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Interaction of the polyene antibiotic etruscomycin with large unilamellar lipid vesicles: binding and proton permeability inducement

Elisabetta Capuozzo * and Jacques Bolard **

Département de Recherches Physiques (LA CNRS No. 71), Université Pierre et Marie Curie, 4 Place Jussieu, 75230 Paris Cedex 05 (France)

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The effect of the polyene antibiotic etruscomycin on the permeability of large unilamellar lipid vesicles was investigated. Proton leakage was induced in egg-yolk phosphatidylcholine (EPC) vesicles only when sterol was present in the membrane; the extent of leakage was limited. High etruscomycin/lipid ratios (R) were necessary ($R > 0.1$). Higher percentages of sterol increased the permeability, slightly more strongly for ergosterol than for cholesterol. Dipalmitoylphosphatidylcholine (DPPC) vesicles were more sensitive to permeability inducement, even in the absence of sterol in the bilayer (inducement for $R > 0.06$). The interactions of etruscomycin with the vesicles were examined by circular dichroism, fluorescence and ^{31}P-NMR. In the range of antibiotic concentration where permeability was induced, $R > 0.1$ for EPC vesicles, $R > 0.06$ for DPPC vesicles, etruscomycin exhibited characteristic circular dichroism spectra independent of the presence of sterol. Under the same conditions, ^{31}P-NMR and fluorescence studies indicated a destruction or a fusion of the vesicle bilayer. At lower etruscomycin concentrations ($R < 0.03$), the etruscomycin circular dichroism spectra were different, indicating that the interaction with membranes containing ergosterol differed from that with membranes containing cholesterol. From correlating the increase in fluorescence intensity with this interaction, as well as from exchange experiments, it was inferred that etruscomycin at a low antibiotic/lipid ratio is more strongly bound to ergosterol-containing vesicles than to cholesterol-containing vesicles. These results and their comparison with the results obtained with other polyene antibiotics indicate that at low R etruscomycin resembles amphotericin rather than filipin in its preferential binding to ergosterol-containing vesicles. At higher R, that is in conditions where permeability is induced, the selectivity is different. The corresponding mechanism seems not to involve the formation of an etruscomycin-sterol channel, since the hydrophobic chain of the complex would be too short to form a channel.

Introduction

The mechanism of action of polyene antibiotics is not well understood, in spite of the impressive quantity of data gathered in recent years. In particular, the exact molecular basis of the selective toxicity for fungi of amphotericin B and nystatin, the two polyenes which are used most frequently,

* Present address: Institute of Biological Chemistry, University of Rome, Center for Molecular Biology, C.N.R., Rome, Italy.

** To whom correspondence should be addressed.

Abbreviations: PC, phosphatidylcholine; DPPC, dipalmitoylphosphatidylcholine; NMR, nuclear magnetic resonance; CD, circular dichroism; FCCP, carbonyl cyanide *p*-(trifluoromethoxy)phenylhydrazone; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

has not been clearly established. These two drugs are clinically useful apparently because they interact preferentially with the sterols of fungal cell membranes, (which contain ergosterol rather than the cholesterol found in animal cells) [1–5]. Indeed, amphotericin B binds more strongly to ergosterol-containing vesicles from egg PC than to cholesterol-containing vesicles [6–9]; and it is possible to observe this differential selectivity for these two sterols in methanol/water solutions [10]. Another polyene antibiotic, filipin, too toxic for use as an antifungal agent, accordingly binds in water more strongly to cholesterol than to ergosterol [11] and similarly to cholesterol-containing vesicles from egg PC more strongly than to ergosterol-containing vesicles [12,13]. Filipin interacts with cholesterol monolayers more than with ergosterol monolayers [1].

These differences between the heptaene amphotericin B and the pentaene filipin led to the proposal that the number of carbon atoms in the hydrophobic part of the polyene molecule may determine the selectivity of the polyene-sterol interaction. However, another structural difference between amphotericin B and filipin suggested as the basis for sterol selectivity is the presence of a sugar (mycosamine) in the amphotericin B molecule that is absent in filipin [14]. To distinguish the contribution of these two factors, it seemed worthwhile to investigate the selectivity of the tetraene etruscomycin (lucensomycin), which contains a mycosamine moiety and has fewer double bonds than amphotericin B. Study of the K^+ released from multilamellar vesicles by etruscomycin has shown that the sensitivity is increased by the incorporation of ergosterol in the membrane, more than by the incorporation of cholesterol [6]. On the other hand, it has been shown that etruscomycin binds more strongly in aqueous solutions to cholesterol than to ergosterol [15].

To obtain more information on the selectivity of etruscomycin for these two sterols, we used fluorescence and circular dichroism to investigate the interaction of this antibiotic with large unilamellar vesicles, which afford a better model for cell membranes than multilamellar vesicles. Fluorescence has been shown to be an excellent method to monitor the interaction of etruscomycin with membranes [16], and circular dichroism, by its sensitivity to the conformational changes of the antibiotic molecule, can be used to detect several bound species in competition [17]. We also used a proton-cation exchange method [18] as well as ^{31}P-NMR to examine the permeability, induced by etruscomycin, of the membrane of large unilamellar vesicles to protons.

