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Incorporation of amphotericin B into large unilamellar vesicles composed of phosphatidylcholine and phosphatidylglycerol

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The spontaneous incorporation of the polyene antibiotic amphotericin B from a micellar solution into phospholipid vesicles was examined as a function of the lipid composition of the vesicles and their physical state. Virtually no insertion of the antibiotic into egg phosphatidylcholine vesicles was observed even when cholesterol was also present in the bilayer. In contrast, rapid incorporation occurred into systems containing an anionic phospholipid such as phosphatidylglycerol or phosphatidylserine with the fastest rates observed for lipids containing the saturated dimyristoyl fatty acyl species. Insertion of amphotericin B into vesicles composed of dimyristoylphosphatidylcholine and dimyristoylphosphatidylglycerol (7:3 mole ratio) was rapid either above, below or within the gel-to-liquid-crystalline phase transition temperature (23°C). The ability of amphotericin B to intercalate into lipid vesicles is discussed in relation to their relative bilayer stabilities.

Keywords: antibiotic; vesicles; amphotericin B, phosphatidylcholine; phosphatidylglycerol.

Introduction

The polyene antibiotic amphotericin B is believed to exert its antifungal activity by forming a complex with ergosterol present in the fungal membrane, which then acts as a selective pore inducing ion conductance and ultimately cell lysis [1]. While the complex, which is reported to consist of a circular array of eight antibiotic and sterol molecules in an alternating sequence [2], can be formed with cholesterol, the selective toxicity of amphotericin B for fungi is believed to result from its greater affinity for ergosterol, which is found exclusively in these organisms [3,4]. Consistent with the formation of such a membrane complex, only low levels of antibiotic binding to sterol-free lipid vesicles have been reported [5].

Systemic fungal infections, notably caused by species of *Candida* and *Aspergillus*, are a major cause of death in patients suffering from leukemia, lymphoma or a number of immunodeficiency diseases including AIDS (acquired immune deficiency syndrome) [6–8]. In the treatment of most such fungal infections the drug of choice is amphotericin B, although its acute and chronic toxicities severely limit its use [9]. A number of workers, however, have reported that amphotericin B toxicity is greatly reduced when it is administered within liposomes [10–12] with little or no reduction in efficacy [13]. Such a liposomal

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Abbreviations: DMPC, dimyristoylphosphatidylcholine; DMPG, dimyristoylphosphatidylglycerol; DMPS, dimyristoylphosphatidylserine; DOPC, dioleoylphosphatidylcholine; DOPG, dioleoylphosphatidylglycerol; EPC, egg phosphatidylcholine; HEPES, N-(2-hydroxyethyl) piperazine-N'-2-ethanesulfonic acid; LUV, large unilamellar vesicles; MLV, large multilamellar vesicles.

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formulation has been applied successfully to the treatment of immunocompromised patients suffering from progressive systemic fungal infections [14]. More recently, a novel lipid preparation with further enhanced selective toxicity of amphotericin B has been characterized [15]. The lipid composition used in this later study as well as the earlier clinical trial, dimyristoylphosphatidylcholine (DMPC) and dimyristoylphosphatidylglycerol (DMPG) efficiently incorporated amphotericin B. The addition of cholesterol or ergosterol does not significantly influence therapeutic efficacy [16]. This observation is surprising in view of the previously mentioned report that the antibiotic does not readily partition into sterol-free vesicles [5]. Here we have examined the direct partitioning of amphotericin B from an aqueous dispersion into phospholipid vesicles as a function of their lipid composition. We show remarkably high rates and extents of antibiotic uptake into vesicles composed of saturated phosphatidylcholine and either phosphatidylglycerol or phosphatidylserine while other phospholipid mixtures show virtually no incorporation. These observations are discussed in the light of earlier work [17] which documents the relative bilayer instability of MLV composed of these same lipid mixtures.

Materials and methods

Egg phosphatidylcholine (EPC), dimyristoylphosphatidylcholine (DMPC), dimyristoylphosphatidylglycerol (DMPG), dioleoylphosphatidylcholine (DOPC), dioleoylphosphatidylglycerol (DOPG) and dimyristoylphosphatidylserine (DMPS) were purchased from Avanti Polar Lipids (Birmingham, AL) while cholesterol (standard for chromatography) was obtained from Sigma. [^{3}H]dipalmitoylphosphatidylcholine was purchased from New England Nuclear while amphotericin B and [^{14}C]amphotericin B were kindly supplied by Squibb, New Brunswick, N.J.

