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The water and ionic permeability induced by polyene antibiotics across plasma membrane vesicles from *Leishmania* sp.

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An osmotic method has been used to study the effect of the polyene antibiotics amphotericin B, nystatin and candicidin on the water permeability of plasma membranes prepared from *Leishmania* sp. The effect of amphotericin B on the permeability of *Leishmania* membranes to a salt such as potassium nitrate was also investigated. A non-linear and saturable enhancement of water and salt permeability was measured with increasing polyene concentrations, which could be adjusted to Hill cooperativity equation. The antibiotic concentrations that induce at 30°C half-maximal effects on the water permeability of *Leishmania* vesicles were 0.021 μM for candicidin, 0.21 μM for amphotericin B and 1.4 μM for nystatin. At 30°C, the concentration of amphotericin B required to induce half of the maximal effect on the permeability of *Leishmania* vesicles to potassium nitrate was 1.8 μM. The temperature dependence for amphotericin B, nystatin and candicidin enhancement of the water permeability of *Leishmania* vesicles was determined by using Q_{10} data at 20 and 30°C. The estimated activation energies at increasing polyene concentrations display the same general pattern for all three polyene antibiotics investigated, that is, a maximal positive value at about the polyene concentrations required for half-maximal effect. The significance of these results for understanding the mechanism of action of polyene antibiotics on natural membranes is discussed.

Introduction

Most of the current ideas about the nature of the structures responsible of the permeability changes induced by polyene antibiotics such as amphotericin B or nystatin in the plasma membrane of sensitive organisms (for a review, see Refs. 1–3) have been derived from studies of polyene effects on the permeability of artificial lipid membranes to ions [4–6] water and small non-electrolytes [7,8] which indicated that addition of amphotericin B or nystatin to both sides of sterol-containing black lipid membranes led to the formation of aqueous pores of about 8 Å in diameter [9,10].

Since amphotericin B and all polyene antibiotics exert its biological effects on membrane permeability from only one side, there has been interest to investigate this type of action by using other lipid membrane model systems [11–13]. Marty and Finkelstein [11] by using planar lipid bilayers prepared without solvent, found that the concentration range of nystatin and amphotericin B that was required to produce a one-sided increase of membrane conductance was greater than that required for the two-sided effect. Van Hoogevest and De Kruijff [13] observed that the magnitude of the potassium leakage induced by amphotericin B across liposomes was smaller when added unilaterally to the external solution than that obtained by incorporating the antibiotic to the lipid phase before the liposomes were formed.

The changes of permeability induced by one-sided addition of amphotericin B to water, urea and salts across liposomes have also been measured by using a rapid mixing osmotic method [14]. This study has revealed that the structures formed rapidly by one-sided addition of amphotericin B to liposomes behaved more like ionic channels than to the aqueous channels known to be formed by two-sided addition of the antibiotic to black lipid membranes. Recently, it was proposed that amphotericin B may form both types of channels in ergosterol-containing liposomes, depending on antibiotic concentration and time elapsed after mixing [15].

The aim of the present work was to investigate the effect of one-sided addition of various polyene antibiotics on the osmotic water permeability of *Leishmania* sp., a polyene sensitive organism [2], which is the causative agent of leishmaniasis, a tropical disease second in importance only to malaria. The presence of ergosterol in the cytoplasmic membrane of *Leishmania* [16] make these organisms sensitive to the action of polyene antibiotics. For this purpose, we have prepared vesicles from the plasma membrane of *Leishmania* sp., suitable for osmotic experiments.