Materials and Methods

L-α-Phosphatidylcholine was prepared from egg-yolk according to Patel and Sparrow [19]. Phosphatidic acid was prepared from egg-yolk according to Roux et al. [20]. Cholesterol and ergosterol were purchased from Carlo Erba and Sigma, respectively. Both sterols were purified by recrystallization in ethanol before use. L-α-Dipalmitoylphosphatidylcholine was supplied by Sigma and used without further purification. FCCP was purchased from Boehringer. 6-Carboxyfluorescein was purchased from Eastman-Kodak and purified according to Ralston et al. [21]. Etruscomycin (lucensomycin) was a kind gift from Farmitalia, Milan, Italy. Large unilamellar vesicles were prepared according to Szoka et al. [22]. Vesicles were prepared above the particular transition temperature (T_c) of the phospholipid.

Permeability studies

Proton efflux was monitored by the pH-stat technique and by ^{31}P-NMR spectroscopy according to an electroneutral proton-cation exchange method [23,24].

(a) pH-stat method. Large unilamellar vesicles were prepared using 40 μmol per ml of lipid mixture of a 200 mM sodium phosphate/100 mM sodium sulfate solution (pH 6.5). The vesicle suspension was subsequently filtered through polycarbonate porous membranes (Nuclepore Corporation, Pleasanton, CA), first using 1 μm and then 0.4 μm pore size.

0.5 ml of the vesicle suspension (6 μmol of lipids) was diluted in 3.5 ml of 375 mM sodium sulfate solution in the titrating vessel of a pH-stat (Radiometer, Copenhagen). The vesicle suspension was equilibrated at 20°C under a nitrogen stream and the pH adjusted to 7.5. Then 10 μl of 1 mM FCCP solution in ethanol were added. Subsequently, the desired amount of polyene in solution

in dimethyl sulfoxide was added. The proton efflux was measured as the volume of 5 mM NaOH solution in 375 mM sodium sulfate necessary to maintain pH 7.5. The total proton concentration inside the vesicles was measured in the same way by addition of Triton X-100. Proton efflux is expressed as a percentage of this total proton concentration.

(b) ^{31}P-NMR method. Large unilamellar vesicles were prepared using 70 μmol of lipid mixture (52% egg yolk PC, 18% egg yolk phosphatidic acid, 30% cholesterol per milliliter of a 200 mM sodium phosphate/200 mM sulfate/1 mM EDTA solution in 40% ^2H_2O (pH 5.50). The vesicles, diluted 4-times in 400 mM sodium sulfate, were filtered as described above. 2.5 ml (about 45 μmol of lipid) were adjusted to pH 7.50. Under these conditions the chemical shifts of the ^{31}P-NMR signals of the external phosphate ions (pH 7.5) and of the internal phosphate ions (pH 5.5) were different [25]. 20 μl of a 100 mM $MnCl_2$ solution were then added to the vesicle suspension; this broadened the external phosphate signals to such an extent that they were no longer detectable. The possible pH variation of the vesicular internal medium upon the addition of polyene antibiotic was then calculated from the chemical shift of the still-observable internal phosphate signal (Ref. 26 and unpublished data). ^{31}P-NMR spectra, with proton noise decoupling, were recorded at 36.4 MHz on a Brucker WH 90. 10 min were necessary for recording each spectrum.

(c) Fluorescence spectroscopy. Egg-yolk phosphatidylcholine vesicles containing 30 mol% cholesterol were loaded with a high concentration of the fluorescent dye 6-carboxyfluorescein (100 mM). At this concentration its fluorescence was self-quenched [27]. These vesicles were centrifuged at $30000 \times g$ and washed, three times. Then they were diluted in the buffer free of the fluorescent dye; the final concentration in lipids was 2 mM. If etruscomycin was then added and induced permeability to 6-carboxyfluorescein, this fluorophore leaked into the aqueous medium and, diluting, fluoresced.

Spectroscopic studies

Vesicles were prepared as described above in 100 mM KCl buffered with 10 mM Hepes, pH 7.5, with minor modifications of the lipid concentrations. Stock solutions of etruscomycin were prepared daily and added either as a $2 \cdot 10^{-2}$ M dimethyl sulfoxide solution or as a suspension in buffer containing less than 0.5% dimethyl sulfoxide. No significant differences were observed between these two procedures.

CD spectra were recorded with a Jobin-Yvon Mark III dichrograph equipped with a Nicolet 1171 signal averager. Spectral wavelengths are reported to ±0.5 nm. Electronic absorption spectra were recorded with a Cary 219 (Varian) spectrophotometer. All experiments were carried out at 22°C. Fluorescence spectra were recorded on an Aminco SPF spectrofluorimeter using 1 cm cells, excitation slit-width of either 1 or 4 nm, and emission slit of 4 nm. Temperature was kept at 20°C. All measurements were carried out after equilibrium had been reached, as checked by repeated measurements; generally a delay of at least 2–3 h was observed between preparation and spectrum recording of samples, except for the 6-carboxyfluorescein release measurements, where the spectra were recorded 15 min after addition of etruscomycin to the vesicle suspension.