Preparation of lipid vesicles

Lipid mixtures were co-lyophilized from benzene/methanol (70:30 v/v) containing trace amounts of [^{3}H]DPPC (20,000 dpm $\cdot$ μmol^{-1}) and the dry lipid stored at -20°C. Unless otherwise stated, the lipid was hydrated at 30°C in distilled water at a concentration of 15 mM and large unilamellar vesicles (LUV) prepared at 30°C, using an "Extruder" (Lipex Biomembranes, Inc. Vancouver, B.C) as detailed previously [18]. Mixtures of DMPC/DMPG dispersed in H_2O at their gel-to-liquid-crystalline phase transition temperature spontaneously form large unilamellar vesicles [17] and in some experiments these were used directly.

Incorporation of amphotericin B into lipid vesicles

Amphotericin B containing trace amounts of [^{14}C]amphotericin B (275,000 dpm $\cdot$ μmol^{-1}) was dispersed in distilled water using a bath sonicator (Laboratory Supplies Co. Inc. Model G112 SPIT) for 15 s. The yellow dispersion was then added to the lipid vesicles to give final lipid and amphotericin B concentrations of 13.5 mM and 1 mM respectively. It should be stressed that this does not represent a solution of amphotericin B. Under these conditions a small fraction of the drug exists as a micellar solution [19] (to approximately 16 μM) with the remainder present as an insoluble colloidal suspension.

The uptake of amphotericin B was followed at various times by centrifuging 500-μl aliquots of the mixture at 13,400 $\times$ g for 2 min in a Fischer Micro-Centrifuge (model 235B). Control experiments verified that this centrifugation removed all insoluble amphotericin B from the supernatant but did not pellet lipid vesicles. Aliquots were then assayed from the supernatant for [^{3}H]DPPC and [^{14}C]amphotericin B by liquid scintillation counting in a Packard 2000 CA.

Isopycnic density gradient centrifugation

Amphotericin B-containing vesicles (200 μl) were layered onto a 3.8-ml continuous sucrose gradient (0–29 wt%) sucrose in 0.15 M NaCl, 10 mM Na_2PO_4 (pH 7.4). The gradient was spun for 24 h at 22°C in a Beckman SW60 rotor at 230,000 g^{av} using a Beckman L8-60 ultracentrifuge. Following centrifugation the gradient was fraction-

ated and lipid and amphotericin B quantified by liquid scintillation counting of [^{3}H]DPPC and [^{14}C]amphotericin B.

Differential scanning calorimetry

Samples were sealed in 75-μl stainless steel pans and inserted into the calorimeter (Perkin-Elmer DSC 2C) at 42°C. The samples were quench-cooled to 0°C prior to heating at 2°C · min^{-1} to 42°C. Following quench-cooling to 0°C the heating thermogram was recorded at least once more. Enthalpies were calculated by reference to an indium standard and are accurate to ±5%. Temperature calibration is accurate to ±0.5°C.

Other experimental procedures

^{31}P-NMR spectroscopy and quasi-elastic light scattering measurements were performed as described previously [17].

Results

As mentioned in the Introduction, amphotericin B has recently been administered clinically in liposomes composed of DMPC/DMPG (molar ratio 7:3) [14]. Initially, therefore, we examined the ability of LUV prepared from this lipid mixture to incorporate the antibiotic directly from an aqueous suspension of the drug. When dispersed in H_2O at a concentration of 1 mM the drug forms a colloidal suspension in equilibrium with a soluble micellar solution, which is present to a concentration of approximately 16 μM. The ability of DMPC/DMPG vesicles to incorporate the antibiotic above, below and within the gel-to-liquid-crystalline phase transition temperature of the lipid ($T_c = 23$°C) is shown in Fig. 1. Surprisingly, even when the lipid is in the gel state, rapid amphotericin B uptake is observed. In the absence of vesicles, however, only low concentrations of antibiotic occur in the supernatant consistent with its low micellar solubility. As described in an earlier publication [17] DMPC/DMPG in low ionic strength media rapidly vesiculates at its T_c and this process and/or the enhanced lateral compressibility of the bilayer [20] may contribute to amphotericin B uptake at 24°C. Vesiculation per se, however, is not a prerequisite for accumulation of the antibiotic, as rapid uptake is also seen both at 15°C and 32°C, temperatures at which only slow breakdown of DMPC/DMPG MLV occurs [17].