Materials and Methods

Preparation of Leishmania membrane vesicles. *Leishmania* sp. strain NR was cultured in a modified LIT medium as previously described [17]. 1.2-liter cultures were harvested after seven days, when the cells had reached late-logarithmic phase (approx. $7 \cdot 10^7$ cells/ml) by centrifugation at 1000 $\times g$ for 10 min at 4°C. The cells were then washed twice with 75 mM Tris-HCl at pH 7.6 containing 140 mM NaCl and 11 mM KCl (buffer A) and once with an hyperosmotic solution containing 400 mM mannitol, 10 mM KCl, 3 mM magnesium acetate and 10 mM Hepes and pH 7.6 (buffer B). After washing the cells with buffer B, the ice-cold pellet of cells were mixed in a mortar on ice with glass beads of 75–150 μM (Sigma Chemical Co.) in a 4:1 ratio. The mixture was ground by gentle rotation for 5–10 min, the progressive rupture of cells being monitored by using a phase contrast microscope. This procedure was stopped when no more than 2–3 intact cells per field could be seen at the microscope. After grinding, 25 ml of buffer B was added and the suspension was then centrifuged at 1000 $\times g$ for 10 min to remove glass beads and unruptured cells. The supernatant from this centrifugation was centrifuged at 5000 $\times g$ for 20 min and the supernatant from this was further centrifuged at 16000 $\times g$ for 40 min. Finally, a microsomal fraction was obtained from the last supernatant by centrifugation at 40000 $\times g$ for 50 min. This fraction was resuspended in small aliquots (0.2–0.5 ml) of buffer B prepared without mannitol and frozen immediately in liquid nitrogen. These frozen vesicles were stored at −70°C until use.

Purification of the microsomal fraction. Purification of the microsomal fraction was performed in a continuous density gradient of 18% Percoll prepared in 0.25 M sucrose and 10 mM Tris-HCl at pH 7.4. For this purpose, 2 ml of buffer B prepared without mannitol were added to the microsomal fraction and layered on top of the Percoll solution. The density gradient of Percoll was autoformed by centrifugation of this preparation at 40000 $\times g$ for 1 h. After centrifugation, two layers of material were observed at 1.036 g/ml and 1.10 g/ml. The 1.036 band correspond to purified plasma membranes. This band was further characterized by electron microscopy and enzymatic analysis after washing of Percoll by centrifugation at 105000 $\times g$ for 1 h.

Electron microscopy. Pellets of Percoll-purified membranes obtained by centrifugation were fixed in 3% glutaraldehyde in buffer A (see above) and postfixed in 1% OsO_4. The fixed pellets were washed with the buffer and dehydrated with a graded series of ethanol solutions. Final dehydration and removal of the ethanol was accomplished by use of propylene oxide. The pellets were embedded in EPON and thin sections were stained in sequence with 1% lead citrate and 1% uranyl acetate and were examined with a Hitachi H-500 electron microscope.

Enzyme analysis. 3′- and 5′-nucleotidase were assayed by incubating 0.2 ml of microsomal fraction or Percoll-purified membranes with 0.7 ml buffer A and 0.1 ml 3′-AMP or 5′-AMP (50 mM) for 2 h at 37°C under constant stirring. The reaction was terminated by adding cold 20% trichloroacetic acid (1 ml). After centrifugation of

this suspension phosphate was determined in the supernatant by the method of Fiske and SubbaRow [18].

Acid phosphatase was measured by the release of *p*-nitrophenol from *p*-nitrophenyl phosphate in the presence of acetate buffer (pH 5) following the procedure of Gottlieb and Dwyer [19].

Succinate-cytochrome *C* oxidoreductase was assayed according to Sottocasa et al. [20].

Extraction of lipids. The microsomal fraction (2 mg/ml) was washed twice with bidistilled water by centrifugation at 40000 × *g* for 1 h. The resulting pellet was resuspended in 2 ml of water and lyophilized for 12–18 h. The dried product was extracted in chloroform/methanol (2:1, v/v) and then filtered through Whatman paper No. 2. Organic solvents were evaporated in a rotary evaporator and the thin lipid film formed suspended in chloroform. Phosphates were determined by the method of Ames and Dubin [21] and ergosterol by the method of Stadtman [22].

Chemical analysis. Proteins were estimated by the method of Lowry et al. [23] with bovine serum albumin as standard.

Osmotic measurements. Osmotic volume changes of *Leishmania* membrane vesicles were followed by measuring the 90° light scattering intensity at 450 nm, in a Durrum stopped-flow spectrophotometer (D-110) as described previously [24]. It was found convenient to use the 4:1 ratio drive syringe, that permits one volume of vesicles to be mixed with four volumes of an hyperosmotic glucose solution prepared in the same buffer in which vesicles were finally suspended (buffer B without mannitol). In all experiments *Leishmania* vesicles were diluted to a protein concentration of 1.2 mg/ml before mixing.

