Biochemical Pharmacology, Vol. 33, No. 22, pp. 3675–3680, 1984.
Printed in Great Britain.

0006-2952/84 $3.00 + 0.00

EFFECT OF SURFACE CURVATURE ON THE INTERACTION OF SINGLE LAMELLAR PHOSPHOLIPID VESICLES WITH AROMATIC AND NONAROMATIC HEPTAENE ANTIBIOTICS (VACIDIN A AND AMPHOTERICIN B)

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(*Received* 18 *January* 1984; *accepted* 25 *June* 1984)

Abstract—The interactions of unilamellar lipid vesicles with vacidin A, an aromatic heptaene antibiotic, and with amphotericin B, a nonaromatic heptaene antibiotic were compared.

Uptake of both antibiotics, monitored by circular dichroism, was found to be faster with small vesicles than with large ones.

By combining permeability measurements (Gary-Bobo and Cybulska, *J. Antibiotics* **35**, 1068 (1982)) and circular dichroism spectra, we found that for vacidin A, the same permeability inducing species is formed regardless of vesicles size. However, at a given concentration of antibiotic, less of the permeability inducing species is formed in the presence of small vesicles than in the presence of large vesicles. This may account for the differences between small and large vesicles in antibiotics-induced permeability. For amphotericin B, the permeability inducing species formed in the presence of small vesicles differs from that formed in the presence of large vesicles.

Aromatic heptaene macrolide antibiotics appear to be promising antifungal agents. Like most other large macrolide polyene antibiotics commonly used in therapy (e.g. amphotericin B), they induce permeability changes in all sterol-containing membranes [1–4]. Several methods may be used to measure permeability to specific ions such as K^+ or Na^+ in cell membranes or lipid vesicles. Lipid vesicles are convenient for the study of the structure–activity relationships because the interaction of membrane with antibiotic, measured spectroscopically, can be correlated to permeability. Initial experiments of this type [5] showed that the aromatic polyene antibiotics vacidin A (Vac)§ and candicidin D are less efficient than amphotericin B (AmB) on small unilamellar vesicles which are obtained by sonication (SUV) [6]. On the contrary, Vac is about 5 times more efficient than AmB [7] in inducing cation permeability in large unilamellar vesicles (LUV) which are obtained by reverse phase evaporation [8].

It was proposed that either Vac interacts more strongly with LUV than with SUV or the aromatic heptaene pores are more efficient than the nonaromatic ones [7]. The first hypothesis seemed more probably both since the physico-chemical properties of SUV and LUV are different and since the two types of vesicle do not interact with drugs at the same rate. This is true for the interaction of such vesicles with cytochrome oxidase [9], phospholipase A [10], phosphatidylcholine exchange protein [11] and α lactalbumin [12]. It was nevertheless important to check the second hypothesis. Verification of this hypothesis would lend insight on the mechanism of action of polyene antibiotics. To decide between these two explanations, we study, in the present work the circular dichroism (C.D.) of AmB and Vac in the presence of unilamellar vesicles. By using this spectroscopic method, the interaction of the drug with the membrane can be monitored directly, whereas with permeability measurements only the overall result of the interaction is observed. Furthermore, as we have shown [13, 14], C.D. appears to be the most suitable spectroscopic method for this type of study because unlike other spectroscopic methods it can reveal the existence of several conformational species of polyene antibiotics for a given type of vesicle and antibiotic/phospholipid ratio.

We have also used electronic absorption spectroscopy to determine whether the polyene antibiotics at low concentration bind to the vesicles: although absorption spectra are not very sensitive to the conformational changes of the polyene antibiotics, differences in the absorption maxima wavelengths between free antibiotic in water and antibiotic bound to phospholipid membranes can be detected. These changes, which can be monitored at antibiotic concentrations as low as 10^{-8} M were used to monitor the drug partition between water and lipid medium at concentrations too low to be detected with C.D.