Results

Permeability studies

The permeability induced by etruscomycin in large unilamellar vesicles was measured by the proton-cation exchange method [18] and monitored by two different techniques: pH-stat and ^{31}P-NMR spectroscopy.

(a) the pH-stat method. We observed that when etruscomycin was added to egg-yolk phosphatidylcholine vesicles the proton efflux developed rapidly, reaching a plateau within 10–15 min. In the presence of pure dipalmitoylphosphatidylcholine vesicles, a plateau was reached within 1 h. The percentage of proton released at these plateaus depended on the etruscomycin concentration. Fig. 1 shows the curves obtained by plotting the percentage of proton efflux vs. either etruscomycin concentration or the molar ratio, R, expressed in moles of antibiotic per mole of lipid. Each curve gives the results obtained with one batch of vesicles containing a given amount of sterol. The following information can be obtained from these plots.

(1) Proton permeability was induced only at antibiotic concentrations higher than 10^{-4} M, corresponding to antibiotic/lipid ratios higher than 0.1.

(2) Dipalmitoylphosphatidylcholine vesicles are more sensitive than egg-yolk phosphatidylcholine vesicles to etruscomycin, even in the absence of sterol in the bilayer.

(3) Proton efflux does not occur in egg-yolk phosphatidylcholine vesicles (or almost not) unless sterol is present in the membrane, and it increases with the percentage of sterol in the membranes.

(4) Ergosterol-containing vesicles are slightly more sensitive to etruscomycin (down to 20%) than cholesterol-containing ones. Furthermore, it can be seen in Fig. 3 that the sigmoidal curves obtained at different sterol concentrations never reach the maximum value.

(b) ^{31}P-NMR spectroscopy. This was used to measure the etruscomycin-induced proton efflux by monitoring the chemical shift of the intravesicular phosphate ion signal. The paramagnetic Mn^{2+} broadened the external phosphate signals. At an antibiotic-lipid ratio $R = 0.43$ sufficient to induce 55% proton leakage, according to the previous results, we observed in 12 min the disappearance of approx. 40% of the initial signal and in 33 min 60% (Fig. 2). Upon addition of amphotericin B at a concentration sufficient to induce the same proton leakage as in the former experiment, the spectra were strikingly different: after antibiotic was added the internal phosphate signal rapidly split in two parts, the first one slowly decreasing in intensity and the second one increasing (unpublished data).

(c) Fluorescence. A 100 mM solution of 6-carboxyfluorescein was sequestered in vesicles. Fig. 3 shows the results obtained by incubating these vesicles with increasing concentrations of etruscomycin. It can be seen that fluorescence intensity measured at 520 nm (wavelength of emission maximum of 6-carboxyfluorescein by excitation at 490 nm) increased with increasing antibiotic-lipid ratio and began to appear in the same concentration range at which we observed a proton efflux. Since the self-quenching largely prevents the dye from fluorescing as long as it remains sequestered in vesicles, it is clear that a considerable amount of dye was released from the vesicles and diffused into the medium. In contrast, when vesicles containing 30% cholesterol were incubated with amphotericin B, carboxyfluorescein was released, though weakly. This occurred only at much higher antibiotic-lipid ratios ($R > 3 \cdot 10^{-3}$) than that observed for amphotericin-induced proton leakage [23].

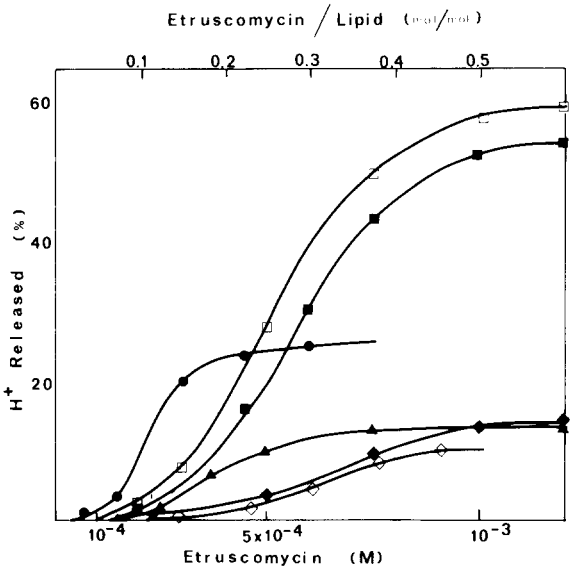

Fig. 1. Proton release from cholesterol- and ergosterol-containing vesicles as a function of either etruscomycin concentration or etruscomycin/total lipids ratio (R). The total lipid concentration was 2 mM at the beginning of the H^+ efflux. This figure gives the mean curves obtained from several experiments. Symbols: egg yolk phosphatidylcholine vesicles containing (◇) 10% cholesterol, (◆) 20% cholesterol, (■) 30% cholesterol, (□) 40% cholesterol, (▼) 10 and 20% ergosterol; ●, pure DPPC vesicles.

