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THE EFFECT OF AMPHOTERICIN B ON THE PERMEABILITY OF LIPID BILAYERS TO DIVALENT TRACE METALS

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In this study amphotericin B released the divalent trace metals Zn^{2+}, Co^{2+}, Cu^{2+}, Ni^{2+}, Mn^{2+}, Fe^{2+}, Cd^{2+} and Pb^{2+} from multilamellar liposomes containing cholesterol. This observation is consistent with amphotericin B channels being permeable to these metals, and it is proposed, therefore, that the antibiotic may be useful in investigating the metabolism of these elements.

The polyene antibiotic amphotericin B achieves its fungistatic and fungicidal activity by interacting with sterol containing membranes to form aqueous channels of about 0.4 nm radius which are permeable to small solutes and ions [1–4]. The permeability of these channels is poorly selective and is inversely related to the molecular size of the solute [1,2]. Additional selectivity is associated with whether the amphotericin B is applied to the membrane bilaterally or unilaterally, although these effects are not mutually exclusive the former is preferentially anion selective whilst a greater degree of cation selectivity accompanies the unilateral application of the antibiotic [1,3–6]. This paradox has been reconciled by the proposals that the sterol-amphotericin B aggregates which constitute a half pore within one layer of a lipid bilayer may be able to span some membranes and that they possess an intrinsic cation selectivity which is lost when two such multimers align to form a complete pore [5,6].

The membrane channels created by unilateral exposure to amphotericin B are permeable to alkali and alkaline earth metals but their permeability to other metals of biological importance has not been reported. Following a clinical observation that oral amphotericin B improved the zinc status in a child with the inherited disorder of zinc absorption, acrodermatitis enteropathica, it was shown that the antibiotic increased the permeability of liposomes containing cholesterol to zinc [7]. In that study, the amphotericin B was incorporated in the lipid bilayer and presumably formed complete pores; in this brief study the effect of the unilateral application of amphotericin B on the permeability of the multilamellar phospholipid vesicles to zinc, and to other trace metals has been investigated.

Liposomes comprising egg phosphatidylcholine and phosphatidic acid (Lipid Products Ltd., U.K.) in the molar ratios 95:5 or egg phosphatidylcholine, cholesterol (Sigma London) and phosphatidic acid in the molar ratios of 61:33:5 were prepared by mixing appropriate aliquots of stock chloroform solutions and evaporating the solvent under O_2-free N_2. The dried lipid was dispersed in a small volume of a 20 mM or 50 mM aqueous solution of a metal chloride containing 5 mM buffer (Bis-Tris or Tris-HCl, Sigma London) at a known pH. In the preparation of Fe^{2+} containing

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Abbreviations: ferrozine, 3-(2-pyridyl)-5,6-bis(4-phenyl-sulfonic acid)-1,2,4-triazine; xylenol orange, *o*-cresol sulfonephthalein-3,3′-bismethyliminodiacetic acid; zincon, 2-carboxy-2′-hydroxy-5′-sulfoformazylbenzene; Bis-Tris, (bis(2-hydroxyethyl)imino-tris(hydroxymethyl)methane; Tris-HCl, tris(hydroxymethyl)-aminomethane hydrochloride.

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liposomes 0.1 mM ascorbic acid was included in the buffered solution. The experimental variables are summarised in Table I. After equilibration under N_2 non-entrapped metal ions were removed by chelation with Chelex 100 (Bio-Rad Laboratory Ltd., U.K.) which had been equilibrated previously with the buffer. Two equal aliquots of an appropriately buffered aqueous solution of a metallochromic indicator were placed in the thermostatically controlled cuvettes of a double-beam spectrophotometer (SP30 UV Pye Unicam Ltd.) and liposomes added to a concentration of 1 mM lipid. All studies were done at 30°C. Freshly prepared stock solution of amphotericin B (Sigma London) in Me_2SO was added to the measuring cuvette to achieve a concentration of 10 μM; an equal volume of Me_2SO was added to the reference cuvette. The increased efflux of entrapped metal ions was measured by monitoring, at a predetermined wavelength, the absorbance due to the formation of extra-liposomal colorimetric metal complexes. A preliminary study indicated that neither amphotericin B nor Me_2SO altered the absorbance of the metallochromic indicator or of preformed colorimetric complexes at the selected wavelength.

