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TRANSFER OF THE POLYENE ANTIBIOTIC AMPHOTERICIN B BETWEEN SINGLE-WALLED VESICLES OF DIPALMITOYLPHOSPHATIDYLCHOLINE AND EGG-YOLK PHOSPHATIDYLCHOLINE

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Amphotericin B transfer between single-walled vesicles of dipalmitoylphosphatidylcholine (DPPC) and of egg phosphatidylcholine, both containing 10 mol% cholesterol, has been studied concurrently by circular dichroism spectroscopy and permeability measurements. At 22°C amphotericin B is rapidly transferred from DPPC to DPPC vesicles as well as from egg phosphatidylcholine to egg phosphatidylcholine vesicles. On the other hand, although amphotericin B is rapidly transferred from egg phosphatidylcholine to DPPC vesicles, it is not transferred from DPPC to egg phosphatidylcholine vesicles. At 48°C, above the transition temperature of DPPC, transfer occurs rapidly both ways. These results are interpreted in terms of difference of association constant of amphotericin B with vesicle membranes in the gel and liquid-crystalline state.

Introduction

Polyene macrolides' interaction with cell membrane, which leads to permeability changes and eventually to membrane disruption, is a complex phenomenon. In the case of amphotericin B, the molecular basis of this action seems to be the formation by amphotericin B-sterol complexes of aqueous channels [1–3] which can exist in a closed or open state with many transitions between these two functional states [4]. Due to the very low aqueous solubility of amphotericin B, which results in its aggregation in aqueous medium, the primary events of the interaction with membranes are in themselves complex and difficult to study: as a matter of fact, spectroscopic studies by electronic absorption [5] or circular dichroism [6] have shown the existence of intermediate steps presumably involving adsorption at the membrane surface. In order to focus on the amphotericin B complexes responsible for membrane permeability, it appears convenient to study the exchange of amphotericin B between membranes, a phenomenon which has been proved to take place by van Hoogevest and de Kruijff [7], thus avoiding the complexity of the primary interaction between amphotericin B aggregates in solution and membranes.

In the present report, the exchange of amphotericin B between unilamellar vesicles made of either dipalmitoylphosphatidylcholine (DPPC) or egg phosphatidylcholine containing 10 mol% cholesterol has been studied concurrently by two different methods: circular dichroism (CD) spectroscopy, which has been proved to be very sensitive to amphotericin B conformational changes and makes possible the observation of numerous species [6], and permeability measurements, which allow us to distinguish among these numerous observable species those which are effectively responsible for permeability induction. As will be shown, the combination of the two types of result

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Abbreviations: DPPC, dipalmitoylphosphatidylcholine; DMSO, dimethyl sulphoxide.

leads to useful information concerning the association between amphotericin B and membranes and the condition of pore formation.

Materials and Methods

L-α-Phosphatidylcholine from egg yolk was prepared according to the method of Patel and Sparrow [8]. L-α-Dipalmitoylphosphatidylcholine (DPPC) was supplied by Sigma, cholesterol by Fluka, FCCP (carbonylcyanide-*p*-trifluoromethoxyphenylhydrazone) by Boehringer and amphotericin B by Squibb France.

Vesicle suspensions were prepared by dissolving weighed amounts of phospholipids and cholesterol in chloroform. After removal of this solvent in vacuo, the appropriate buffer solution was added to give the desired final concentration of lipid and the sample was quickly vortexed and sonicated to clearness under nitrogen, the temperature being maintained above the transition temperature of the phospholipid.

It is known that vesicles prepared this way are predominantly unilamellar. In our particular case this has been checked by ^{31}P-NMR spectroscopy [9]. Assuming that the vesicles are about 22 nm in diameter and made of about 2000 lipid molecules [10,11], the concentration of amphotericin B can be expressed as number of molecules per vesicle, as well as in mol per mol lipid.

Results

Circular dichroism studies

It has been already shown [6] that the amphotericin B spectrum in aqueous solution depends upon its concentration: namely, above the critical micelle concentration (10^{-7} M) a strong dichroic doublet at 319 nm appears which is characteristic of amphotericin B aggregates. Upon addition of vesicles, this signal disappears simultaneously with the appearance of the characteristic spectrum of amphotericin B in vesicles. The rate of transfer from solution to vesicles depends upon concentration and, at a high amphotericin B/vesicle ratio, can take up to 1 h to be completed. In all the experiments described below, it was ensured, by monitoring its specific signal, that there was no amphotericin B present in aggregated form in solution during the exchange between vesicles.