MATERIALS AND METHODS

L-α-Phosphatidylcholine and L-α-phosphatidic acid were prepared from egg yolk according to Patel

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§ Abbreviations: DMSO, dimethylsulfoxide; HEPES, 4(2-hydroxyethyl)-1-piperazine ethane sulfonic acid; SUV, small unilamellar vesicles; LUV, large unilamellar vesicles; AmB, amphotericin B; Vac, vacidin A.

and Sparrow [15]. Cholesterol was purchased from Fluka and recrystallized in ethanol before use. AmB was a generous gift from Squibb France. Vac was isolated and purified in the Department of Pharmaceutical Technology, Technical University, Gdańsk (Poland).

All the vesicles were prepared with the same mixture of lipids: L-α-phosphatidylcholine, phosphatidic acid and cholesterol in a molar ratio of 70:10:20. The presence in the vesicles of a certain amount of negatively charged egg yolk phosphatidic acid did not affect the results, as has been already described for the interaction between AmB or Vac and egg yolk phosphatidylcholine SUV containing only 20% cholesterol [13, 14].

The influence of the curvature of the vesicles on the interaction with drugs can only be studied with well defined vesicles. In particular, multilamellar vesicles have to be discarded because in these liposomes a large portion of the phospolipid molecules constitutes the inner bilayers and is therefore not available to interact with the drug. This gives an effective ratio of antibiotic/lipid higher than the calculated one. To correct this, after sonication the SUV were filtered through a Sepharose 4B gel according to the procedure already described [5]. LUV were prepared according to the Papahadjopoulos method [8] in 10^{-2} M HEPES buffer at pH 7.4. This was not done when our results were compared with those of Gary-Bobo and Cybulska because in this case we followed exactly their procedure. The final phospholipid concentration was determined colorimetrically [16].

Under these conditions the SUV collected from the trailing half of the second fraction of the Sepharose 4B chromatography are well defined [6, 17], with an outer radius of 124 Å for a phosphatidylcholine, phosphatidic acid and cholesterol mixture in a molar ratio of 76:4:20 [17]. The absorption at 300 nm is stable for at least 8 hr, which indicates the absence of fusion or aggregation, as found in studies on phosphatidylcholine SUV above the phase transition temperature [18–20]. Sequential extrusion of the LUV through 0.4 and 0.2 μm Nucleopore membranes gives a fairly uniform population of vesicles [8, 12, 21] with a mean diameter of 1600 Å, as determined both by freeze fracture electron microscopy and dynamic light scattering [21]. The absorption of the LUV at 300 nm is also stable for at least 8 hr following preparation, which indicates the absence of agreggation.

The concentrations of the polyene antibiotics were determined from their absorption in DMSO and calculated for pure compounds. The antibiotics were first dissolved in DMSO and then dispersed in HEPES buffer. These suspensions were added either by small increments ($2–5 \times 10^{-7}$ M) or all at once. The final DMSO content was always lower than 0.5%. The spectra were recorded at 22°, 1 hr after the antibiotics were added.

Circular dichroism spectra were recorded with a Jobin-Yvon Mark III dichrograph equipped with a Nicolet 1171 signal averager or with a Jobin-Yvon Mark V dichrograph. In the figures $\Delta\varepsilon$ is the differential molar dichroic absorption coefficient (10^3 cm^2/mole); the spectra of the suspensions of vesicles without antibiotic were subtracted from those with antibiotic. Electronic absorption spectra were recorded with a Cary 219 (Varian) spectrophotometer. The precautions used in recording the spectra have been previously described [13]. Light scattering should not perturb the CD spectra of the antibiotics embedded in the bilayers of the vesicles because the size parameter X of the LUV and SUV are respectively 1.25 and 0.2 ($X = 2\pi a/\lambda$, where a is the radius of the sphere and λ the wavelength, here 400 nm). Under these conditions the CD spectrum for spheres is similar to the intrinsic CD spectrum [22]. Furthermore at 500 nm where there is no antibiotic absorption, the absorption spectra are unperturbed, even after 6 hr. Increased turbidity is interpreted as aggregation or fusion of the vesicles [18–20]; the absence of changes in turbidity here means that these phenomena do not occur to an appreciable extent. This is in agreement with a previous observation [23] that at most 90% of the complex lecithin-cholesterol vesicles–amphotericin B remains in the supernatant after centrifugation at 100,000 g. It has been observed by electron microscopy [24] that lecithin-cholesterol vesicles swell in the presence of amphotericin B. The differences in the experimental conditions (ratio antibiotic/lipid $R = 1.2 \times 10^{-2}$, incubation 20 hr at 4° for electron microscopy) may explain this discrepancy. Also, the aggregated state of the free aromatic polyene antibiotics in solution and the corresponding Duysens effect influence the intensity of the absorption bands, which is approximately half that of the antibiotics embedded in the vesicles.