To study the polyene-induced changes of the osmotic water permeability of *Leishmania* vesicles the maximal rates of initial light scattering intensity changes $(dI/dt)_o$ were measured from the slopes recorded 50 to 100 ms after mixing, due to the presence of an injection artefact. For this purpose, vesicles were mixed with a 500 mM glucose solution prepared in buffer B but without mannitol. Before mixing, amphotericin B, nystatin or candicidin dissolved in dimethylformamide were added in microliter amounts to the glucose-containing solution maintained at a constant temperature. In all cases the organic solvent final concentration was less than 0.5% by volume. Control experiments indicated that at this concentration dimethylformamide had no effect on light scattering changes. The polyene concentrations were expressed as μM, calculated using the molecular weights of the pure compounds [25].

The effect of amphotericin B on the permeability to KNO_3 of *Leishmania* vesicles was measured by using the 'maximum slope' method as described previously [24]. All measurements were done with water continuously circulating through the drive syringes and mixing cuvette at constant temperature (20 and 30°C).

Results

Characterization of Leishmania membrane vesicles

We have prepared a subcellular fraction from *Leishmania* sp. promastigotes which has enzymatic characteristics of plasma membranes. Gottlieb et al. [19] have proposed to consider acid phosphatases as marker enzymes of the plasma membranes of *Leishmania*, since cytochemical studies of isolated membranes of this organism have shown that this enzyme was confined to the external face of the membrane. The data presented in Table I indicate a 4-fold enrichment of the specific activity of acid phosphatases in the Percoll-purified membrane fraction, a value which

TABLE I

MARKER ENZYME DISTRIBUTION DURING PURIFICATION OF PLASMA MEMBRANE VESICLES FROM *LEISHMANIA* sp.

S.A., specific activity (nmol/min per mg protein). The number in parenthesis represents the relative enzyme activity with respect to the homogenate. n.d., not detectable. H, homogenate; MF, microsomal fraction; PP, percoll-purified membrane fraction; M, mitochondria.

	Acid phosphatase	3'-Nucleotidase	5'-Nucleotidase	Succinate-cytochrome *c* oxidoreductase
H	38.7(1)	61.2(1)	1.8(1)	15.8(1)
MF	95.2(2.5)	129.8(2.1)	1.8(1)	2.6(0.16)
PP	147.3(3.8)	215.6(3.5)	2.3(1.3)	n.d.
M				87.0(5.5)

is comparable to the 5-fold increase reported by Gottlieb and Dwyer [19] for a sucrose-gradient purified membrane fraction of *Leishmania donovani*. The specific activities of 3′- and 5′-nucleotidases in the crude (2.1) and purified (3.5) *Leishmania* membrane fraction (Table I) are also comparable to that obtained for *Leishmania donovani* [26], including the absence of enrichment of 5-nucleotidase. On the other hand, succinate-cytochrome *C* oxidoreductase activity, an indication of mitochondrial contamination, was very small in the non-purified membrane fraction and no activity was detected in the Percoll-purified membrane fraction (Table I).

The purity of the *Leishmania* vesicles after and before the Percoll step was also assessed by fine-structure electron microscopic examination (Fig. 1). The majority of the vesicles appeared spherical with an average diameter of 0.24 ± 0.11 μm.

The sterol composition of the membrane vesicles isolated by the present method from *Leishmania* sp. was quantitated on the basis of phospholipid and protein content (see Methods). The ergosterol to phospholipid molar ratio was 0.27 whereas the phospholipid to protein ratio was calculated to be 0.18 μmol of phospholipid/mg protein.

Osmotic behaviour of Leishmania membrane vesicles

After mixing of *Leishmania* vesicles with an hyperosmotic glucose solution a rapid increase of light scattering intensity at 90° was observed (inset to Fig. 2). The initial increase in ligh scattering corresponds to the shrinkage of vesicles due to the efflux of water from the vesicles. It has been previously calculated by Latimer and Pyle [27] that as small particles shrink, 90° scattering increases. This theoretical prediction agrees qualitatively with the present behaviour of *Leishmania* vesicles and the observed behaviour of liposomes [15].