It has been already shown [6], that amphotericin B in phospholipid vesicles exhibits distinct spectra depending upon its concentration and the physical state of the phospholipid (gel or liquid crystalline state). In the case of phospholipid in the gel state (such as DPPC with or without cholesterol at 22°C), at a concentration of amphotericin B of $0.5 \cdot 10^{-3}$ mol/mol lipid (one molecule per vesicle) a positive peak is observed ($\Delta\epsilon = +8$) at 417 nm and for 25 amphotericin B molecules per vesicle ($12.5 \cdot 10^{-3}$ mol/mol) this positive peak ($\Delta\epsilon = +22$) is observed at 420 nm. In the liquid-crystalline state (with egg phosphatidylcholine, for instance) a peak is observed at 415 nm ($\Delta\epsilon = +8$) with one amphotericin B molecule per vesicle, and at 420 nm ($\Delta\epsilon = -30$) with 25 amphotericin B molecules per vesicle. On the basis of these differences the transfer of amphotericin B from vesicle to vesicle can be monitored.

In the following experiments, egg phosphatidylcholine vesicles contained 10% molar cholesterol (unless otherwise indicated); DPPC vesicles contained either 0 or 10% cholesterol. They were prepared in a $5 \cdot 10^{-2}$ M Tris-HCl buffer, pH 7.5, and CD spectra were recorded as previously described [6]; all experiments were carried out at 22°C.

Exchange of amphotericin B between vesicles in the same physical state

Vesicle suspensions were loaded with ten amphotericin B molecules per vesicle ($5 \cdot 10^{-3}$ mol/mol lipid), adding the desired amount of its $1 \cdot 10^{-3}$ M solution in DMSO. The spectra were recorded and then a suspension of the amphotericin-free suspension of vesicles of the same nature was added in such a way that the final concentration of amphotericin B decreases to 0.25 mol/mol (0.5 molecule per vesicle). This experiment has been carried out with egg phosphatidylcholine vesicles (liquid crystalline state) and with DPPC vesicles (gel state). The cholesterol content of DPPC vesicles has no influence. In both cases (Fig. 1) the initial spectrum typical of a high amphotericin B/vesicle ratio is transformed rapidly into a spectrum typical of a ratio lower than 1. Therefore amphotericin B distributes itself between all vesicles present. The half-time for the exchange was found to be about 30 s (insert Fig. 1).