Circular dichroism studies

The experiments were performed by adding to vesicles either small amounts of the etruscomycin solution in dimethyl sulfoxide or small amounts of the antibiotic suspended in buffer. In the former case the spectra were recorded at intervals and we did not observe further changes after 2 h. Identical spectra were obtained in both types of experiments for a given R value.

In Hepes buffer, at pH 7.5, the spectrum of etruscomycin ($7 \cdot 10^{-6}$ M) was characterized by three positive bands at 292, 305 and 320 nm whose $\Delta\varepsilon$ values were 4, 10 and 9, respectively (Fig. 4a).

The interaction of etruscomycin with the vesicles was monitored by the changes in the circular di-

chroism spectrum of free etruscomycin. In the presence of cholesterol-containing egg-yolk phosphatidylcholine vesicles at an R value of $7 \cdot 10^{-3}$ (expressed as moles of antibiotic per mole of lipids), the free etruscomycin bands at 292, 305 and 320 nm shifted to 295 ($\Delta\varepsilon = +9$), 308 ($\Delta\varepsilon = +13$) and 323 ($\Delta\varepsilon = 7$), as shown in Fig. 4b. The same red-shift was observed with DPPC and egg-yolk phosphatidylcholine vesicles not containing sterol. However, upon addition of ergosterol-containing vesicles a more pronounced red-shift was observed: at the same R value ($7 \cdot 10^{-3}$) the free bands at 305 and 320 nm shifted to 315 ($\Delta\varepsilon = 12$) and 330 nm ($\Delta\varepsilon = 10$). At a higher antibiotic concentration ($3 \cdot 10^{-4}$ M), corresponding to an R value of 0.2, a new spectrum appeared which had the same characteristics whether or not sterol was present in the membrane. This spectrum consisted of two negative peaks at 326 and 312 nm and two positive peaks at 305 and 295 nm with a shoulder at 285 nm (Fig. 5b, c). However, while this spectrum appeared with DPPC vesicles or sterol-containing vesicles for $R > 7 \cdot 10^{-2}$, with egg-yolk phosphatidylcholine it appeared for $R > 0.2$. Although the appearance of this spectrum correlated with permeability inducement, its intensity did not depend on the amount of sterol present in the bilayers.

Exchange of etruscomycin molecules between vesicles

The difference between the CD spectra of

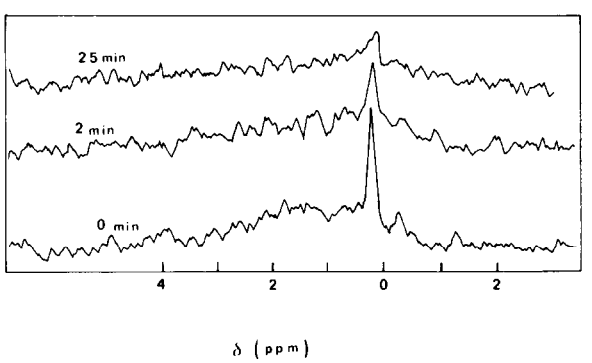

Fig. 2. Time dependence of the ^{31}P-NMR spectra of lipid vesicles (total lipid concentration 18 mM; internal phosphate concentration 200 mM; internal pH 5.5) after the addition of 7.75 mM etruscomycin. The signals of the external phosphate ions were broadened by the presence of 0.4 mM $MnCl_2$. The times given are the beginning of the recording of spectra.

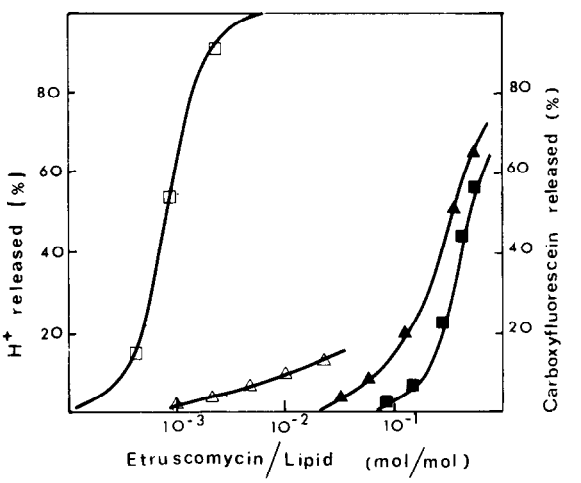

Fig. 3. Comparison of 6-carboxyfluorescein and proton releases from 30% (mol/mol) cholesterol-containing egg yolk phosphatidylcholine vesicles as a function of amphotericin B or etruscomycin concentrations. For the incubation, the total lipid concentration was 2 mM. The suspension was then diluted 200-times for recording the fluorescence spectra. Symbols: 6-carboxyfluorescein release in the presence of (△) amphotericin B or (▲) etruscomycin. Proton release in the presence of (□) amphotericin or (■) etruscomycin. The dose-response curve for proton release in the presence of amphotericin B was drawn from Gary-Bobo and Cybulska [23] with 20% cholesterol-containing vesicles.

etruscomycin in the presence of ergosterol-containing vesicles or cholesterol-containing vesicles was used to monitor this exchange. If cholesterol-containing vesicles were loaded with etruscomycin at $R = 0.05$ and then added to the same amount of ergosterol-containing vesicles, the CD band observed initially at 323 nm shifted in less than 15 min to 330 nm, which indicated that etruscomycin was totally transferred to the ergosterol-containing vesicles. If ergosterol-containing vesicles were loaded with the antibiotic, no CD change was observed when cholesterol-containing vesicles were added, indicating that no exchange occurred.