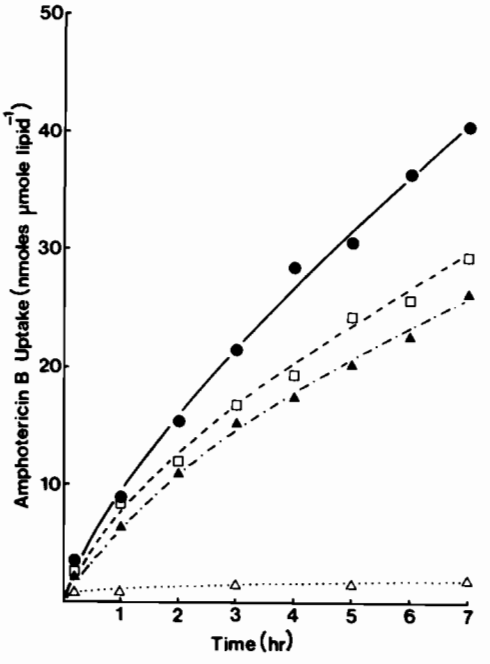

Fig. 1. Influence of temperature on the uptake of amphotericin B into DMPC/DMPG LUV. Vesicles were incubated with the drug as described under Materials and methods at 15°C, –·–▲–·–; 24°C, – – □ – –; and 32°C, —●—. In addition, the solubilization of amphotericin B in the absence of vesicles at 32°C is shown, ···△···.

To confirm that the amphotericin B solubilized is associated with the lipid, aliquots of drug-loaded vesicles were examined by isopycnic density gradient centrifugation. In this procedure continuous gradients are subjected to high centrifugal forces for an extended time period (24 h) allowing all of the components in the sample to migrate to their equilibrium density positions. Shown in Fig. 2 is the profile of DMPC/DMPG vesicles incubated with amphotericin B for 16 h at 24°C. Prior to loading on the gradient any unincorporated drug was removed by low-speed centrifugation (see Materials and methods). Am-

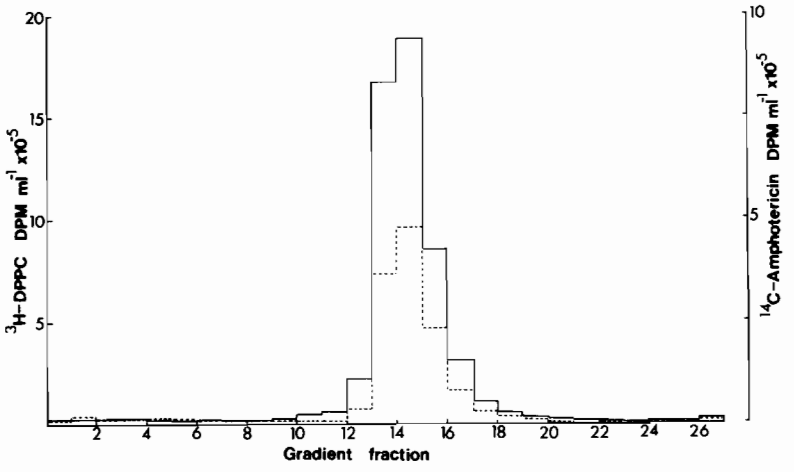

Fig. 2. Isopycnic density gradient profile of DMPC/DMPG LUV loaded with 5 mol% amphotericin B. Vesicles were incubated with amphotericin B for 24 h at 24°C as described under Materials and methods. Unincorporated drug was removed by low-speed centrifugation and the vesicles then fractionated on a 0–29 wt% continuous sucrose gradient as described under Materials and methods. The gradient is shown from top to bottom, left to right. The DPM arising from [^{3}H]DPPC, —; or [^{14}C]amphotericin B, - - -; are shown for each fraction.

photericin B and lipid comigrate on the gradient to a position intermediate between that of the free lipid and the pellet of colloidal drug, indicating that the antibiotic is tightly associated with the vesicles. The homogeneous population of vesicles seen is in sharp contrast to the heterogeneous mixture produced when amphotericin B is incorporated into DMPC/DMPG MLV from organic solvent [15] where antibiotic-free vesicles coexist with systems containing high mole ratios of drug.