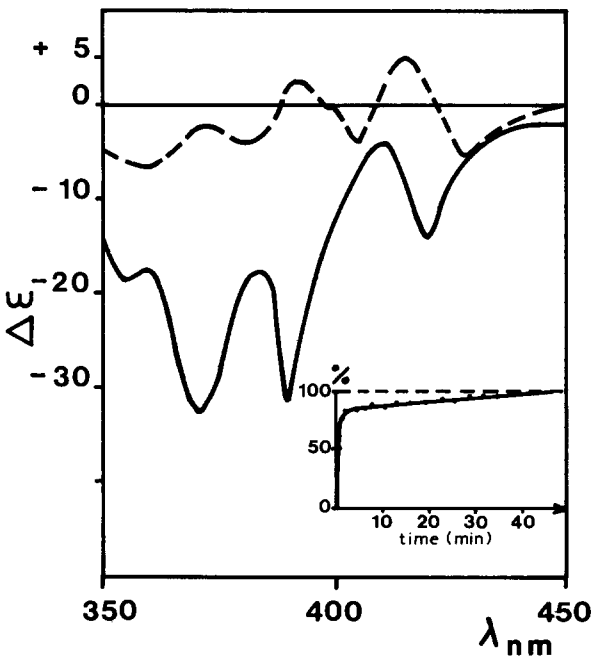

Figure 1

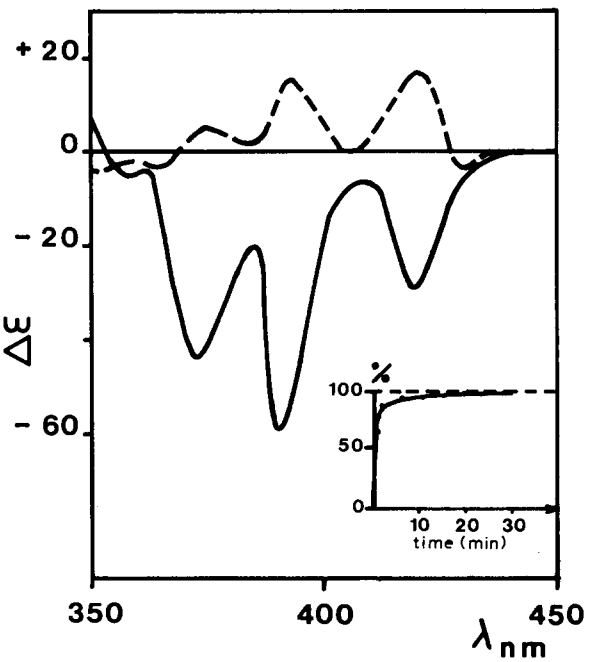

Figure 2

Fig. 1. Amphotericin B exchange between egg phosphatidylcholine 10 mol% cholesterol vesicles at 22°C: ———, CD spectrum of $5 \cdot 10^{-6}$ M amphotericin in 1 mM vesicle suspension (ten molecules/vesicle); - - - - - -, CD spectrum obtained 1 h afterwards, using 1 vol. of the precedent suspension with 1 vol. 20 mM vesicle suspension (final concentration: 0.5 amphotericin B molecules/vesicle). $\Delta\epsilon$, differential molar dichroic absorption coefficient (10^3 cm^2 · mol^{-1}). Inset: rate of appearance of the second spectrum, as percentage of its final amplitude.

Fig. 2. Amphotericin B exchange between vesicles in different physical states at 22°C. First experiment: ———, CD spectrum of $2 \cdot 10^{-4}$ M amphotericin B in 10 mM egg phosphatidylcholine 10% cholesterol vesicle suspension (100 molecules/vesicle). - - - - - -, CD spectrum obtained 1 h after mixing 1 vol. of the preceding suspension with 1 vol. 10 mM DPPC vesicle suspension. Inset: rate of appearance of the second spectrum in percentage of its final amplitude. Second experiment: - - - - - -, CD spectrum of $5 \cdot 10^{-6}$ M. Amphotericin B in 1 mM DPPC vesicle suspension; 1 h after mixing 1 vol. of the preceding suspension with 1 vol. 10 mM egg phosphatidylcholine 10% cholesterol vesicle suspension the CD spectrum remains identical. $\Delta\epsilon$, differential molar dichroic absorption coefficient (10^3 cm^2 · mol^{-1}).

Exchange of amphotericin B between vesicles in different physical states

In a first series of experiments, a suspension of DPPC vesicles (in the gel state) was added to a suspension of amphotericin B-loaded egg phosphatidylcholine vesicles ($10 \cdot 10^{-3}$ mol/mol; 20 molecules/vesicle) (in the liquid-crystalline state). These experiments were carried out with three phospholipid concentrations: 0.1, 1 and 10 mM. In the three cases, one observes the rapid transformation (half-time about 30 s) of the spectrum typical of amphotericin B in vesicles in the liquid-crystalline state into the spectrum of amphotericin in vesicles in the gel state (Fig. 2). The kinetics are concentration-dependent and at a concentration of 10 mM phospholipids, a two-step process is observed. The cholesterol content of DPPC vesicles has no influence.

In a second series of experiments, the reverse was done: a suspension of egg phosphatidylcholine vesicles was added to DPPC vesicles preloaded with 20 amphotericin B molecules/vesicle. In this case no detectable change was observed in the spectrum, which remained typical of amphotericin in phospholipid in the gel state. The same result was obtained in absence of cholesterol in the vesicle.

