

where $x = (\alpha_t - \alpha_t^0)/(\alpha_0 - \alpha_t^0)$ and k_t is the rate constant. The results of the application of this equation are given in Fig. 1 b. An excellent fit is obtained for each experiment, with correlation coefficients of 0.999. The same excellent fit is obtained with vesicles filtered through 0.4 μm or even 1 μm pores. It was observed, however, that the flux rate decreased with increasing vesicle size, since flux rates depend strongly upon the surface/volume ratio of the vesicles.

Figures 2a, b and c, show typical NMR spectra obtained after addition of valinomycin to vesicles of various sizes (filtered through 0.2, 0.4 and 1.0 μm pores). Spectra were recorded in the presence of $MnCl_2$. The paramagnetic manganese ions, present only in the external medium, induce a shorter relaxation of nearby phosphate ions; the linewidth of the external medium phosphate signal in the presence of manganese ions is broadened to such an extent that it becomes undetectable under these conditions. In addition, the phosphate signal of the membrane phospholipids cannot be seen due to their low concentration and to their high correlation time.

Therefore, only signals arising from intravesicular phosphate ions are detected. Upon addition of valinomycin (in the presence of FCCP) the phosphate signal, initially centered at $\delta = +0.25$ ppm (which corresponds to pH 5.50) progressively shifts until it reaches $\delta = +2.20$ ppm, which corresponds to the equilibrium pH, equal to 7.50. Two important points must be stressed: firstly, the area of the internal phosphate signal remains unchanged during the transport process, indicating that phosphate ions do not leak out of the vesicles. This means that even at the highest concentration used ($3.5 \cdot 10^{-3} M$) valinomycin does not induce a detergent-like effect. It promotes only a Na^+ permeability which is compensated by proton efflux mediated by protonophore and a change of the internal pH occurs. Secondly, it can be seen that during its shift towards 7.50, the signal broadens significantly when compared to the

initial or final signal. This broadening reflects in part the fact that the recording time is not completely negligible in comparison with the flux rate; this results in a certain degree of "blurring" of the signal. But the broadening appears to be mostly due to a pH distribution which is the result of a distribution of the vesicle size and hence of a distribution of ion flux rates. This is shown in Figs. 2a to 2c. As expected, the flux rate, which strongly depends upon the surface/volume ratio, increases with decreasing mean vesicle diameter. In order to compare spectra at about the same flux rates, the molar ratio, valinomycin/lipid, was varied. In fact the phosphate signal can be clearly observed throughout the whole process only with vesicles filtered through 0.2 μm pores. With vesicles filtered through 1.0 μm pores the broadening is such that the signal rapidly disappeared into the noise. It is interesting to note that in this last case, the proportion of multilayered vesicles which are permeabilized by valinomycin at the slowest rate, is large enough to be distinguished. The changes occurring with time in the intravesicular pH can be monitored precisely only with vesicles filtered at 0.2 μm; the accuracy is poorer with vesicles filtered at 0.4 μm. The kinetic analysis of the proton–cation exchange rate measured by NMR in vesicles filtered at 0.2 μm yielded results comparable to those obtained by pH-stat method. However, the accuracy obtained using the NMR data is not as good as that obtained by the pH-stat method.

Permeability induced by amphotericin B and gramicidin

Figures 3a and b show time courses of proton efflux measured by the pH-stat method (using vesicles filtered through 0.2 μm pores) after addition of increasing concentrations of amphotericin B and gramicidin respectively. The curves obtained are quite similar for both antibiotics but at the same time are very different from that obtained with valinomycin. Upon ionophore addition the proton flux develops very rapidly until it reaches a plateau value which thereafter remains stable with time. The time to reach the plateau is practically the same at all ionophore concentrations and is usually less than 10 min. In contrast, the amount of protons released at the plateau, expressed as a percentage of the total intravesicular titratable protons obtained after Triton X-100 addition, increases with the ionophore concentration. 100% proton release is obtained only at very high ionophore concentration, that is at ionophore/lipid molar ratios, R, greater than $5 \cdot 10^{-3}$ for amphotericin B and 10^{-3} for gramicidin. None of these curves can be analysed in terms of first-order