In Fig. 2 the reciprocal of the maximum extents of shrinkage exhibited by *Leishmania* vesicles after mixing with increasing glucose concentrations are

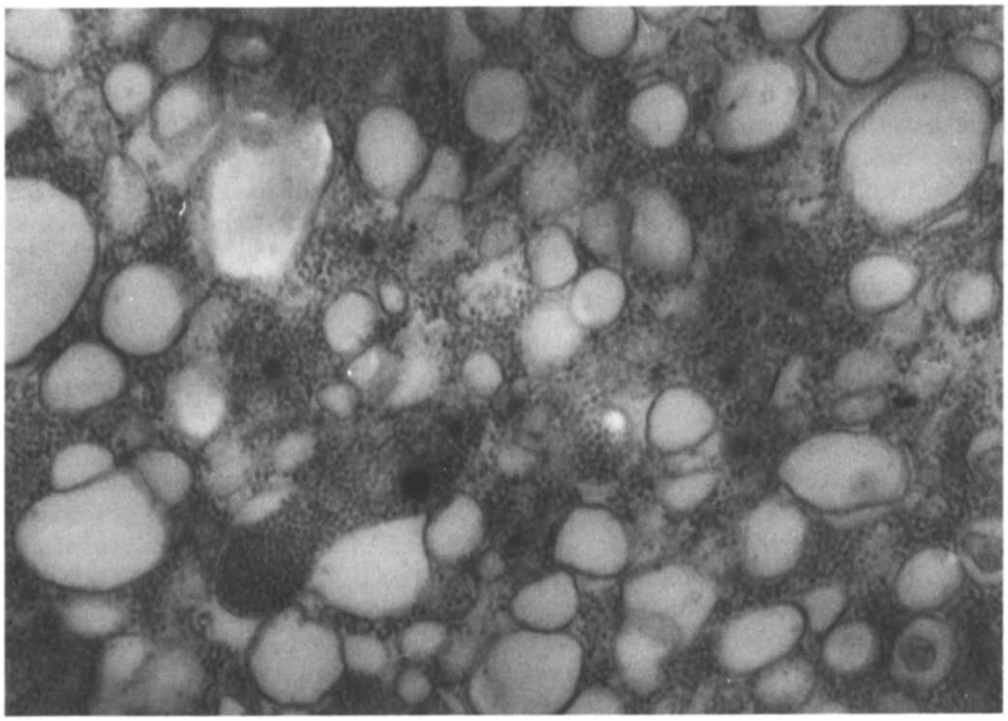


Fig. 1. Electron micrograph of thin section of the *Leishmania* vesicles. Plasma membranes isolated by Percoll-gradient were concentrated to a pellet by centrifugation, fixed and sliced (see Methods). Vesicles diameter range from 0.13 to 0.35 μm. Magnification × 45 000.

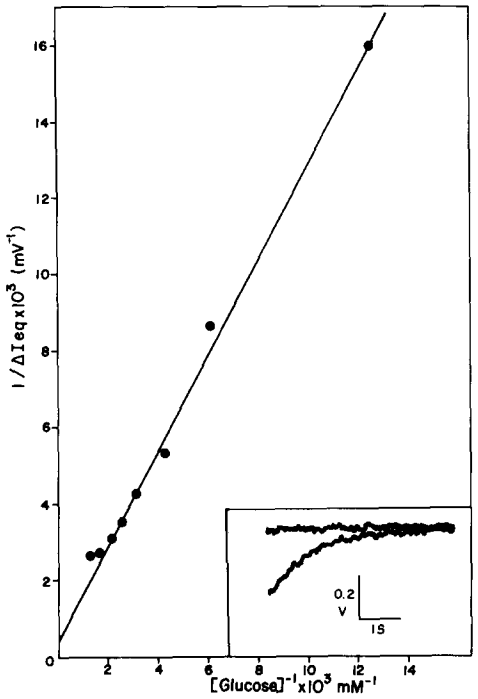


Fig. 2. Relationship between reciprocal of the 90° light scattering intensity changes at equilibrium ($1/\Delta I_{eq}$) of *Leishmania* vesicles and reciprocal of glucose concentration (mM^{-1}). Inset: time-course of the changes of light scattering after rapid mixing of *Leishmania* vesicles with an hyperosmotic glucose solution (500 mM).