Permeability studies

Sonicated vesicles of either egg phosphatidylcho-

line or DPPC are rigorously impermeable to hydrophilic ions such as phosphate or sulphate and it has been previously shown by ^{31}P-NMR spectroscopy that in phosphate buffer these vesicles are able to maintain more than a 4 pH unit difference across their membranes for several hours or several days, depending upon lipid composition and temperature [9]. It must be pointed out that, due to their impermeability to hydrophilic anions, any cation movement across the vesicle bilayer can take place only by strictly electroneutral exchange. The addition of a proton carrier such as FCCP to vesicles submitted to a large proton gradient does not result in any net proton flux: another path must be provided for the exchanging cation. Under these conditions, the permeability to cation induced by amphotericin B on vesicles submitted to a pH difference between intravesicular and external medium can be conveniently measured by monitoring the proton flux occurring in exchange through FCCP, provided this proton flux is not rate limiting, and this condition is easily fulfilled, at least at low and moderate concentrations of amphotericin B.

The vesicles used in the following experiments carried out on this basis were obtained by sonicating a mixture of 27 μmol phospholipid and 3 μmol cholesterol in 1 ml 400 mM sodium phosphate buffer, pH 5.20. After sonication, the vesicle suspension was dialysed at equilibrium against 500 vol. isotonic sodium sulphate. The proton flux was measured using pH-stat equipment (Radiometer) as follows:

1 ml dialysed vesicles suspension was diluted with 3 ml dialysing medium in the titrating vessel of the pH-stat. After equilibration under a constant nitrogen stream, the pH of the suspension was adjusted to 7.40. Thus a pH difference of 2.2 units was established between intravesicular and external medium. 20 μl of a $1 \cdot 10^{-2}$ M FCCP solution in ethanol was added. Then amphotericin B was added and the proton efflux elicited was measured against time as the amount of a 4 mM NaOH solution in isotonic sulphate necessary to maintain pH 7.40.

Amphotericin B was introduced according to the two following procedures:

Procedure 1: directly as 60 μl of a 1 mg/ml solution in DMSO, that is, about $2 \cdot 10^{-3}$ mol/mol (four amphotericin B molecules per vesicle);

Procedure 2: at the end of the experiment carried out according to procedure 1, the pH equilibrium being reached between intravesicular and external medium, the vesicle suspension was withdrawn and stored in a syringe. A new vesicle suspension was prepared as before, including FCCP addition, and the syringe contents were added to it. Therefore the final concentration of amphotericin B thus added as preloaded vesicles was only $1 \cdot 10^{-3}$ mol/mol lipids (two molecules per vesicle).

It has been checked in preliminary experiments that: (1) in the absence of ionophore, the proton efflux is practically negligible, amounting at most to about 0.1–1% of the total titratable protons per h, depending upon lipid composition and temperature; (2) FCCP added alone is not able to promote any significant increase of the basic flux even at concentrations as high as $1 \cdot 10^{-3}$ M; (3) the proton efflux mediated by FCCP in presence of amphotericin B is maximal and independent of FCCP concentration above $2 \cdot 10^{-5}$ M; (4) the total amount of titratable proton was measured after Triton X-100 addition: the intravesicular volume calculated on this basis was found to be equal to 1.3 ± 0.2% of the volume of the stock vesicle suspension. Under the influence of FCCP plus amphotericin B, more than 95% of this amount is titratable.

Exchange of amphotericin B between vesicles in the same state

The effects at 22°C of amphotericin B added according to procedures 1 and 2 are described in Figs. 3 (for DPPC vesicles) and 4 (for egg phosphatidylcholine vesicles). The proton efflux observed is given as percentage of the total titratable protons (after Triton X-100 addition) versus time. The No. 1 curves of Figs. 3 and 4 represent the proton efflux obtained respectively on DPPC and egg phosphatidylcholine vesicles by direct addition of amphotericin B according to procedure 1. The No. 2 curves in the same figures represent the proton efflux obtained by addition, according to procedure 2, of preloaded DPPC and egg phosphatidylcholine to vesicles of the same nature.