To confirm that amphotericin B truly intercalates within the bilayer and is not simply adhering to the surface, the ultraviolet spectral characteristics of the antibiotic following its accumulation by DMPC/DMPG vesicles were analyzed. Amphotericin B was incubated for 24 h at 24°C in the presence or absence of DMPC/DMPG vesicles, as described under Methods and materials and insoluble drug then removed by centrifugation. When spectra are recorded in dimethylformamide the two samples show identical characteristics with maxima at 412 nm, 388 nm and 368 nm (Fig. 3A). In contrast, the UV spectra obtained for the two aqueous dispersions are quantitatively and qualitatively different (Fig. 3B). The increase in absorbance at 413 nm and 387 nm and the shift in the maxima at 420 nm to 413 nm, seen for the sample incubated in the presence of DMPC/DMPG vesicles (Fig. 3B) has previously been interpreted as evidence for insertion of the polyene antibiotic into membranes [21,22]. Additional evidence for insertion of amphotericin B into the vesicle bilayer was obtained by differential scanning calorimetry. Thermograms for both drug-loaded (5 mol% amphotericin B) and drug-free vesicles are shown in Fig. 4. DMPC/DMPG alone shows a pre-transition at around 15°C and the main gel-to-liquid-crystalline transition at 23°C. In the presence of amphotericin B the pre-transition is virtually eliminated and a marked reduction in the enthalpy of the main transition is apparent. These observations are consistent with the antibiotic interpolating into the bilayer with the mycosamine moiety in contact with the phospholipid headgroups and the macrolide ring buried in the region of the fatty acyl chains. This would account for its influence on both the pre- and main gel-to-liquid-crystalline transitions. Again the present DSC results can be contrasted with those reported for systems of DMPC/DMPG/amphotericin B pre-

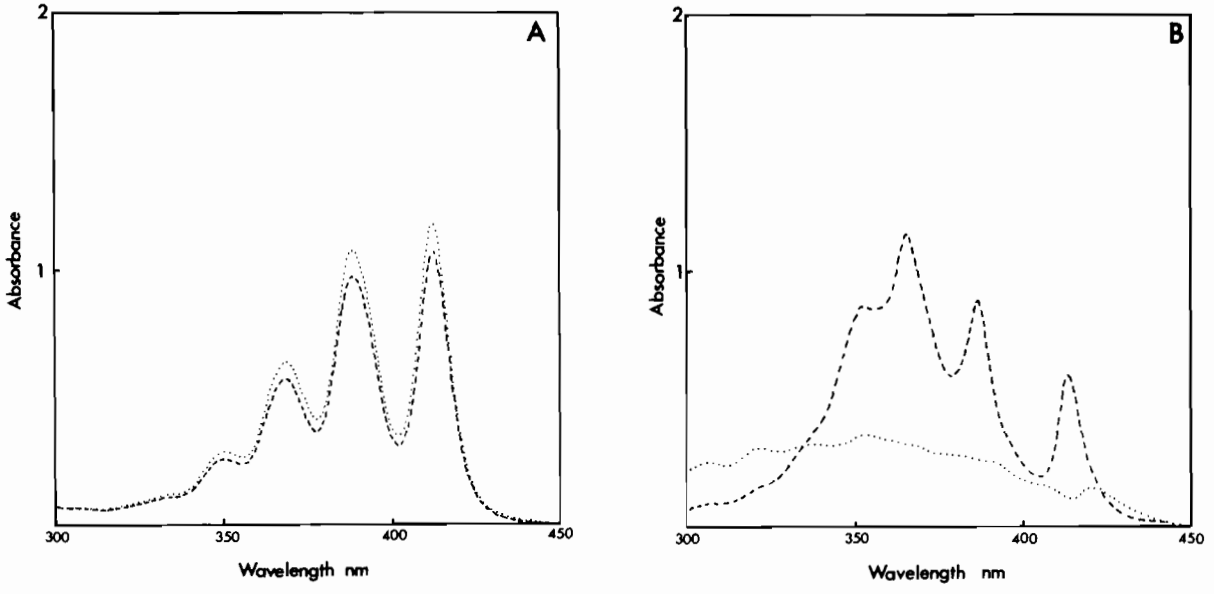

Fig. 3. Absorption spectra of amphotericin B. In A the spectra of amphotericin B incubated with (- - -) and without (····) DMPC/DMPG vesicles and subsequently dissolved in dimethylformamide are shown (final amphotericin B concentrations were 8.6 μM and 9.3 μM, respectively). Spectra obtained for the same samples in aqueous media are shown in B. The final concentrations of antibiotic were 17.4 μM with vesicles (- - -), and 14.8 μM in absence of vesicles (····).