Electronic absorption studies

The binding of etruscomycin to vesicles was accompanied by changes in its absorption spectrum: the antibiotic spectrum in aqueous solution was characterized by three bands, at 292, 305 and 320 nm. Upon addition of vesicles, a small red-shift occurred, associated with a reduced intensity of the absorption bands, especially that at 320 nm.

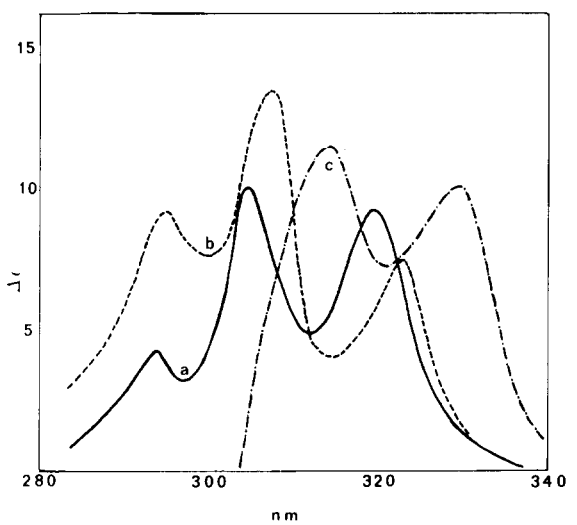

Fig. 4. CD spectra at 25°C of $7 \cdot 10^{-6}$ M etruscomycin in the presence of 1 mM cholesterol or ergosterol egg yolk phosphatidylcholine vesicles ($R = 7 \cdot 10^{-3}$). Spectra have been recorded approximately 1 h after the mixture. The background due to the dichroic scattering of the vesicles has been subtracted. a, free etruscomycin in aqueous medium; b, 20 mol% cholesterol; c, 20 mol% ergosterol; $\Delta\varepsilon$ is the differential molar dichroic absorption coefficient (10^3 cm^2·mol^{-1}). This value was determined from the intensity of the signal recorded on the dichrograph, referred to the total etruscomycin concentration. With ergosterol-containing vesicles the dichroism due to ergosterol is very important between 300 and 280 nm and has not been drawn.

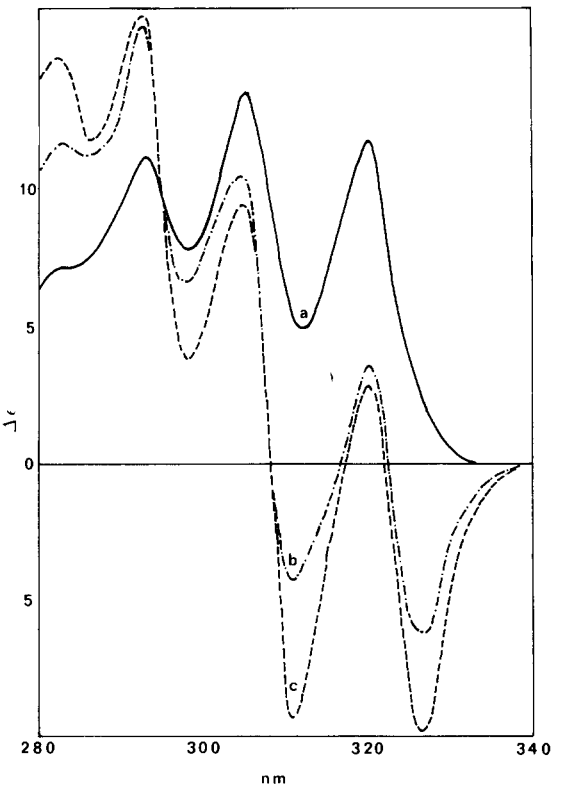

Fig. 5. CD spectra at 25°C of $3.5 \cdot 10^{-4}$ M etruscomycin in the presence of 1.5 mM cholesterol or ergosterol egg yolk phosphatidylcholine vesicles ($R = 0.23$). Spectra were recorded 15 min after the mixture. Other conditions as in Fig. 4. a, free etruscomycin in aqueous medium; b, 20 mol% cholesterol; c, 20 mol% ergosterol.

At low antibiotic concentration ($R = 7 \cdot 10^{-3}$), the interaction of etruscomycin with membranous ergosterol differed from that observed with membranous cholesterol: the two bands at 305 and 320 nm shifted to 310 and 325 nm in the presence of ergosterol-containing vesicles, while a smaller red-shift (3 nm) was observed with those containing cholesterol. This difference was not observed at high antibiotic/lipid ratios ($R > 0.1$): the same spectra with a 3 nm red-shift were observed with pure EPC, pure DPPC, cholesterol-containing and ergosterol-containing vesicles.