In both cases, a comparable flux rate is obtained, which demonstrates, as previously shown by Hoogevest and de Kruijff [7], that amphotericin B can be transferred between vesicles of the same lipid composition. The proton efflux obtained by procedure 2 is

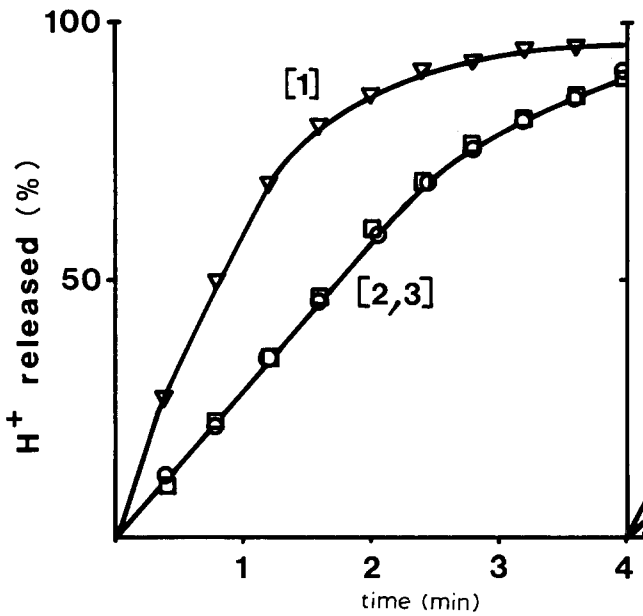

Figure 3

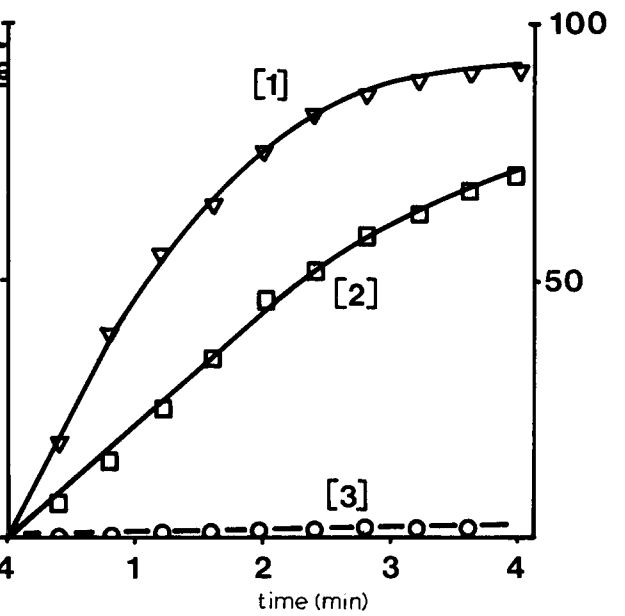

Figure 4

Fig. 3. Proton release from DPPC 10% cholesterol vesicles at 22°C. Curve 1: direct addition of amphotericin B; curve 2: addition of amphotericin B preloaded DPPC 10% cholesterol vesicles; curve 3: addition of amphotericin B preloaded egg phosphatidylcholine 10% cholesterol vesicles. Amphotericin B concentration, $2 \cdot 10^{-3}$ mol/mol lipid. Ordinate: percentage of total titratable proton released.

Fig. 4. Proton release from egg phosphatidylcholine 10% cholesterol vesicles at 22°C. Curve 1: direct addition of amphotericin B; curve 2: addition of amphotericin B preloaded egg phosphatidylcholine-10% cholesterol vesicles; curve 3: addition of amphotericin B preloaded DPPC-10% cholesterol vesicles. Amphotericin B concentration, $2 \cdot 10^{-3}$ mol/mol lipid.

slower than by procedure 1. This difference is at least partly ascribable to the difference in the number of amphotericin molecules present per vesicle, which in procedure 2 is half of that in procedure 1.

Exchange of amphotericin molecules between vesicles in different physical states

The results of this experiment, carried out by procedure 2 is described in the No. 3 curves of Figs. 3 and 4. The No. 3 curves of Fig. 3 represent the efflux obtained when at 22°C preloaded egg phosphatidylcholine vesicles are added to DPPC vesicles; it can be seen that this efflux is not significantly different from the efflux obtained by transfer of amphotericin B from DPPC to DPPC vesicles. The reverse experiment is described by curve 3 in Fig. 4. It can be seen that when preloaded DPPC vesicles are added to egg phosphatidylcholine vesicles no proton efflux occurs. Therefore the transfer of amphotericin B is not symmetrical; it occurs from egg phosphatidylcholine to DPPC vesicles but not from DPPC to egg phosphatidylcholine vesicles.