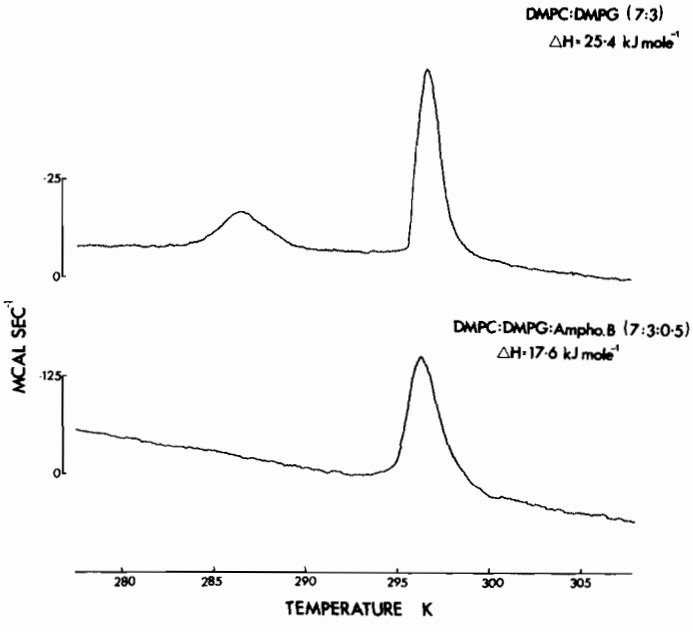

Fig. 4. Differential scanning calorimetric analysis of DMPC/DMPG MLV with and without amphotericin (5 mol%). MLV of the lipid alone were formed by dispersion of lyophilized DMPC/DMPG in 150 mM NaCl, 20 mM HEPES (pH 7.6). The amphotericin B-containing sample was prepared by incubating the lipid mixture with the drug at 24°C for 24 h in H_2O. The ionic strength of the sample was then brought to 150 mM NaCl, 20 mM HEPES (pH 7.6), the mixture lyophilized and then rehydrated in H_2O. The enthalpy of the main transition is shown for each sample.

pared from organic solvent which at 5 mol% antibiotic still exhibit a significant pretransition, presumably arising from the large population of antibiotic-free vesicles [15].

Having demonstrated that amphotericin B will spontaneously insert into DMPC/DMPG vesicles, we next sought to characterize what effect lipid vesiculation had on this process. First the influence of vesicle lamellarity on the rate of uptake of the antibiotic was studied. LUV were prepared by the extrusion technique (see Methods and materials) while MLV were formed by hydrating DMPC/DMPG at 4°C [17]. In Fig. 5 the rate of uptake of amphotericin B into these two systems is shown. Clearly over the first 2 h uptake into LUV is considerably faster than into MLV. This is consistent with the much greater surface area available for uptake for the smaller vesicles. After 2 h, however, the uptake rates are parallel, suggesting that the MLV have vesiculated to form LUV. This was confirmed by quasi-elastic light scattering which gave a mean vesicle diameter of 180 nm for the "MLV" sample following the 7-h incubation.