Amphotericin B increased the efflux of all the entrapped metals studied; namely Zn^{2+} (Fig. 1), Cu^{2+}, Co^{2+}, Ni^{2+}, Mn^{2+} (Fig. 2), Fe^{2+} (Fig. 3), Cd^{2+} and Pb^{2+} (Fig. 4) from liposomes containing cholesterol *. The initial rates of efflux for each cation clearly differ. This model, however, does not permit reliable comparison of these observations. The differences, for example, cannot be interpreted on the basis of relative ionic size (Table I) [8], that is on the assumption that large ions such as Pb^{2+} and Cd^{2+} would permeate the amphotericin B channels less readily than smaller ones such as Ni^{2+} or Co^{2+}, because this approach ignores the greater surface charge density and water of hydration associated with the latter elements which would reduce their mobility and, possibly, their permeability through the pores. Further variability in the efflux rates may have

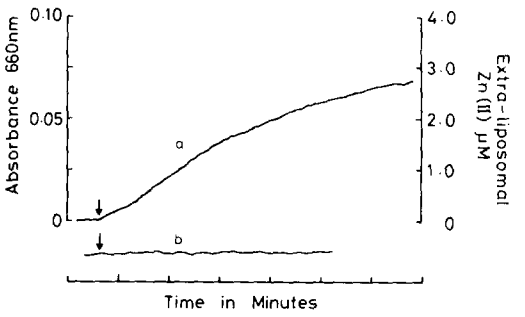

Fig. 1. The efflux of Zn^{2+} from (a) liposomes containing cholesterol and from (b) cholesterol free liposomes after the addition of 10 μM amphotericin B (↓). See Table I for experimental details.

been derived also from the relative size of the cation activity gradients maintained across the pores. These gradients would have been dependent on the molarity of the intra-liposomal metal solution and the relative affinities of the colorimetric reagents for the extra-liposomal cations. Furthermore, the permeabilities demonstrated in this study would have been influenced by the negative surface charge endowed on the lipid bilayers by their phosphatidic acid content. The zeta potential of representative liposomes containing cholesterol, measured by microelectrophoresis [9] at pH 7.2 in a preliminary study, was −5.3 mV, but this would have been modified by the pH of the various experimental systems. This charge may have bound the ions to the membrane surface [10,11] and thereby reduced their mobility but alternatively, however, by inducing cations to aggregate on the membrane it may have enhanced any inherent cation selectivity of the channel [3]. This latter characteristic may explain the discrepancy between our observations and that of another study in which amphotericin B failed to alter the permeability to Fe^{2+} of liposomes containing the cationic amphipath stearylamine [12]. We are not aware, however, of any systematic studies on the effect of surface charge on the permeability of membranes or of polyene induced membrane pores to the divalent cations investigated in this study.

For all metals except Pb^{2+} the total efflux induced by amphotericin B stopped after 5–10 min and approximated to 25% of the total liposomal metal content as judged by the increase in

* The use of symbols for ions in the text, e.g. Zn^{2+} assumes that this is the permeable species and the use of the oxidation states in the figures indicates that the absorbance is that of the colorimetric metal complex.

TABLE I

MOLARITY, BUFFERING AGENT AND pH OF THE METAL CHLORIDE SOLUTIONS IN WHICH LIPOSOMES WERE PREPARED

Liposomes were suspended at 1 mM (total lipid) in the metallochromic reagent indicated and the efflux of metal induced by 10 μM amphotericin B was measured by monitoring the increased absorbance, due to the formation of colorimetric metal complexes, at the wavelength shown. (Zincon and ferrozine were purchased from Sigma London, and xylenol orange from Hopkin and Williams Ltd. U.K.)

Metal chloride	Ionic crystal radii (Å)	Molarity (mM)	Buffer (5 mM)	pH	Metallochromic reagent	Absorbance wavelength (nm)
Zn^{2+}	0.74	50	Tris-HCl	6.9	Zincon (0.3 mM)	660
Cu^{2+}	0.72	50	Bis-Tris	5.0	Zincon (0.3 mM)	600
Co^{2+}	0.74	50	Tris-HCl	6.9	Zincon (0.3 mM)	656
Cd^{2+}	0.97	20	Bis-Tris	5.5	Xylenol orange (0.5 mM)	580
Mn^{2+}	0.80	20	Bis-Tris	5.5	Xylenol orange (0.5 mM)	585
Ni^{2+}	0.72	50	Bis-Tris	5.5	Xylenol orange (0.5 mM)	610
Pb^{2+}	1.21	20	Bis-Tris	5.5	Xylenol orange (0.5 mM)	580
Fe^{2+}	0.76	50	Bis-Tris and 0.1 mM ascorbate	5.5	Ferrozine (0.5 mM)	562

absorbance after lysis of the vesicles by Triton X-100 (0.5%). This incomplete release is similar to earlier observations of the K^+ efflux caused by amphotericin B from multilamellar liposomes and is consistent with the proposal that the antibiotic interacts only with the outermost lipid bilayer to release cations entrapped in the outer aqueous sphere of the vesicles [6]. Qualitative, though this model was, the completed efflux of Pb^{2+} was only 15% of the entrapped metal content, and the ex-