The transfer experiments were then performed at 48°C, that is above the transition temperature of DPPC. The resulting proton fluxes are described in Figs. 5 and 6. In Fig. 5, curve 1 describes the proton flux obtained on DPPC by direct addition (procedure 1) and curve 2 by addition of preloaded egg phosphatidylcholine vesicles (procedure 2). In Fig. 6, curve 1 describes the proton flux obtained on egg phosphatidylcholine vesicles by direct addition, curve 2 that obtained by addition of preloaded DPPC vesicles. At this temperature, the proton efflux obtained is the same in both cases and the transfer of amphotericin B occurs from DPPC to egg phosphatidylcholine as well as from egg phosphatidylcholine to DPPC vesicles. This result demonstrates that DPPC vesicles in the liquid-crystalline state are able to trans-

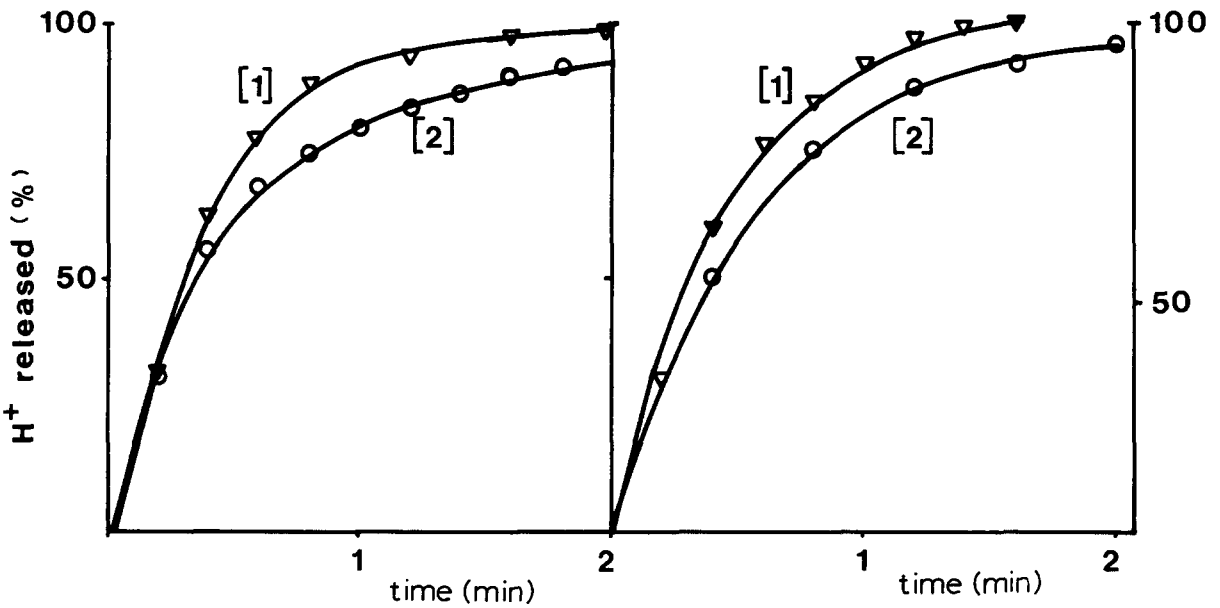

Figure 5

Figure 6

Fig. 5. Proton release from DPPC 10% cholesterol vesicles at 48°C. Curve 1: direct addition of amphotericin B; curve 2: addition of amphotericin B preloaded egg phosphatidylcholine 10% cholesterol vesicles. Ordinate: percentage of total titratable proton released. Amphotericin B concentration, $2 \cdot 10^{-3}$ mol/mol lipid.

Fig. 6. Proton release from egg phosphatidylcholine 10% cholesterol vesicles at 48°C. Curve 1: direct addition of amphotericin B; curve 2: addition of amphotericin B preloaded DPPC 10% cholesterol vesicles. Amphotericin B concentration, $2 \cdot 10^{-3}$ mol/mol lipid.