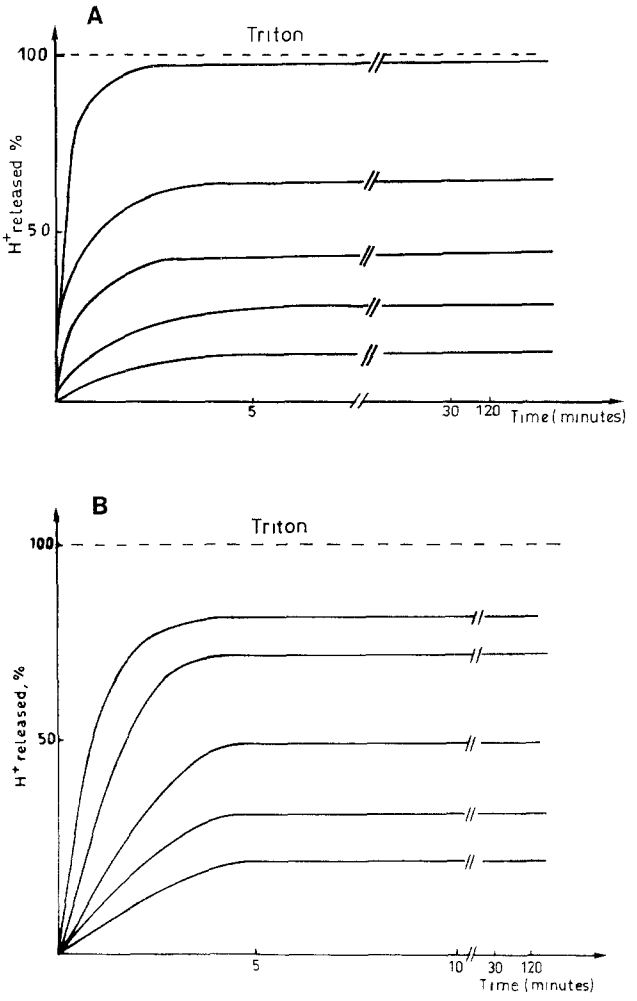

Fig. 3A and B. Proton permeability of LUV's in the presence of channel-forming antibiotics. **A.** Typical time-course of proton efflux measured by pH-stat method for amphotericin/lipid ratios (R) ranging from 10^{-5} to $5 \cdot 10^{-3}$. **B.** Time course of proton release in the presence of gramicidin D for R ranging from 10^{-5} to 10^{-3}

kinetics as was done in the case of valinomycin. These results can be interpreted as follows: upon ionophore addition, two vesicle populations are formed, the relative proportion of which depends upon the concentration of ionophore: one population of very rapidly permeabilized vesicles in which the internal pH is equal to the pH of the medium and another, which apparently is not permeabilized at all and keeps its initial pH. Such a situation should be easily detected by the NMR method.

The corresponding NMR spectra obtained on vesicles filtered through 0.2 μm pores are shown in Figs. 4a and 4b for amphotericin B and gramicidin respectively. These spectra are strikingly different from those obtained previously with valinomycin. After ionophore addition the internal phosphate signal rapidly splits into two parts: one remains

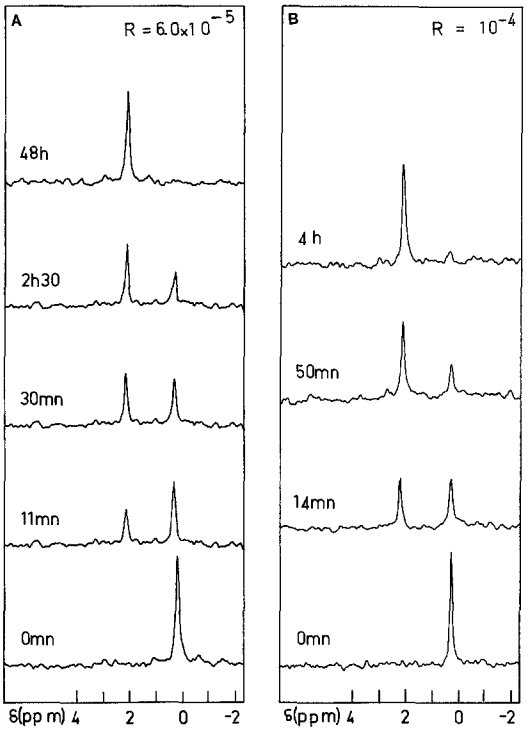

Fig. 4 A and B. Proton permeability in the presence of channel-forming ionophores measured on LUV's by ^{31}P-NMR. **A.** amphotericin B at $R = 6 \cdot 10^{-5}$; **B.** gramicidin D at $R = 10^{-4}$. Indicated times are the midpoints of the accumulation period