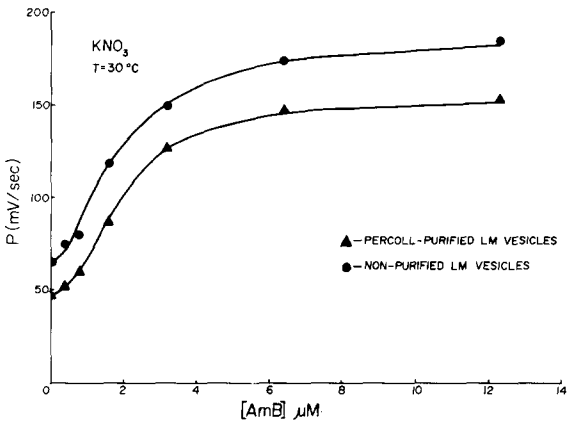


Fig. 3. The effect of amphotericin B on the KNO_3 permeability across *Leishmania* vesicles before and after Percoll purification. *Leishmania* vesicles (1.2 μg/ml) suspended in buffer B without mannitol (see Methods) were rapidly mixed (1:4, volume ratios) with an hyperosmotic KNO_3 solution (600 mosM) containing amphotericin B at increasing concentrations. Ordinate: rate of change of swelling after the maximum shrinkage (dV/dt (in mV/s). Absissa: amphotericin B concentration (in μM). All experimental values shown are an average of at least five determinations. The maximal standard deviation was less than the size of the symbols . Temperature 30°C.

plotted vs. the inverse of glucose concentration. As predicted by the Boyle- Van't Hoff equation an ideal osmotic behaviour was observed. The deviation from linearity that can be observed at glucose concentrations higher than 800 mM, are not unexpected since glucose was slightly permeable through *Leishmania* vesicles.

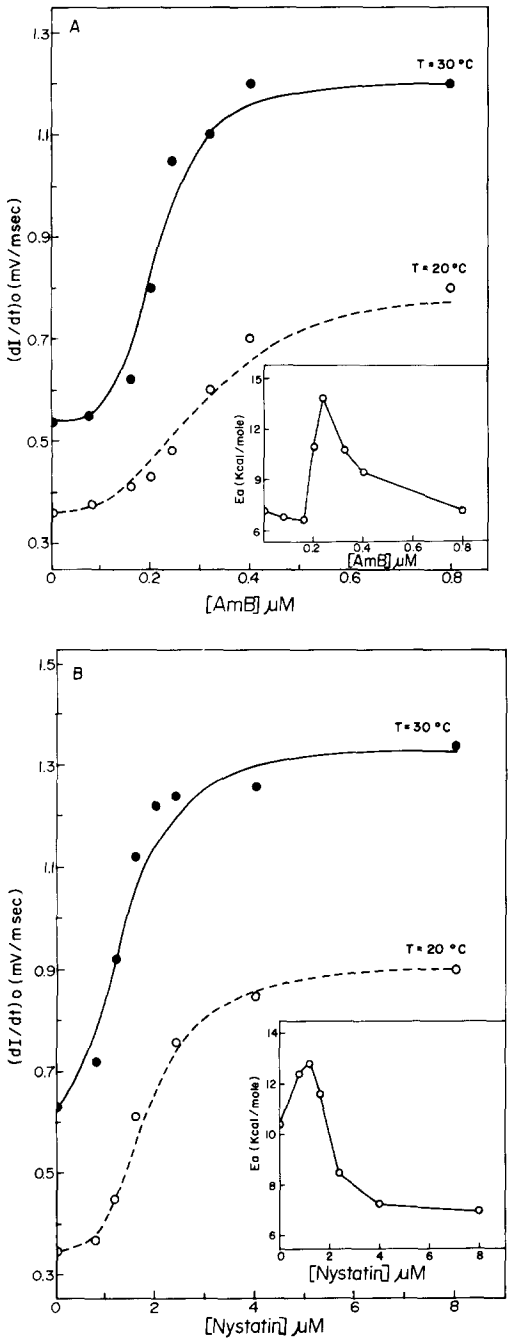
The effect of amphotericin B on the permeability of Leishmania membrane vesicles to potassium nitrate

One of the results of the action of polyene antibiotics such as amphotericin B and nystatin on sensitive organisms is a decrease in intracellular potassium ions. It follows that it is of some importance to investigate the sensitivity of the membrane vesicles prepared by the present method to this type of effect. This was done by measuring the effect of increasing concentrations of amphotericin B on the permeability of potassium nitrate across Percoll-purified and non-purified vesicles (microsomal fraction, see Methods).