RESULTS

Measurements and comparisons of the interactions of AmB and Vac with the SUV and the LUV were performed under two different conditions. First the procedure described by Cybulska and Gary-Bobo in their permeability experiments [7] was followed: SUV at a concentration of 3 mg/ml of total lipid was compared to LUV at a concentration of 0.3 mg/ml. Second we compared SUV, filtered on Sepharose 4B, to ultrafiltered LUV, using both types of vesicles at the same final concentration, 1.30 mM (1.33 mg of total lipids/ml). In both cases the results were the same, in the concentration range studied the important parameter is the antibiotic/lipid ratio and not the absolute concentrations.

In all the cases studied, (antibiotic/phospholipid ratios, smaller than 5×10^{-3}), when the polyene antibiotics were mixed with the vesicles, the CD spectra of the free antibiotics disappeared and were superseded by new ones. In the spectra given below we subtracted from the observed spectra the scattering contribution of the vesicles, which was very weak with SUV and more significant with LUV.

Dependence of the AmB CD spectra on the AmB/lipid ratio R and the size of the vesicles

With SUV we obtained results identical to those already described for SUV without the negative charges of phosphatidic acid [13]: when the molar ratio between antibiotic and total lipids was low ($R < 10^{-4}$), no permeability was observed. The CD

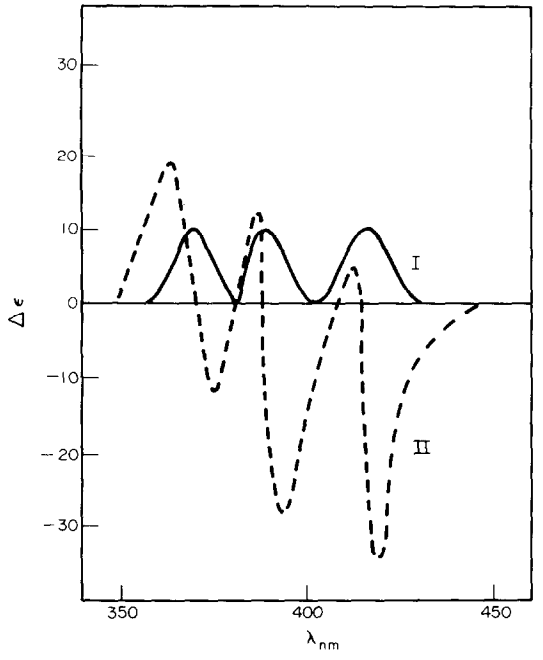

Fig. 1. Circular dichroism spectra of amphotericin B in the presence of cholesterol containing SUV (phosphatidylcholine, phosphatidic acid and cholesterol in a molar ratio 70:10:20; final lipid concentration 3 mg/ml). I: ratio antibiotic/lipid R: 10^{-4}; II: ratio antibiotic/lipid R: 8×10^{-4}. $\Delta\varepsilon$ is the differential molar dichroic absorption coefficient (10^3 cm^2/mole).

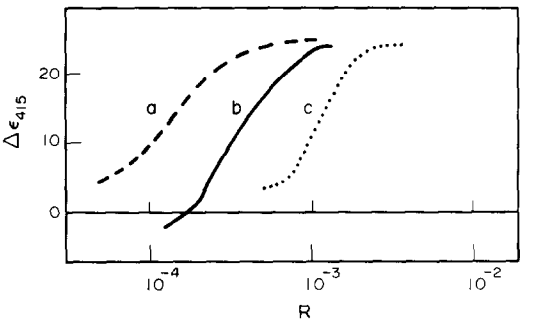

Fig. 2. Differential molar dichroic absorption coefficient at 415 nm of amphotericin B and vacidin A in the presence of phospholipid vesicle 1.33 mg/ml of total lipid as a function of the molar ratio R antibiotic/total lipid. (a) vacidin A in the presence of LUV; (b) amphotericin B in the presence of SUV; (c) vacidin A in the presence of SUV.

spectrum consisted of three positive bands (type I, attributed to AmB in monomeric form either adsorbed or dissolved in the vesicle bilayer). As R increases, the membranes became increasingly permeable and a new CD spectrum appeared, consisting of alternatively positive and negative bands (type II) (Figs. 1 and 2).