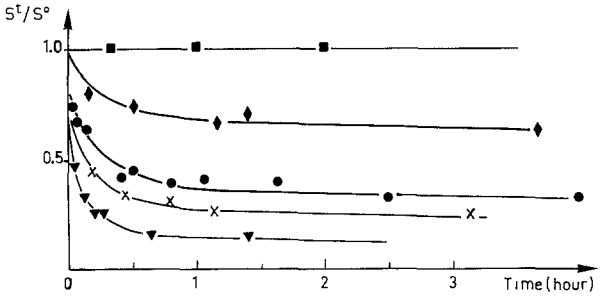

Fig. 5. Time-course of the disappearance of the initial pH signal in LUV's in the presence of amphotericin B. Ordinate: S_t/S_0, where S_t is the area of the signal measured at the time t, and S_0 is the area measured before amphotericin B addition. R ranging from $3 \cdot 10^{-6}$ to $2 \cdot 10^{-4}$

centered at $\delta = +0.25$ ppm corresponding to the initial pH value, and the other is centered at $\delta = 2.20$ ppm corresponding to the equilibrium pH value. At longer times, the signal at $\delta = +0.25$ ppm slowly decreases in intensity whereas the second signal increases and eventually the internal signal is completely lost. Within the limit of the accuracy of the measurement, the sum of the intensities of the two signals was always approximately equal either to the initial signal intensity, or to the final signal at equilibrium.

No signals were detected between 0.25 ppm and 2.20 ppm. In contrast with the situation observed with valinomycin, there were no progressive shifts nor a broadening of the phosphate signal. The relative proportion of the two signals at the beginning of the experiment, and also the rate with which this proportion varies with time, depend upon ionophore concentration. In Fig. 5, the decrease in intensity of the signal at $\delta = +0.25$ ppm is given as a function of time at different amphotericin B concentrations. One observes a rapid decrease followed by a much slower phase. The same result is obtained with gramicidin. These time courses are, in general, consonant with the curves in Figs. 4a and b, for the proton efflux measured by the pH-stat method. However, it is clear that the stability of the plateau value observed in the pH-stat experiment was only apparent. In fact, the proton flux keeps going but with a rate too slow to be measured using the pH-stat method. Therefore, some corrections have to be introduced to the interpretation given above on the basis of pH-stat measurements. Amphotericin B and gramicidin induce in vesicles a permeability so intense that pH equilibration is reached in too short a time to allow intermediate states to be observed by NMR spectroscopy. However, at low ionophore concentrations and shortly after its addition, this process of permeability is incomplete and results in an heterogeneous situation: at the beginning only a part of the vesicle population, which increases with increasing ionophore concentration, is involved in the permeability process. The remaining part will be permeabilized but at a much slower rate.

An important point should be stressed. The mixing procedure of a small volume of concentrated ionophore with a large volume of vesicle suspension, does not seem to be the source of the heterogeneity. When vesicles are preincubated with ionophore for a time which allows for the ionophore distribution in the vesicle suspension before establishing the proton gradient, the results obtained are not significantly different.

Discussion

As compared to the very sensitive and accurate pH-stat method of monitoring proton fluxes across the vesicular lipid bilayer, the ^{31}P-NMR method seems to be very slow and inaccurate. Nevertheless, the NMR method provides information which could not be obtained otherwise and which is essential for the correct interpretation of the data obtained by the pH-stat method. This information derives from the ability of NMR to simultaneously monitor both total proton concentration and the pH distribution. The

NMR method also allows subpopulations of vesicles to be distinguished on the basis of internal pH.

Data obtained using the NMR method verify that the ionic fluxes measured are the result of true permeability induced by ionophores and not due to vesicle destruction by any detergent-like effects. The advantage of the NMR method is that it allows this important point to be checked on the same sample in which actual permeability measurements were made.

The second important source of information derived from NMR method, concerns the heterogeneity of the vesicle population subjected to ionophore action. In the present work it has been shown that this heterogeneity has two different origins: heterogeneity of vesicle size, related to the way vesicles are prepared and the apparent heterogeneity of the ionophore-vesicle interaction, related to the mode of action.