It can be seen in Fig. 3 that both membrane fractions exhibited a similar response to increasing amphotericin B concentrations. Thus, at concentrations of amphotericin B higher than about 0.2 μM, a non-linear increase of salt permeability could be measured. The sigmoid curves (continuous lines in Fig. 3) were traced in both cases by fitting experimental points to the equation of Hill. The values calculated for the cooperativity order n were $n = 2.3$ ($r = 0.99$) for purified membrane vesicles and $n = 1.8$ ($r = 0.99$) for non-purified membrane vesicles. It was calculated that half of the maximal effects of amphotericin B on KNO_3 permeability of non-purified and purified vesicles were obtained at concentrations ($C_{1/2}$) of 1.8 μM and 1.9 μM, respectively.

The effect of polyene antibiotics on osmotic water permeability of Leishmania vesicles

In the light of the results described in the last section indicating that no significative differences arise between the osmotic salt permeability of



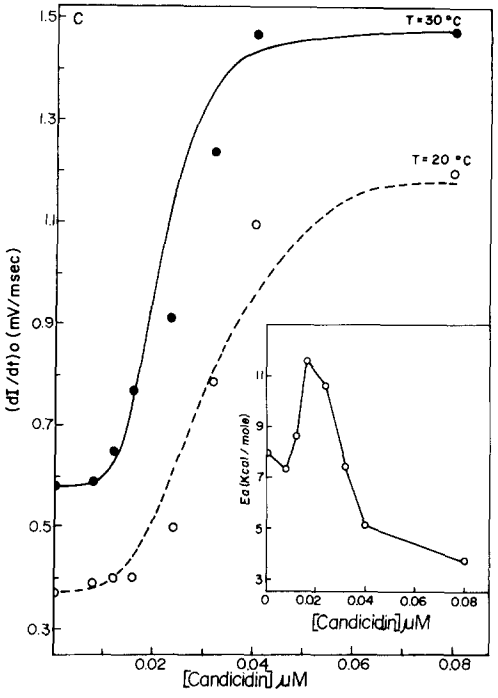


Fig. 4. The effect of increasing concentrations of different polyene antibiotics on the water permeability of *Leishmania* vesicles at 20 and 30 °C. Ordinate: Initial changes of 90° light scattering intensity changes. Absissa: polyene antibiotic concentration (μM). (A) Amphotericin B, (B) nystatin, (C) candicidin. Vesicles (1.2 mg protein/ml) suspended in buffer B without mannitol (see Methods) were rapidly mixed (1:'4, volume ratios) with a 500 mosM glucose solution containing polyene antibiotic at increasing concentrations. In all experiments, the final (after mixing) lipid concentration was about 0.05 μmol/ml (0.24 mg protein/ml). Plotted values are the means of at least eight determinations at each concentration. The maximal standard deviation was ±0.1 mV/ms. Insets: Effect of increasing polyene antibiotic concentration on the total activation energies for osmotic water permeability of *Leishmania* vesicles. Plotted values were estimated from Q_{10} data at 20 and 30 °C (in kcal/mol). Abscissa: polyene concentration (in μM).

non-purified and purified membrane vesicles and, since for osmotic experiments relatively large amounts of material were needed, in all the following experiments non-purified *Leishmania* vesicles were used.

Fig. 4 illustrates the effect of increasing concentrations of amphotericin B, nystatin and candicidin on the initial light scattering changes exhibited by *Leishmania* vesicles after mixing with hyperosmotic glucose solutions (see Methods). In this figure, measurements at two temperatures 20 and 30 °C are shown for all polyene antibiotics tested.