The same type I spectrum was observed with LUV for $R < 10^{-4}$. However, at higher R, the spectrum was not the same as that with SUV (Fig. 3). Instead of the self-associated free AmB spectrum transforming into the type II spectrum in approximately 15 min, one first observes a spectrum with a negative band at 417 nm and weak $\Delta\varepsilon$, and progressively, 1 or 2 hr later, a new spectrum appears with a positive band at 430 nm. During the same time, the excitonic doublet of free AmB decreases in intensity and is blue shifted (from 342 to 332 nm). It is difficult to quantitate these changes because there are no well defined steps, but these new characteristics appear only when R is higher than 10^{-4}, that is, in the same range as the appearance of type II AmB spectrum with SUV.

Dependence of the Vac CD spectra on the Vac/lipid ratio R

We have already described [14] the C.D. characteristics of the interaction of Vac with SUV: in the presence of SUV that contain 20% cholesterol the intense spectrum of free aggregated Vac disappeared and was superseded by spectra of bound Vac which varies with R. At low R (approximately 5×10^{-4}) weak positive bands ($\Delta\varepsilon \sim 5$) between 415 and 330 nm were observed. This type I′ spectrum is assigned to antibiotic molecules adsorbed in mono-

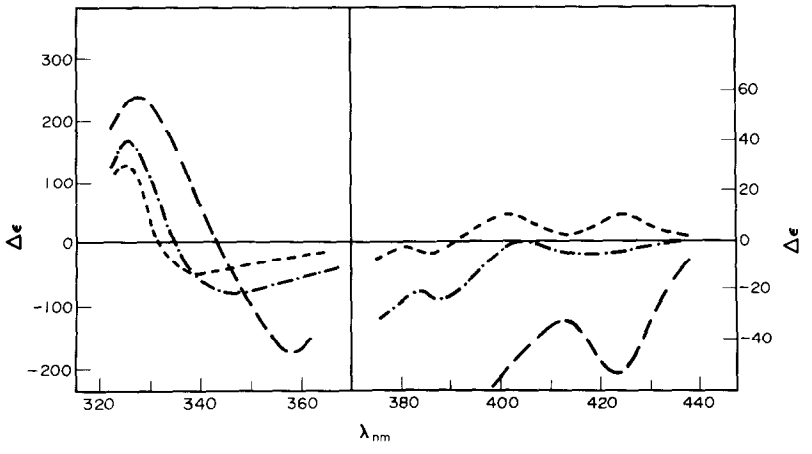

Fig. 3. Circular dichroism spectra of amphotericin B (2×10^{-6} M) in the presence of LUV (1.33 mg of total lipid/ml) and as a function of time: ———, $\Delta t = 2$ min; —·—·, $\Delta t = 48$ min; – – – –, $\Delta t = 4$ hr.

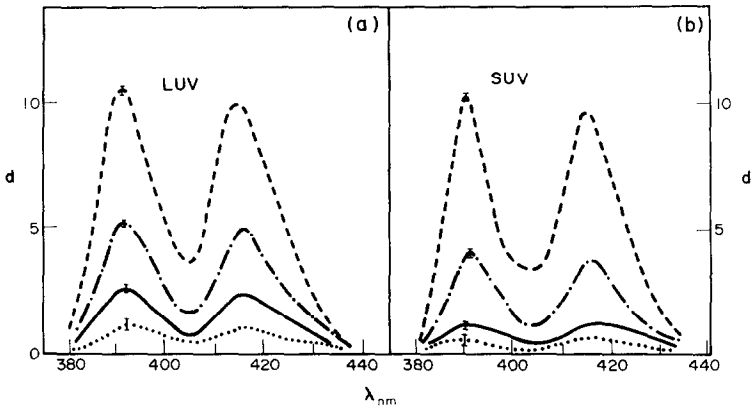

Fig. 4. Comparison of the circular dichroic spectra of Vacidin A in the presence of LUV or SUV (1.33 mg of total lipid/ml) as a function of the molar ratio antibiotic/lipid R. (a) in the presence of LUV; (b) in the presence of SUV; ———, $R = 32 \times 10^{-4}$; —·—·, $R = 16 \times 10^{-4}$; ———, $R = 8 \times 10^{-4}$; ....., $R = 4 \times 10^{-4}$.