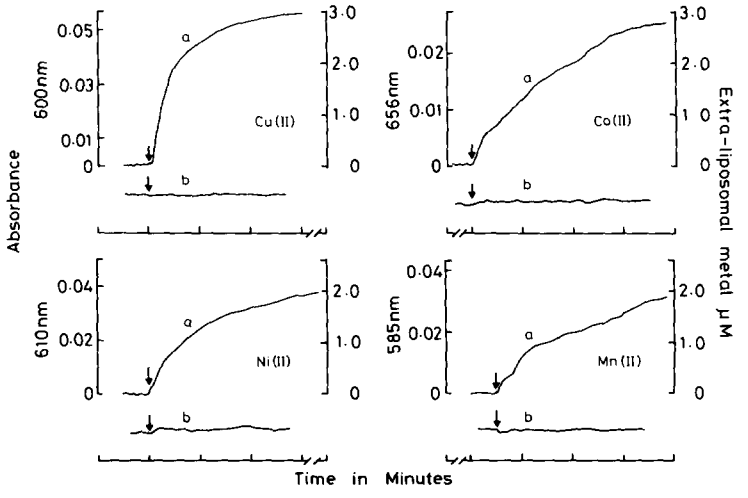

Fig. 2. (Left). The effect of 10 μM amphotericin B ↓ on the relase of Co^{2+}, Cu^{2+}, Ni^{2+} and Mn^{2+} from liposomes (a) with and (b) without incorporated cholesterol.

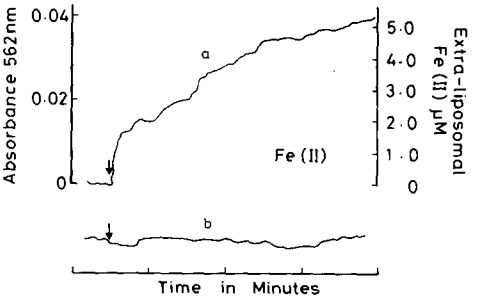

Fig. 3. (Right). The release of Fe^{2+} (a) from liposomes containing cholesterol, and its absence, (b) from cholesterol free liposomes following the addition of 10 μM amphotericin B ↓.

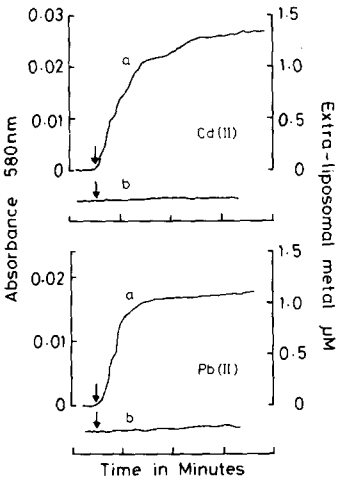

Fig. 4. The efflux of Cd^{2+} and Pb^{2+} induced by 10 μM amphotericin B (↓) (a) from liposomes containing cholesterol and (b) from liposomes containing no cholesterol.

ample illustrated was the best replicate result obtained. The reason for this reduced efflux relative to the other metals is not clear but all of the possible factors limiting the rate of efflux could have contributed to this phenomenon.

These results indicate that amphotericin B channels are permeable to a number of divalent cations and suggest that when interacting with biological membranes containing sterols amphotericin B and possibly other polyene antibiotics may affect the intracellular concentrations of both essential and nonessential, but potentially, toxic, divalent trace metals. While this effect on biological membranes remains to be demonstrated an example of its possible clinical exploitation has been observed in an inborn error of zinc absorption [7]; but perhaps, more importantly, these results indicate that amphotericin B may be as valuable in investigating the metabolism of trace metals as it is in the study of Na^+ and K^+ transport in epithelia and other biological systems (for example see Refs. 1, 13 and 14).

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