It can be observed in Fig. 4 that amphotericin B (Fig. 4A), nystatin (Fig. 4B) and candicidin (Fig. 4C) induced a nonlinear, cooperative enhancement of water permeability but at different

TABLE II

COOPERATIVITY NUMBERS (n) AND HALF-MAXIMAL CONCENTRATIONS ($C_{1/2}$) FOR POLYENE-INDUCED WATER PERMEABILITIES ACROSS *LEISHMANIA* VESICLES

n values and $C_{1/2}$ were calculated by using the Hill's equation.

Antibiotic	n (mean ± S.D.)		$C_{1/2}$ (μM)	
	20°C	30°C	20°C	30°C
Amphotericin B	2.8 ± 0.3	4.5 ± 0.3	0.32	0.21
Nystatin	3.5 ± 0.2	2.6 ± 0.5	2.0	1.4
Candicidin	3.4 ± 0.7	4.8 ± 0.8	0.031	0.021

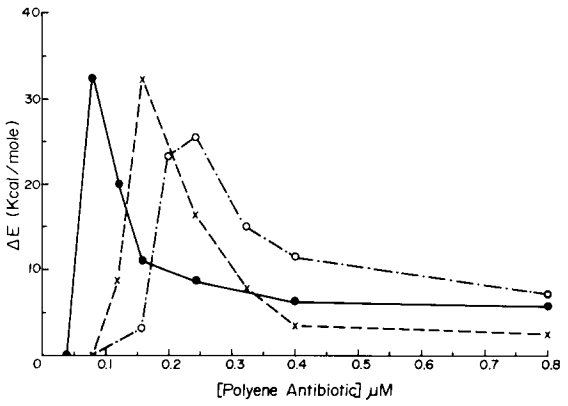


Fig. 5. Activation energies (in kcal/mol) for polyene-induced water permeation across *Leishmania* vesicles estimated from Q_{10} data at 20 and 30°C. At each temperature, polyene-induced water permeabilities were calculated by substracting the initial slopes shown by control vesicles exposed to hypertonic glucose solutions without drug–from the corresponding initial slopes from polyene-treated vesicles. Plotted values are the means of two separate experiments each done with at least eight determinations at each polyene concentration. The maximal standard deviation was about ±10 kcal/mol. ○, amphotericin B (×1 μM); ●, nystatin ($\times 10^{-1}$ μM); ×, candicidin (×10 μM).

polyene concentration ranges. The experimental points were fitted in all cases to the equation of Hill.

The polyene concentrations $C_{1/2}$ required for half-maximal increase of water permeability were calculated from the fitted curves (Table II). At 20 and 30°C, $C_{1/2}$ values were 0.031 μM and 0.021 μM for candicidin, 0.32 μM and 0.21 μM for amphotericin B and 2.0 and 1.4 μM for nystatin, respectively. It can seen in Table II that the cooperativity order n values range from about 3 to 5 for the three antibiotics investigated.

The experimental data presented in Fig. 4 also indicate that the osmotic water permeabilities of *Leishmania* vesicles treated with the differential polyene antibiotics increased with a rise of temperature at all polyene concentrations tested. From the corresponding Q_{10} values, activation energies for water permeation across polyene-treated *Leishmania* vesicles were estimated (insets to Fig. 4). The estimated E_a values for water permeation across un-treated vesicles (8.6 ± 2 kcal/mol) are very similar to that measured for water permeation across closed lipid vesicles [28].

The results shown in the insets of Fig. 4 are replotted in Fig. 5 as follows: for each polyene concentration and temperature, initial light scattering changes from control vesicles have been sustracted from the initial light scattering changes from polyene-treated liposomes and using average data of at least two separate experiments, the contributions to the total water activation energies of the presence of polyene molecules at increasing concentrations were calculated. It can be seen in Fig. 5 that such calculations, yield a maximal positive value of about 25–30 kcal/mol, at approximately the polyene concentrations at which half of the maximal effects on water permeability were measured.

Discussion

The osmotic measurements carried out in the present work show clearly that rapid mixing of *Leishmania* vesicles with hyperosmotic glucose solutions containing amphotericin B, nystatin or candicidin at increasing concentrations, led to a non-linear, saturable enhancement of the water permeability of such vesicles. The overall magnitude of the measured changes of water permeability are small when compared with the 20-fold increments induced by two-sided addition of nystatin or amphotericin B to black lipid membranes [7,8]. However, the observed polyene-induced maximal enhancement of about 2-fold in the water permeability of *Leishmania* vesicles is similar to that found before after one-sided ad-

dition of amphotericin B to ergosterol-containing liposomes, at similar polyene concentrations and temperature [14].