meric form on the surface of the lipid bilayer. As R progressively increases, stronger bands were observed (two positive bands at 415 and 392 nm and two negative ones at 383 and 365 nm representing the type II′ spectrum that is assigned to polyene–cholesterol complexes). When the vesicles contained 20% cholesterol, the $\Delta\varepsilon$ values of these bands were respectively +22, +23, −8 and −14.

Contrary to what is observed with AmB, the C.D. spectra of Vac in the presence of either SUV or LUV are the same. However, the R values at which the type II′ spectrum begins to appear are not the same. With SUV, the type II spectrum appears at around $R = 7 \times 10^{-4}$, with LUV around $R = 10^{-4}$ (Figs. 2 and 4).

With cholesterol-free SUV or LUV, type I′ spectra are observed. However, the binding constant is weaker with LUV than with SUV: with LUV, as soon as $R > 2 \times 10^{-3}$ the spectrum of free aggregated Vac with a negative peak at 418 nm begins to reappear, indicating that the LUV are saturated (Fig. 5). With SUV this limit is reached at $R > 10^{-2}$ which indicates that their capacity of sorption is greater.

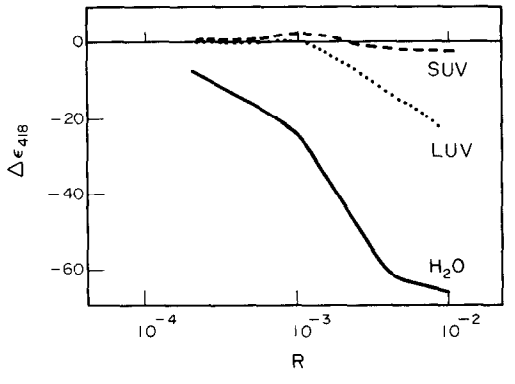

Fig. 5. Differential molar dichroic absorption coefficient at 418 nm of vacidin A in the presence of phospholipid vesicles without cholesterol (1.33 mg/ml of total lipid) as a function of the molar ratio R antibiotic/lipid: – – – –, in the presence of SUV; . . . . . in the presence of LUV; ———, free vacidin A in buffer at the corresponding concentration. The increase of $\Delta\varepsilon$ when the concentration increases is due to the progressive formation of self-aggregated vacidin A [21].

Comparison of the kinetics of interaction of AmB and Vac with SUV and LUV

The interaction of both antibiotics at a concentration of 1.5×10^{-5} M with vesicles at a concentration of 1.3 mM ($R = 1.5 \times 10^{-3}$ was followed as a function of time.

In Fig. 6 the interaction of AmB is monitored, first by the disappearance (SUV) or decrease (LUV) of the excitonic doublet of free self-associated amphotericin (positive maximum at 328 nm) and second by the decrease of the negative band at 423 nm of free self-associated amphotericin. The rate of disappearance of free AmB is definitely slower with LUV than with SUV.

In Fig. 7 the interaction of Vac is monitored by the disappearance of the positive peak of the excitonic doublet at 342 nm or of the negative peak at 418 nm of free self-associated Vac. Here again the reaction is faster with SUV.

Exchange of Vac molecules between SUV

Since we demonstrated such an exchange with AmB and SUV [18], we also tried to demonstrate it with Vac.

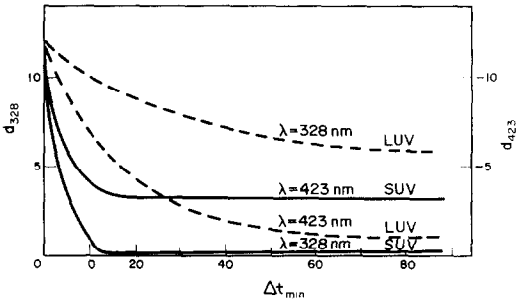

Fig. 6. Comparison of the decrease of the circular dichroism of amphotericin B (1.5×10^{-6} M) at 423 and 328 nm in the presence of SUV and LUV (1.33 mg total lipid/ml) as a function of time: ———, LUV; ———, SUV.