Fluorescence studies

It is known that the fluorescence of etruscomycin in aqueous solutions at neutral pH is extremely low and increases considerably upon addition of cholesterol-containing membranes [16]. Therefore, fluorescence is an extremely sensitive method for evaluating the binding of etruscomycin to vesicles containing different cholesterol concentrations. We followed the fluorescence increases as a function of molar percentage of sterol in the membranes. The measurements were carried out maintaining constant the total lipid concentration of both the vesicle suspension (0.2 mM) and etruscomycin (1.4 μM) and therefore operating at a steady R value of $7 \cdot 10^{-3}$.

As can be seen in Fig. 6, upon addition of egg-yolk phosphatidylcholine vesicles the fluorescence at 405 nm remained extremely low if compared to that observed in the presence of sterol-containing vesicles.

Upon addition of cholesterol-containing vesicles, the fluorescence intensity increased considerably, at least for a cholesterol content higher than 10%.

For ergosterol-containing vesicles two different

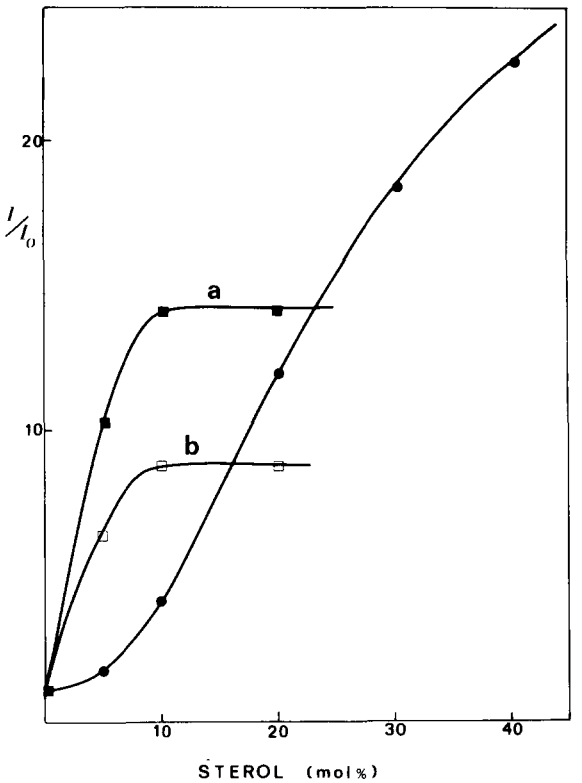

Fig. 6. Relative fluorescence intensity (I/I_0) at 20°C of etruscomycin in the presence of increasing concentrations (mol%) of cholesterol (●) or ergosterol (□, ■) -containing vesicles. I_0 is the fluorescence intensity of antibiotic in the presence of pure egg yolk phosphatidylcholine vesicles and I that of antibiotic in the presence of sterol-containing vesicles. The etruscomycin and lipid concentrations were kept constant at $1.4 \cdot 10^{-6}$ M and $2 \cdot 10^{-4}$ M, respectively. a, excitation wavelength: $\lambda = 310$ nm; b, excitation wavelength: $\lambda = 308$ nm. The very weak fluorescence at 405 nm due to the vesicles in the absence of etruscomycin was negligible.

curves were obtained, as shown in Fig. 6, one by excitation at 310 nm (wavelength of electronic absorption maximum) and the other by excitation at 308 nm. Under both conditions we observed a pronounced increase of fluorescence intensity, especially when 5% ergosterol (mol%) was present in the membrane. However, the fluorescence intensity reached a plateau at 10% ergosterol (20–40 μM), at a lower sterol concentration than that observed in the presence of membrane cholesterol. The curve obtained with cholesterol-containing vesicles had a sigmoidal shape, while that obtained with ergosterol-containing vesicles was hyperbolic.

Discussion

The most striking features of etruscomycin-induced permeability in large unilamellar vesicles were that high concentrations of antibiotic were necessary and that only a limited amount of protons was released. Under the experimental conditions used here, that is 2 mM total lipids, the minimum antibiotic concentration necessary to observe some proton leakage from egg yolk PC vesicles was $3 \cdot 10^{-4}$ M, or an antibiotic/lipid ratio of 0.15. This result is comparable to that given for etruscomycin-induced leakage of dichromate ions from dichromate-loaded sonicated vesicles [28]. Indeed, in the latter case the vesicles become permeable only when 40–50% of the cholesterol sites are occupied by the polyene. In contrast, K^+ is released from multilamellar vesicles even at an antibiotic/lipid ratio of 0.035 [6]. This discrepancy may be explained by the fact that in multilamellar vesicles the antibiotic easily penetrates only the first external bilayers. Therefore the situation is similar to that of our experiments because the actual antibiotic/lipid ratio is much higher than 0.035 for the external leaflets and permeability is probably induced in the external aqueous compartments.