We next examined the influence of vesicle composition on the uptake of amphotericin B. In all of these experiments LUV were prepared from the hydrated lipid by the extrusion technique to allow direct comparison between different lipid mixtures. The extent of antibiotic incorporation into vesicles as a function of their phosphatidylglycerol content is shown in Fig. 6. While LUV composed of DMPC alone show little uptake, a rapid increase in the extent of accumulation is observed as the DMPG content is raised until at about 15 mol% DMPG the rate levels off (Fig. 6). To determine whether the saturated nature of its fatty acyl groups is partly responsible for the inability of DMPC to accumulate amphotericn B, uptake by EPC vesicles was examined (Fig. 7). Clearly the presence of unsaturated fatty acids alone is insufficient to allow insertion of the antibiotic. In addition, the inclusion of cholesterol in the vesicle membrane

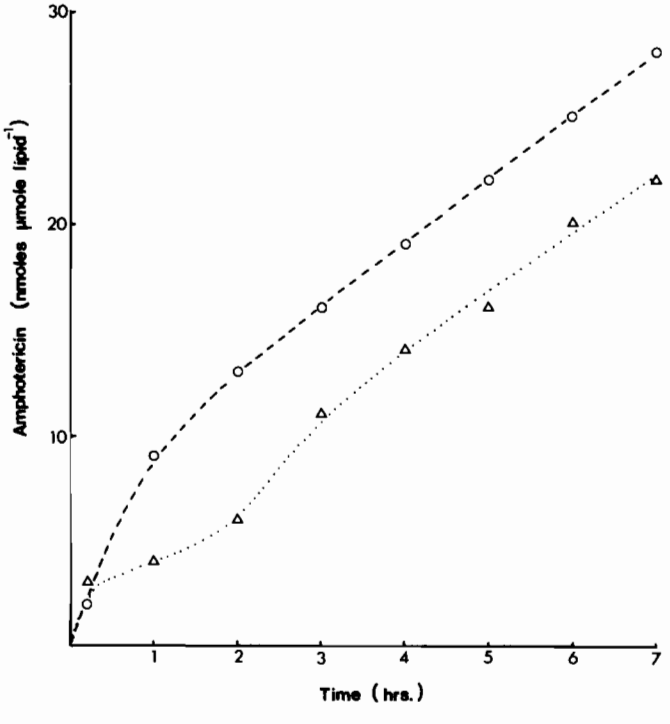

Fig. 5. Influence of liposome morphology on uptake of amphotericin B. MLV, ···△···; or LUV, - -○- -; were formed as described under Materials and methods and incubated at 24°C with amphotericin B.

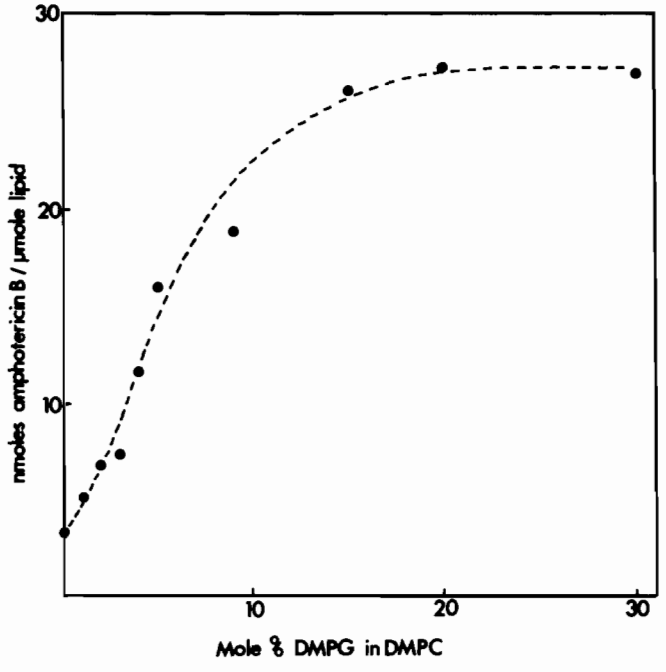

Fig. 6. Influence of lipid composition on uptake of amphotericin B. LUV were prepared from DMPC or DMPC/DMPG mixtures as shown and incubated with the drug for 6 h at 24°C.