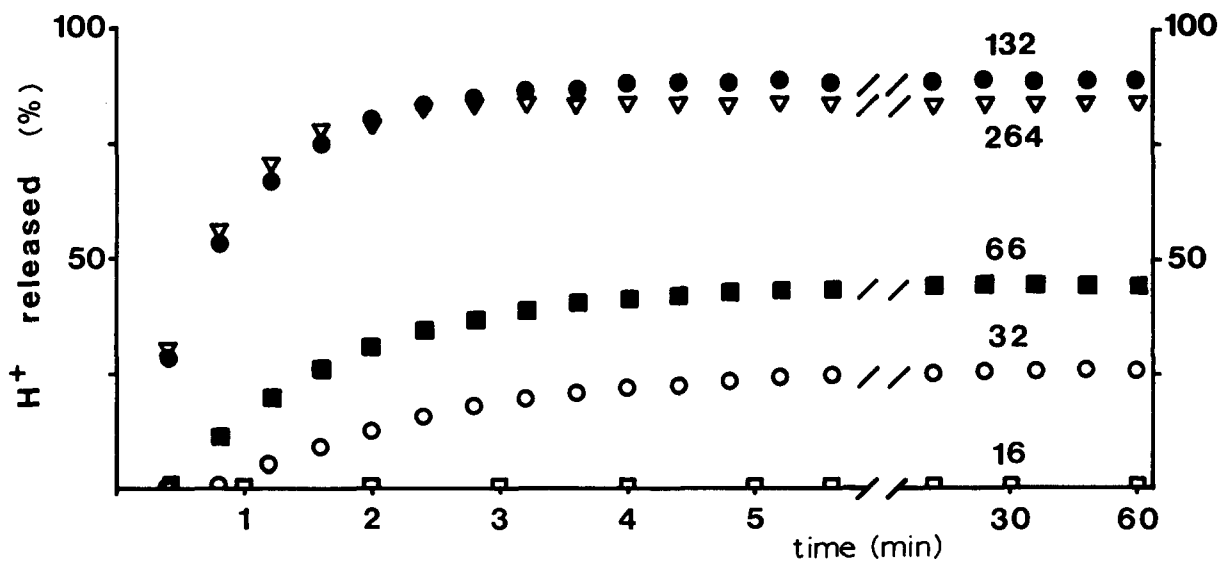

Fig. 7. Proton release from egg phosphatidylcholine 10% cholesterol vesicles by addition of DPPC 10% cholesterol vesicles preloaded with increasing amount of amphotericin B. Temperature, 22°C. The figures indicate the number of amphotericin B molecules/DPPC vesicle. Ordinate: percentage of total titratable proton.

fer amphotericin B to egg phosphatidylcholine vesicles.

Exchange experiments between preloaded DPPC vesicles (in the gel state, at 22°C) and egg phosphatidylcholine vesicles were carried out at a function of amphotericin B molecules/DPPC vesicle ratios. In these experiments it was ensured by circular dichroism spectroscopy that even at ratios as high as 240, no amphotericin aggregate was present in the aqueous medium. The results are given in Fig. 7. It seems that proton efflux begins to appear beyond 32 amphotericin B/DPPC vesicle. With a further increase of this ratio, the proton efflux rate increases, as well as the percentage of titratable protons which can be released, this reaching 100% for a ratio of 132 ($66 \cdot 10^{-3}$ mol/mol lipid).

The exchange can possibly proceed in two different ways: either through amphotericin B molecules in the aqueous medium or by direct contact between vesicles during collision, which in turn might induce fusion. Several experiments have been carried out in order to test these possibilities.

(1) Exchange experiments were studied as a function of vesicles dilution, from 0.5 to 15 μmol lipid per ml (the lipid concentration routinely used being 7.5 mol/ml). The results obtained both by spectroscopic and permeability methods are not significantly affected by the dilution in this range.

(2) Exchange experiments between DPPC or egg phosphatidylcholine vesicles negatively charged by addition of 5 mol% of phosphatidic acid: the only difference with uncharged vesicles appears to be a slight increase in the half-time of the proton release, both in the direct and 'indirect' (exchange) experiments.

(3) Fusion experiments: it is known that DPPC vesicles below the transition temperature undergo aggregation and fusion. As compared with the exchange process, which is completed within 1–2 min the fusion process is very slow, taking place over several hours and even days [12]. However, amphotericin B might act as fusogen; fusion between DPPC vesicle at 22°C was followed comparatively in the absence and presence of amphotericin B, using the methods of Wilschut and Papahadjopoulos [13, 14].