With DPPC vesicles, permeability began to be induced at slightly lower etruscomycin concentrations, that is at a ratio of approx. 0.06. Furthermore, in this case, the presence of sterol molecules in the bilayers was not necessary. The induction of permeability in gel-state vesicles has already been observed for filipin, amphotericin B methyl ester [5] and amphotericin B [26,29].

These results differ from those observed with amphotericin B; with this heptaene antibiotic DPPC vesicles are less sensitive to permeability induction than egg yolk PC vesicles [26] and the minimum antibiotic concentration needed is much lower ($R \approx 10^{-4}$).

Circular dichroism recording and permeability measurements were carried out using the same experimental conditions in order directly to compare the two sets of data. We have already dealt with the problems arising in the CD interpretations from the possible artefacts due to scattering from the vesicle suspensions [8,38]. Whatever may be the origin of the etruscomycin CD changes, the

data clearly demonstrated the existence of a definite threshold beyond which a new phenomenon appears.

Etruscomycin-vesicle interaction at low antibiotic / phospholipid ratio

For $R < 0.1$ (in egg yolk PC vesicles), almost no permeability was induced. Etruscomycin, however, was bound to the vesicles as seen from the wavelength shift of the CD bands relative to those of free etruscomycin. This confirms the results on binding obtained by fluorescence [16,30]. Under these conditions (actual $R = 0.03$) it was shown by ^{1}H-NMR [36] that the non-polar region of the bilayer seems not to be significantly perturbed by the polyene, while a perturbation of the dynamic structure of the *N*-methyl groups is detected. These results are in agreement with the absence of permeability inducement.

Since the peaks measured by CD are not located at the same wavelength, the conformations of etruscomycin bound to cholesterol-containing vesicles and to ergosterol-containing vesicles are clearly different. In contrast, no qualitative difference was observed between cholesterol and ergosterol binding to amphotericin.

The shape of the curves giving the increase of the etruscomycin fluorescence intensity as a function of the percent of sterol in the membranes (Fig. 6) is also indicative of this difference: the curve for cholesterol has a sigmoidal shape, whereas the curve for ergosterol reaches a plateau at a sterol concentration of 10%.

From the curves giving the increase of the etruscomycin fluorescence intensity upon addition of increasing amounts of vesicles containing 10% sterol (data not shown), it appears that etruscomycin has a higher affinity for ergosterol-containing than for cholesterol-containing vesicles. Assuming that the binding corresponds to the formation of a sterol-antibiotic complex in the bilayer, with a 2 : 1 stoichiometry [28], the Scatchard plot of these fluorescence data as a function of the sterol concentration is linear and gives a binding constant of $8 \cdot 10^5$ M^{-2} for ergosterol and of $4 \cdot 10^5$ M^{-2} for cholesterol. This preferential affinity of etruscomycin at low R for ergosterol-containing vesicles is further confirmed by the transfer of the antibiotic from the cholesterol-containing vesicles to the ergosterol-containing vesicles. The rapid exchange of amphotericin B among phospholipid vesicles has already been demonstrated [18,31]. Here the transformation of the CD spectrum of etruscomycin bound to cholesterol-containing egg yolk PC vesicles into the CD spectrum of etruscomycin bound to ergosterol-containing egg yolk PC vesicles demonstrates that the same phenomenon occurs, thus confirming that etruscomycin at low R binds preferentially to ergosterol-containing vesicles.

Etruscomycin-vesicles interaction at antibiotic / phospholipid ratios higher than 0.1

As soon as the etruscomycin concentration is sufficient to induce some H^+ permeability ($R = 0.06$ for DDPC vesicles and $R = 0.1$ for egg yolk PC vesicles), a new CD spectrum begins to appear. For $R = 0.07$ in DPPC vesicles, $R = 0.2$ in pure egg yolk PC and 0.07 for cholesterol-containing vesicles this spectrum is totally formed. It consists of two negative and one positive bands, and bears a resemblance to that of species III observed with amphotericin B, independently of the wavelength shift [8]. Although this spectrum is correlated with the induction of permeability, its intensity does not depend on the amount of sterol in the bilayers, in contrast to the etruscomycin-induced proton leakage. Thus, if the permeability inducement is sterol-dependent, it is so indirectly, insofar as the increase in sensitivity of the vesicles to etruscomycin, observed as the sterol content increases, results from an intrinsic modification of the membrane. The sterols may act indirectly by making the membranes more sensitive to the destructive effects of the antibiotics.

This CD spectrum, observed even with pure phospholipid vesicles, indicates the formation of a binary complex of etruscomycin and phospholipid.