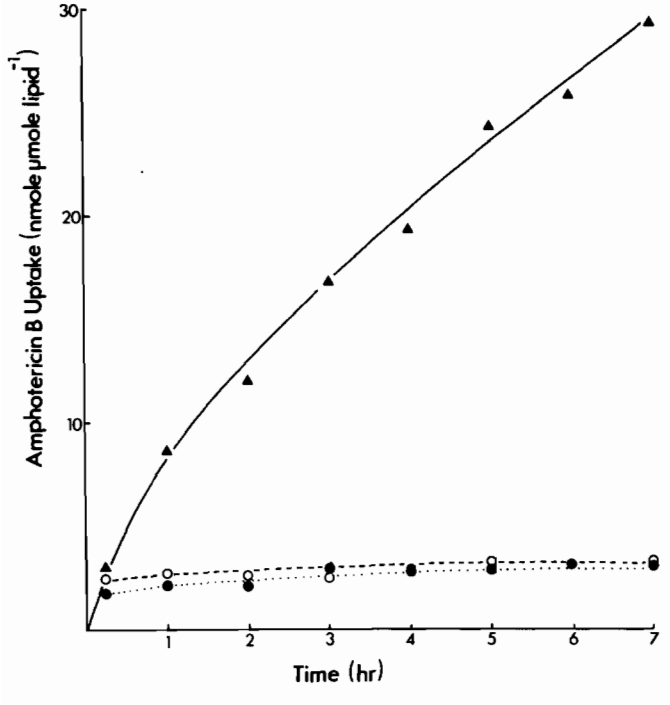

Fig. 7. Influence of lipid composition on uptake of amphotericin B. Drug accumulation at 24°C by LUV prepared from EPC, - -○- -; EPC/cholesterol (7:3), ···●···; or DMPC/DMPG (7:3), –▲– is shown.

has little influence on the rate of drug uptake (Fig. 7). The observation that cholesterol does not influence the rate of incorporation of amphotericin B is unexpected and perhaps also informative. Amphotericin B can readily be incorporated into a variety of phospholipid or phospholipid/sterol systems by cosolubilization in organic solvent or by addition of the antibiotic in dimethylsulfoxide to an aqueous lipid dispersion [16,23,24]. The inability of the antibiotic to incorporate from an aqueous suspension may indicate, therefore, its inability to penetrate the bilayer in the absence of a solvent vector. Additionally, this might account for the incorporation observed with lipid mixtures which spontaneously vesiculate and which presumably exhibit transitory bilayer defects. To investigate this further, a number of mixtures of neutral and acidic phospholipids were prepared. We have shown earlier that, compared to DMPC/DMPG, MLV prepared from DMPC/DOPG (7:3), DOPC/DMPG (7:3) or DOPC/DOPG (7:3) are stable in low ionic strength media, as indicated by the appearance of only a small isotropic component in their ^{31}P-NMR spectra. In addition LUV prepared from these mixtures are relatively impermeable to Mn^{2+} ions [17]. The lipid mixture DMPC/DMPS (7:3) appears to be intermediate in stability between DMPC/DMPG and the mixtures containing the dioleoyl lipid species in that while it does not spontaneously vesiculate it does show high permeability to Mn^{2+} [17]. The ability of these lipid mixtures to accumulate amphotericin B is compared in Fig. 8. Despite the fact that most of the lipid in the DMPC/DMPS vesicles will be in the gel state at 24°C, its uptake rate is second only to DMPC/DOPG vesicles. In comparing this data with that for DMPC/DMPG

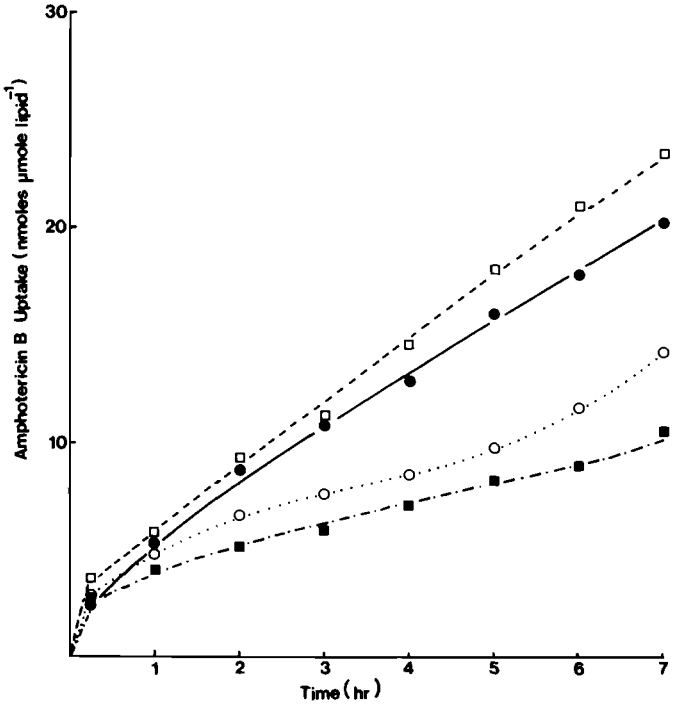

Fig. 8. Influence of lipid composition on uptake of amphotericin B. Shown is the rate of drug uptake at 24°C by LUV prepared from DMPC/DOPG (7:3), - -□- -; DOPC/DMPG (7:3), --■--; DOPC/DOPG (7:3), ···○···; and DMPC/DMPS (7:3), —●—.