The distribution of sizes of the vesicles prepared by reverse phase evaporation is known to be very large. However filtration through porous membranes of definite pore size efficiently reduces this heterogeneity (Szoka and Papahadjopoulos 1980). It has been shown recently by dynamic laser light scattering that after filtration through 0.2 μm pores, vesicles are fairly homogeneous with a mean diameter of 0.16 ± 0.01 μm; this result was confirmed by freeze fracture examination and calculation of the expected internal volume (Louni et al. 1983).

The influence of vesicle size heterogeneity on permeability development by valinomycin is shown in the NMR spectra of Fig. 2. The fact that the broadening of the intravesicular phosphate signal was marked even in the case of relatively uniform vesicles, demonstrates the great sensitivity of the NMR method in terms of permeability distribution according to vesicle size, at least as long as the driving forces of ion fluxes are large (permeability distribution means that the pH gradient disappears with different rates in vesicles of different sizes).

The size heterogeneity effect was not detected in the pH-stat measurements. The fitting of experimental time courses of proton efflux to those calculated (Fig. 1 b) according to the permeability equation was almost as good for vesicles uniform in size, as for heterogeneous ones. This is expected if the vesicle size distribution is Gaussian.

The permeability distribution is not only related to the heterogeneity in vesicle size but also to the distribution of ionophore molecules in the vesicle population. The latter effect seems to be less important for carrier type ionophores than for channel-forming ones. The flux rate induced by any ionophore depends on two factors: the number of functioning permeability units per vesicle and the ion transporting efficiency of these units.

In the case of valinomycin even at the lowest antibiotic-lipid ratio (corresponding to about 0.5 valinomycin molecules per vesicle) all vesicles in the population seem to be immediately involved in the permeability process (Fig. 2). According to the statistical distribution law the majority of vesicles have no valinomycin molecules in the membrane. Progressive discharge of the proton gradient in the whole population suggests that valinomycin molecules are exchanged between vesicles relatively quickly compared to the ion flux rate which they induce. In this case the limiting factor is not the ionophore distribution but its ion transporting efficiency. (It is measured by the kinetics of proton flux which is a first-order rate process).

The situation is completely different in the case of channel-forming ionophores. The rate of ion translocation mediated by gramicidin is three orders of magnitude higher than that mediated by valinomycin (McLaughin and Eisenberg 1975). Even with only one pore per vesicle, equilibration of the ion gradients is extremely fast. Results obtained for low concentrations of amphotericin B (Figs. 4a and 5) and gramicidin D (Fig. 4b) show, that, only part of the vesicle population goes to equilibrium very fast. This phenomenon reflects the statistical distribution of the ionophore molecules among the vesicle population: only part of the population has enough ionophore molecules to form permeability units. This step is observed in the short time experiments carried out by the pH-stat method (Fig. 3) or by fluorescence (Clement and Gould 1981 b). NMR experiments show that on a longer time scale the proton gradient is discharged in the whole population of vesicles. The permeability induction is slower but it is always an all-or-none process and no intermediate steps are observed. This slower phase reflects the rate of formation of new permeating units. The low rate of new pore formation may be due to a slow rate of exchange of ionophore molecules between vesicles or a low probability of meeting a sufficient number of molecules in a condition appropriate for their association into a multimolecular structure, or both. It can be concluded that in the case of channel-forming ionophores the rate of proton efflux, measured by pH-stat or NMR methods, reflects the rate of formation of permeating units and not their ion transporting efficiency.

The all-or-none process observed for channel-forming ionophores agrees with the unit conductance concept first established by Hladky and Haydon (1972). On the black film system, the channel-forming ionophores are easily distinguished from the mobile carriers by their unit conductance characteristics. In the vesicular system, channel-forming ionophores may be distinguished from the

mobile carriers not only by the very high permeability of these channels (which allows a very fast equilibrium of the vesicular content), but also by the slowness of the redistribution between vesicles of ionophore molecules and of their organization into channels.

Finally, the results reported here indicate that in the interpretation of the permeability experiments carried out on cells to test the antibiotic activity of ionophores, the mode of permeability induction should be also taken into account, especially when the antibiotic activity is measured using dose-response curves.

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