Mechanism of permeability inducement by etruscomycin

From studies of the effects of etruscomycin on the release of K^+ and glucose-6-phosphate dehydrogenase from liposomes, it was tentatively concluded [32] that this antibiotic 'probably' acts in the same manner as amphotericin B or nystatin by creating aqueous pores. However, in the study on liposomes the K^+ leakage was very low, which

made the conclusions less evident than those for amphotericin B or nystatin. This assumption was later confirmed [6] by the fact that K^+ release from erythrocytes and hemolysis do not occur in the same concentration range of etruscomycin. On the other hand, the difference between K^+ release and hemolysis has not been observed by other authors, for example Kotler-Brajtburg et al. [33], though it should be noted that in their studies a different buffer was used. Nor was an increase in the Na^+ content of erythrocytes in NaCl buffer observed in the presence of sublytic concentrations of etruscomycin, in contrast with the results obtained with other heptaene antibiotics [34]. Therefore the existence of etruscomycin pores remains controversial. Our results indicate that at high antibiotic-lipid ratios etruscomycin induces permeability by causing vesicle destruction of fusion. Indeed, we have observed the etruscomycin-induced release of carboxyfluorescein in the same antibiotic concentration range as the proton leakage (Fig. 3). If we assume that the carboxyfluorescein molecule is too large to pass through the channels thought to be created by the antibiotic, its release may be considered a good indicator of vesicle disruption or fusion [27]. For instance, it is well established that amphotericin-induced proton leakage occurs at low antibiotic-lipid ratios through the formation of channels; in our experiments such leakage was observed at a much lower concentration than the carboxyfluorescein release (Fig. 3). Our ^{31}P-NMR experiments are in agreement with this conclusion: manganese ions were added to the vesicle suspension in order to broaden the external phosphate peaks and differentiate them from the internal peaks. Actually, at high

TABLE I

COMPARISON OF ERGOSTEROL AND CHOLESTEROL EFFECTS ON BINDING AND PERMEABILIZING PROPERTIES OF AMPHOTERICIN B, FILIPIN AND ETRUSCOMYCIN

The following systems were considered: (i) *Saccharomyces cerevisiae* (ergosterol-containing membranes) and erythrocytes (cholesterol-containing membranes) [33]: K^+ leakage, cell death or hemolysis. (ii) *Mycoplasma mycoides* grown in ergosterol- or cholesterol-containing medium [5]. (iii) Ergosterol- or cholesterol-containing egg yolk PC unilamellar vesicles: permeability inducement (Refs. 5, 6, 8, present study); binding (Refs. 6, 8, 9, 13, present study). (iv) Affinity for sterols in mixtures of water and organic solvent which mimic the lipidic environment [10,15]. (v) Affinity for sterols in water as measured by inhibition of effect on erythrocytes and yeast cells [3–5]. The sign + indicates that the effect of ergosterol is stronger; the sign − indicates that the effect of cholesterol is stronger.

	Amphotericin B	Filipin	Etruscomycin
Saccharomyces cerevisiae/ erythrocytes	+	+	no difference
Mycoplasma mycoides	+ (amphotericin B methyl ester)	−	
Vesicle permeability inducement	+	−	+ (slightly)
Vesicle binding	+	−	at low R: + at high R: no difference
Water/organic solvent mixture binding	+	± (depending on solvent composition)	± (depending on solvent composition)
Water binding	+	−	−

etruscomycin ratios, all signals disappeared, which indicated that the paramagnetic manganese ions were in close proximity to all the phosphate ions. This may be interpreted either as a penetration of Mn^{2+} into the vesicles or as a disruption of the vesicles. The first explanation, which would imply the formation of pores permeable to Mn^{2+}, does not correspond to what is observed with other polyene antibiotics (Ref. 26 and unpublished data). The second explanation, in contrast, is strongly supported by our experiments on carboxyfluorescein release. Furthermore, the vesicle destruction at antibiotic: sterol ratios close to 1 : 1 has already been demonstrated with amphotericin B, nystatin and filipin [35] by the use of ^{1}H-NMR and the upfield shift of outside ions under the influence of $K_3Fe(CN)_6$ or the broadening of the choline methyl peaks under the influence of Mn^{2+}.

The mechanism by which permeability is induced by amphotericin B (pore formation) therefore appears to differ from that for filipin or etruscomycin (membrane disruption or fusion), in agreement with the classification of the polyene antibiotics proposed by Brajtburg et al. [37].

Relationship between the structure of the polyene antibiotics and their selective toxicity

One aim of this study was to compare the interaction of cholesterol and ergosterol with different polyene antibiotics in order to determine which part of the antibiotic molecule is responsible for its preferential affinity for ergosterol and its corresponding selective toxicity for fungi. The heptaene amphotericin B may be compared with both the pentaene filipin and the tetraene etruscomycin. Filipin is devoid of the substituent mycosamine found in both amphotericin B and etruscomycin.

Actually from the results given in Table I and which summarize the data from the literature and our present results it seems not possible to say that the length of the polyene chain is the factor which determines the selective sensitivity of ergosterol-containing membranes to amphotericin B-induced permeability. On the other hand it appears that the binding strength and efficiency in permeability inducement may differ; such discrepancies have already been noted for heptaene antibiotics [9].

Finally, our results emphasize the fact that the relationship between functional data and quantitative information such as binding constants is not straightforward; before drawing any conclusion, it is important to be first qualitatively sure that the species whose binding is determined, is actually responsible for the phenomenon studied. Neglecting this preliminary analysis could lead to irrelevant conclusions.

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