vesicles (see Fig. 7) it is apparent that while some correlation exists between the relative bilayer stabilities of the different lipid mixtures and their ability to accumulate amphotericin B, the tendency is not pronounced.

Discussion

Amphotericin B is a cylindrically shaped molecule which can be considered to possess both hydrophobic and hydrophilic surfaces [25]. When oriented within a lipid bilayer the opposed hydrophilic surfaces of several antibiotic molecules are believed to create an aqueous channel, thus leading to its antifungal activity [2]. In aqueous media the amphipathic nature of the drug results in the formation of micelles above its critical micellar concentration of 2×10^{-7} M [19]. Presumably, in such micelles the hydrophobic surfaces are now oriented facing each other, i.e. in the opposite configuration to the membranous complex. Whether incorporation of amphotericin B into vesicles occurs via fusion of such micelles with the bilayer or whether it involves insertion of antibiotic monomers is not known. The uptake of amphotericin B into vesicles composed of EPC, even in the presence of cholesterol, proceeds relatively slowly. That this slow accumulation does not reflect an inability of these vesicles to bind amphotericin B is attested by the observation that high antibiotic concentrations can be achieved by co-solubilization of the drug and lipid in organic solvent followed by drying and hydration in aqueous media or by the addition of amphotericin B in a solvent vector to an aqueous lipid dispersion [16,23,24]. In contrast to EPC, other lipid mixtures show very rapid accumulation of the antibiotic. Vesicles composed of DMPC/DMPG (7:3) sequester the drug even when the lipids are in the gel state. When either of the components of this mixture is employed alone, however, only the DMPG vesicles demonstrate a comparable rate of uptake (data not shown). This observation is notable in view of the fact that DMPG alone or DMPC/DMPG mixtures can spontaneously vesiculate while DMPC forms stable MLV [17]. Vesiculation would require transient defects in the bilayer which would be expected to continue to arise even following LUV formation. Such defects might facilitate access of amphotericin B to the hydrophobic bilayer interior permitting rapid antibiotic accumulation. Such an uptake mechanism would also explain why accumulation is possible even when the lipids are in the gel state, where penetration of the bilayer would normally occur much less readily. While vesiculation may promote antibiotic accumulation, it is clearly not a requirement; lipid mixtures which do not vesiculate, such as DMPC/DOPG, DMPC/DMPS and DOPC/DOPG, exhibit appreciable drug uptake rates. In the case of DMPC/DMPS these two lipids are poorly miscible in the gel state and as the mixture is cooled below the phase transition temperature of the phosphatidylserine (38°C) this component begins to segregate into gel state domains. Consequently, on reheating, thermograms show a broad transition extending from close to the T_c of pure DMPC to almost the transition temperature of DMPS [17]. The high Mn^{2+} permeability of this lipid mixture may arise due to bilayer defects at the phase domain boundaries of these two phospholipid species and it is possible that amphotericin B insertion may also occur at these interfaces. No such membrane discontinuities should exist, however, for DMPC/DOPG vesicles at 24°C and yet drug accumulation occurs at a rate only slightly slower than for DMPC/DMPG vesicles. The situation is further complicated by the fact that the presence of different lipid phase domains is not sufficient by itself to allow amphotericin B intercalation. In the case of DMPC vesicles, for example, little drug uptake is observed at 24°C even though both gel and liquid-crystalline domains should coexist at this temperature. Upon introduction of an acidic phospholipid to DMPC bilayers, however, a rapid increase in the rate of accumulation is observed. Whether the negative surface charge facilitates the interaction of amphotericin B with the membrane interface thereby promoting insertion or whether charge repulsion increases the surface area per phospholipid molecule favouring penetration remains to be determined.

Acknowledgements

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