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INTERACTION OF THE POLYENE ANTIBIOTICS WITH LIPID BILAYER VESICLES CONTAINING CHOLESTEROL

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SUMMARY

The interaction of the polyene antibiotics, amphotericin B, nystatin and filipin with cholesterol-containing single bilayer lipid vesicles has been characterized using gel permeation chromatography and proton magnetic resonance. All three antibiotics bind to vesicles at low concentrations without causing a large amount of vesicle destruction. The strength of binding as determined by gel permeation studies is greater for filipin and amphotericin than for nystatin. Nystatin and amphotericin B at these low concentrations induce a rapid loss of internal vesicle contents consistent with pore formation. Filipin induces no leakage beyond that expected from partial vesicle destruction or general detergent action.

At antibiotic levels above 1 : 1 antibiotic : cholesterol ratios the NMR results show all three antibiotics to cause extensive vesicle destruction. The onset of this behavior, which appears to be independent of the total antibiotic concentration, indicates a well defined antibiotic : cholesterol interaction stoichiometry. Despite the fact that cholesterol is required for antibiotic activity, the NMR spectra prior to vesicle destruction show no changes indicative of an antibiotic-induced reversal of cholesterol restriction of phosphatidylcholine mobility. The contrast with polyene antibiotic behavior in more extended bilayers is discussed.

INTRODUCTION

Filipin, nystatin and amphotericin B are members of a class of cyclic antibiotics having as one portion of their ring a conjugated double bond system and as another a region rich in hydroxyl groups [1]. These unusual molecules, referred to as polyene antibiotics, are of biological interest because they selectively attack fungi and other organisms having a sterol-containing membrane [2]. Previous studies indicate that their behavior is linked to an ability to alter the permeability or integrity of cell membranes [3].

A mechanism of action has been proposed in the case of low concentrations of nystatin or amphotericin B interacting with an extended membrane having cholesterol

or similar sterols as a membrane constituent. Transmembrane pores are supposedly formed that allow the passage of a variety of cations, anions and small organic molecules [4–6]. Pore formation, which requires aggregation of approx. 16 polyene as well as approx. 16 cholesterol molecules [1] explains the basis of their selective toxicity to sterol-containing organisms. Filipin's mechanism of action can be defined with less certainty. It also requires a sterol interaction but apparently forms large disruptive aggregates rather than simple transmembrane pores [7, 8].

In all cases, the mode of polyene interaction with membranes can be complex. Depending on the local environment, or effective concentration of polyene, interactions can vary from simple binding [9, 10], to pore formation, to destructive detergent-like action that does not require the presence of a sterol [6]. It is likely that interaction proceeds stepwise through two or more of these phases, but in many cases only the final most destructive mode can be observed [11]. In the case of filipin's interaction with black lipid films, for example, rupture of the membrane occurs before any changes in permeability can be detected.

Most of the studies which have led to the present description of polyene action have been based on systems such as whole cells, black lipid films or liposomes. Although studies on these systems have proven very useful, it is difficult to define or control the composition or extent of the interacting membrane surface. In liposomes, for example, because of their multilamellar nature, one finds that on the time scale of most experiments only the outer bilayer is exposed to the effect of added polyene. Given the variable modes of polyene interaction, these uncertainties have made quantitative and in some cases qualitative evaluation of the resulting data difficult.

An alternate system which can be examined in the presence of polyenes is the phospholipid vesicle. This structure, which is in the form of a small (300 Å) spherical shell, has only a single bilayer wall so that polyenes added have ready access to the outer half of all bilayer surfaces present.

The vesicle system also offers several experimental advantages. The size and surface area distributions of vesicle preparations can be easily evaluated by analytical gel chromatography. Qualitative estimates of the binding of the polyene antibiotics to the vesicle surface can be made by separation of antibiotic-treated vesicles from the preparative solution by gel permeation columns. Alterations in bilayer structure can be monitored by high-resolution NMR spectroscopy. In the most extreme case the collapse of a vesicle structure to a multilayer one leads to a large increase in linewidth and an apparent intensity loss of NMR resonances which can be used to monitor vesicle breakdown. With less severe alterations, such as aggregation of cholesterol into clusters or antibiotic pores, a reversal of the 2- to 4-fold broadening of the phosphatidylcholine resonances produced by phosphatidylcholine-cholesterol interactions may occur. Finally, permeability changes can be examined via NMR by using paramagnetic shift reagents to determine the leakage of ions having a proton resonance from the interior volume of the vesicle.