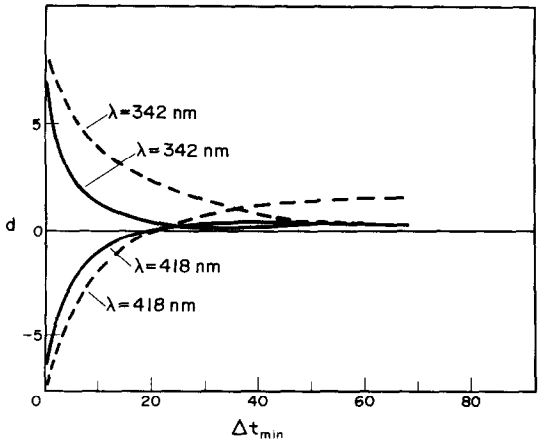

Fig. 7. Comparison of the decrease of the circular dichroism of vacidin A (1.5×10^{-6} M) at 418 and 342 nm in the presence of SUV and LUV (1.33 mg total lipid/ml) as a function of time: -----, SUV; ———, LUV.

Vac (0.8×10^{-5} M) was allowed to react with SUV at the concentration giving an R of 8×10^{-3} (1 mM). Under these conditions we observed the type II′ spectrum. When the reaction was completed, the sample was divided in two parts. The first was diluted with an equivalent volume of buffer, the second with an equivalent volume of a concentrated suspension of vesicles, 11 mM. At this concentration of vesicles the final R value (6.67×10^{-4}) corresponded to conditions where type I′ would be observed if an exchange of Vac existed. Indeed, a type I′ spectrum was observed; in the first sample, however, type II′ was still observed.

Absorption spectra of AmB or Vac in the presence of vesicles

We will only consider the first absorption band, that is the first intense band of vibronic progression representing the transition $1^1Ag \rightarrow 1^1B_u$. In free AmB this band is located at 409 nm for the monomeric form (below the critical micellar concentration of 2×10^{-7} M) and at 420 nm for the self associated form (above the critical micellar concentration). In free Vac the corresponding bands are respectively at 402 and 413 nm.

Table 1 shows the absorption wavelengths for an antibiotic/lipid ratio R of 10^{-4}, with lipid concentration at either 0.1 mM or 1 mM, for antibiotic concentrations of 10^{-8} M and 10^{-7} M, respectively. The results for R = 10^{-2} at a lipid concentration of 1 mM are also presented in Table 1.

For AmB and SUV, in all the cases considered, the absorption band is shifted while for AmB and LUV the shift occurs only for values of R higher than 10^{-4}. For Vac the same shift is observed with both SUV and LUV.

DISCUSSION

(a) *Interaction of vacidin with vesicles*

Permeability–circular dichroism relationship. We have already [14] related the Vac-induced permeability in the SUV to the appearance of a type II′ CD spectrum (two strong positive bands at 415 and 392 nm and two negative bands at 383 and 365 nm): the curves giving $\Delta\varepsilon$ and the percentage of proton release, as a function of R, have the same sigmoidal shape with a mid-point at about the same R value (the C.D. results are the same whether the experiments are performed in 10 mM HEPES buffer or in 400 mM SO_4Na_2). Under our experimental conditions the midpoint of the variation is approximately located at R = 1.5×10^{-3}; that is, about 4.5 molecules of Vac per vesicle are necessary to release 50% of the protons [7].

The same relationship is observed with LUV although the appearance of a type II′ spectrum begins to occur at an R value of $\sim 10^{-4}$, which is lower than that observed with SUV. For LUV, the midpoint of the variation is located approximately at R = 2×10^{-4}, i.e. about 30 molecules of Vac per vesicle for a 50% proton release [7]. Actually the corresponding absolute concentration of Vac is 7 times lower with LUV than with SUV.