The increase in the fluorescence signal due to the complexation of terbium ions (in one vesicle population) by dipicolinic acid (in the other vesicle population) upon mixing of the two vesicle populations was found to be 4–5% of the maximum fluorescence obtained by detergent addition, after 90 min. Amphotericin B addition up to $20 \cdot 10^{-3}$ mol/mol lipid, either directly as DMSO solution, or indirectly (preloading either one of the two populations) does not result in a significant increase in the fluorescence signal.

Therefore the negative results obtained in the three types of experiment indicate that under our experimental conditions, amphotericin B exchange cannot be accounted for in terms of direct contact and fusion of vesicles.

Discussion

The results obtained by circular dichroism spectroscopy and permeability measurements demonstrate that the exchange of amphotericin B is relatively fast between vesicles in the same physical state, but is clearly asymmetrical between vesicles in different states. The fact that the state of the lipids is involved rather than a more specific interaction related to the different chemical nature of the hydrocarbon chains is shown by the experiments carried out at 48°C, a temperature at which no significant difference is observed between the exchange of amphotericin B from egg phosphatidylcholine to egg phosphatidylcholine or DPPC to DPPC on the one hand and from egg phosphatidylcholine to DPPC or DPPC to egg phosphatidylcholine on the other.

Between vesicles in the same state, the exchange occurs rapidly, with a half-time of about 30 s as seen by the evolution of CD spectra. This rate is quite compatible with the development of proton fluxes observed under the same conditions. This result confirms and expands the results already obtained by van Hoogevest and de Kruijff [7] on vesicles in the liquid-crystalline state.

This exchange seems to occur through the aqueous medium. It has been shown on black films [15] that the membrane/solution equilibration of amphotericin B is fast. It must be pointed out that since the CD signal characteristic of amphotericin B aggregates in solution is not detectable under our conditions, the amphotericin B concentration in solution is not greater than $1 \cdot 10^{-7}$ M, which means that the pool of

amphotericin B molecules in solution is small as compared to the total amount present.

Therefore, when amphotericin B is added to vesicles either directly as DMSO solution or by exchange, it distributes itself rapidly and evenly throughout all the vesicles present, and this is confirmed by the evolution of the CD spectra. However, an important feature of the proton effluxes induced by exchange is that, at low amphotericin B/vesicle ratios, this proton efflux is not only slower, but imcomplete, involving only a fraction of the titratable protons, that is only a fraction of the vesicle population. This can be explained only by considering, in accordance with the now classical pore model [3], that much more than one amphotericin molecule is required in one vesicle to elicit permeability; therefore, as the dilution of amphotericin B increases, the probability of having enough molecules per vesicle, and of pore formation, becomes vanishingly small and the proton efflux stops.

The exchange of amphotericin B between vesicles in different physical states is found to be asymmetrical: although at 22°C amphotericin in egg phosphatidylcholine vesicles is rapidly transferred to DPPC vesicles, the reverse is not true, at least at low amphotericin B/vesicle ratios. However, transfer occurs when this ratio increases. In order to interpret these results, one has to consider that the binding of amphotericin with phospholipids in the gel state is much higher (about 200-times) than with phospholipid in the liquid crystalline state, as has already been shown for nystatin [16].

Any kinetic effect seems to be excluded: Chen and Bittman [5] have shown that, on the contrary, the initial rate of association decreases as the interaction between phospholipid hydrocarbon chains within the vesicles becomes stronger, namely, when passing from the liquid crystal state to the gel state.

Finally, the comparison of CD spectra and permeability measurements allows a useful distinction to be made among the former: the species observed either by electronic absorption [5] or by CD [6] are numerous and their evolution in time complex. It appears that the species recorded by spectroscopy in the very first moment of the reaction of amphotericin B with vesicles are not responsible for the induced permeability; on the contrary, those presented here are effectively related to pore formation. A remarkable point is that two clearly distinct spectra are obtained for DPPC in the gel state and for egg phosphatidylcholine. This might correspond to two different types of ionophoretic entity. The proton efflux measurements presented here give no information about this possible difference: however, work in progress in our laboratory seems to indicate that there are differences in the selectivity of the pores induced in the two phospholipids.

Acknowledgment

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