The measured changes of water permeability with increasing polyene concentrations (Figs. 4A, 4B, and 4C), were fitted to a sigmoid curve by using the equation of Hill and the corresponding n values and polyene concentrations required for half maximal effect were thus calculated. For all the polyene-induced water permeability n range from about 3 to 5 (Table II), suggesting a common mechanism underlying such changes.

Concerning such a mechanism, it is important to note that the measured changes of water permeability of *Leishmania* vesicles occurred in a narrow polyene concentration range. Thus, most of the amphotericin B-induced enhancement of water permeability occurred between 0.2 to 0.4 μM (Fig. 4A); beyond about 0.5 μM amphotericin B, no further increment could be demonstrated. By contrast, the onset of the ion permeability induced by amphotericin B across *Leishmania* vesicles (Fig. 3) occurred at concentrations very near the amphotericin B concentration at which half of the maximal effect on water permeability was measured (Table II) and extends up to amphotericin B concentrations as high as 3.2 μM (Fig. 3). On the other hand, the fitting of the amphotericin B-induced changes by using the equation of Hill yields n values of about 2 (see Results). Such n values are about half of those calculated for the corresponding amphotericin B-induced water permeability changes (Table II), eventhough the overall magnitude of the changes induced by amphotericin B in the permeability of *Leishmania* vesicles to potassium nitrate was about 2–3-fold greater than those measured for osmotic water permeability (compare the ordinate scales of Fig. 3 and Fig. 4A).

These observations can be taken as a clear indication that water and ionic enhancement of membrane permeability by a polyene antibiotic such as amphotericin B, under our experimental conditions, are two distinct physical phenomena. In this sense, it is suggested that the relatively modest osmotic water permeability enhancement exerted by the polyene antibiotics investigated across *Leishmania* vesicles may partly originate from a disturbance of the membrane permeability

TABLE III

ANTIFUGAL AND ANTIPROTOZOAL ACTIVITY RANGE OF POLYENE ANTIBIOTICS

Antibiotic	Minimium inhibitory concentrations (μM)		
	Candida albicans [a]	*Saccharomyces cerevisiae* [a]	*Leishmania* sp. promastigotes [b]
Amphotericin B	0.54	0.27	0.12 (0.21) [c]
Nystatin	3.3	3.24	1.76 (1.2)
Candicidin	0.033	0.016	0.006 (0.021)

[a] Taken from Refs. 2, 32 and 33.

[b] Taken from Ref. 34.

[c] The values in parenthesis correspond to half-maximal values ($C_{1/2}$) of polyene-induced water permeabilities across *Leishmania* vesicles at 30 °C (see Table II).

produced by the insertion of polyene molecules into the membrane phase, previous to the formation of the ionic channels, responsible of the potassium permeability.

The basic support of this interpretation is the fact that *Leishmania* vesicles maintained its full osmotic response in all the amphotericin B concentration range where the potassium permeability is expressed (Fig. 3), thus indicating that the ionic pathways created by the polyene molecules in the membrane are not simple aqueous channels.

The finding that the osmotic water permeability changes induced by low concentrations of amphotericin B, nystatin or candicidin display a positive temperature dependence (insets to Fig. 4) contrasts with the negative temperature dependence reported for polyene-induced membrane permeability alterations under other experimental conditions [5,29–31]. Such a positive temperature dependence may correspond to the energy required for polyene molecules for insertion into the membrane barrier. A similar positive temperature dependence of amphotericin B-induced water permeability changes has been observed in ergosterol-containing liposomes [14] but no explanation was given.

Finally, the relevance of the water and ionic permeability changes reported here for understanding the mechanism of action of polyene antibiotics on biological membranes is indicated by the fact that the concentrations of amphotericin B, nystatin and candicidin, exerting half of the maximal effect of osmotic water permeability of

Leishmania vesicles, do show a correlation with the minimal concentrations of such antibiotics required to inhibit growth of *Leishmania* promastigotes in vitro [34] and of other sensitive organisms (Table III).

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