From the similarity of the CD spectra in both cases we can conclude that the permeability inducing species are similar. Furthermore, the shift of the permeability dose–response curves parallel the appearance of the type II′ spectrum when going from SUV to LUV. Thus, the reason that smaller amounts of Vac induce permeability in the LUV than in the SUV is that the species which induces permeability is more easily formed in the LUV. The alternative explanation is that one pore in a LUV releases many more cations than one pore in a SUV [7] because the internal content of a LUV is much higher than that of a SUV; it seems therefore unreasonable. Moreover, if this second explanation were true, one would observe permeability dose–response curves starting

Table 1. U.v.-visible absorption: wavelength of the first intense band of the vibronic progression of the transition $1^1Ag \rightarrow 1^1Bu$ or Amphotericin B or Vacidin A free in water or in the presence of vesicles

	Amphotericin B			Vacidin A		
	C = 10^{-8} R = 10^{-4}	C = 10^{-7} R = 10^{-4}	C = 10^{-5}	C = 10^{-8} R = 10^{-4}	C = 10^{-7} R = 10^{-4}	C = 10^{-5}
Free	409		420	402		413
SUV	410	412	413	407	409	410
LUV	409	409	413	407	409	411

The wavelengths are given at ± 0.5 nm.
R: antibiotic/total lipid molar ratio; C: antibiotic concentration (M).

from the same point for both the SUV and the LUV but having different slopes (steeper with LUV), which was not the case.

Presence of two types of bound vacidin. The real $\Delta\varepsilon$ of the permeability inducing species (type II′) is higher than that observed under our experimental conditions; in fact, with higher cholesterol contents we observed a $\Delta\varepsilon$ at least two times higher [14]. Therefore, only a portion of the Vac molecules goes to form the permeability inducing species while the remaining molecules are dispersed on the bilayer, under monomeric form with small C.D. (type I′). As far as can be detected by absorption, no Vac molecules remain in solution, since both the absorption and the C.D. spectra of free Vac disappear. The type I′ species was also observed in the vesicles of pure lecithin. We can assume then, that in the cholesterol-containing vesicles this species occurs through binding to the regions of pure lecithin. The type II′ species occurs through binding either to cholesterol-rich regions or to the boundaries between cholesterol-rich and pure lecithin regions. It is interesting to note that the CD spectrum of Vac (type II) closely resembles that of candicidin, another aromatic heptaene antibiotic, in aqueous solution, in the presence of cholesterol [25].

Origin of the difference between LUV and SUV to permeability induced by vacidin. In the case of cholesterol-containing vesicles we have seen that Vac induces proton leakage more easily in LUV than in SUV. For instance, at $R = 5 \times 10^{-4}$ pores exist in the LUV but not in the SUV as demonstrated by C.D. and by permeability measurements. The absence of free Vac in solution in both cases means that the difference between LUV and SUV does not originate from a difference in the partition coefficient of Vac between water and membranes. The greater affinity of Vac for pure lecithin SUV than for pure lecithin LUV (type I′ species) may explain this difference, at least in part. This observation is in agreement with the generally observed stronger binding of ligand to vesicles with higher curvature, for instance binding of the phosphatidylcholine exchange protein [11]. As we have shown, there is a competitive equilibrium between type I′ and type II′ species. If the formation of species II′ has the same constant with either LUV or SUV, as the formation constant of type I′ species decreases, the type II′ species, which induces permeability, is formed.

(b) *Interaction of amphotericin B with vesicles*

The existence of at least two competing species of bound AmB can be demonstrated in the same manner as with Vac. Studies of AmB induced permeability in black lipid membranes indicate that the equilibrium among three AmB species may explain the observed kinetics [26].

However, the interaction of AmB with LUV is not the same as with SUV. The conformations of the two permeabilizing species are different although they are formed at approximately the same R. On the other hand, at low R (10^{-4}) AmB binds to SUV but not to LUV, since with SUV the absorption bands are shifted relative to those of free AmB whereas with LUV they are not.

Vac and AmB display similar permeability and C.D dose–response curves. Furthermore molecules of both antibiotics are exchanged between vesicles. These observations indicate that the difference in the permeabilizing effects of the antibiotics for Na^+ ions may be explaining by differences of the number of pores formed.

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