These advantages led to the present study of the interaction of nystatin, amphotericin B and filipin with unilamellar phospholipid vesicles containing cholesterol. Of course, data on vesicles may not compare on a one-to-one basis with those on more extended membranes. It has been shown that the vesicle bilayer has a higher internal fluidity [12] and it has even been demonstrated that some antibiotics, valinomycin and alamethicin for example, bind differently to vesicles than to multi-

layers [13]. For this reason we have attempted to examine each stage of interaction, simple binding, induced leakage and structural transformation, independently.

MATERIALS AND METHODS

Filipin complex (86 % pure) lot No. 8393-DEG-11-8 was the generous gift of the Upjohn company. Nystatin, grade B, lot No. 200973, and amphotericin B, grade A, lot No. 900812, were purchases from Calbiochem, Los Angeles. All the antibiotics were stored under N_2 in a desiccator at −10 °C until used. The cholesterol was lot No. 732063 from Fisher, melting point 147.3–148.5 °C. All the above were used without further purification. Phosphatidylcholine was purified from fresh egg yolks by the method of Singleton et al. [14] and stored under N_2 at −10 °C until used. Sepharose 2B lot No. 8522 and 9722 came from Pharmacia, Uppsala, Sweden. All other chemicals were reagent grade.

Thin-layer chromatography was performed on 250 μn Silica gel G plates. The antibiotics were developed with methylene chloride/methanol 80 : 20 and visualized with 2,7-dichlorofluorescein spray. Both filipin and nystatin showed 10–20 % impurities. Phosphatidylcholine developed with $CHCl_3/CH_3OH/H_2O$, 65 : 25 : 4, showed no chemical degradation due to the sample preparation procedure.

Vesicle dispersions were prepared by dissolving weighed amounts of phosphatidylcholine and cholesterol in chloroform and then removing the solvent in vacuo. The samples were heated to 50 °C for 2 h to complete the process. The appropriate buffer was added to give a 5 % w/v phosphatidylcholine solution. After dispersion of the lipid by vortexing the sample was degassed and capped under N_2, then sonicated to clearness in a Branson model E sonication bath maintained at 30 °C. The vesicle solution was filtered through a 0.22 μn millipore filter and diluted to the required concentration just before use. Liposome samples were prepared in a similar manner, but omitting the sonication and filtering steps. The antibiotics were weighed and dissolved in sufficient Me_2SO to give a 5 % v/v $Me_2SO/^2H_2O$ final solution. The vesicle solution was added and vigorously shaken. The samples were stored under N_2 at 4 °C when not actually being analyzed. The composition of the buffer solution used in the NMR and column chromatography experiments was 0.01 M Tris/0.10 M KCl/0.001 M $Na_2S_2O_3$/0.02 % w/v NaN_3/5.0 % v/v Me_2SO titrated to pH 7.5 with conc. HCl. For the NMR experiments [U-^{2}H]Me_2SO was used in the ^2H_2O analog of the above buffer.

Gel permeation chromatography experiments were carried out on a Sepharose 2B column about 30 cm in height by 2.6 cm internal diameter. The total volume was about 200 ml, and the typical sample volume was 10 ml. The sample was eluted at 40 ml/h and continuously detected with a Waters Associates differential refractometer. Fractions were collected every 5 ml and analyzed later by ultraviolet absorbance in a 1-cm cell versus a solvent blank. The absorbance was measured at one of the long-wavelength polyene absorbance maxima to reduce light scattering contributions to the absorbance from the vesicles. The column was calibrated with pure egg yolk lecithin vesicles of assumed diameter 240 Å [15] and a series of small proteins. The void volume was determined from the refractive index profile of multilayer particles, the total volume was determined by the change in refractive index that always occurs at that point because of a slight change in buffer composition, and the vesicle elution

volume was taken as the maximum in the refractive index curve for the vesicle fraction. The vesicle sizes were calculated using the equation of Ackers [16].

^1H NMR measurements on vesicle systems were done on a Varian HA100 spectrometer operating at 100 MHz and 27 °C. To improve signal-to-noise ratios the samples were time averaged over approx. 20 scans while locked on the ^2HOH resonance. All intensities were measured relative to the intensity of Me_2SO in the solvent. Under the conditions employed, the resolution was about 1 Hz and the intensities were accurate to 10 %. Measurements on liposome systems which require a very wide sweep range were done on a Bruker HX270 spectrometer operating at 270 MHz.

For vesicle permeability measurements the vesicle stock solution was sonicated in buffer containing 0.15 M tetramethylammonium chloride. After sonication the vesicles were filtered through a Sephadex G-100 column reducing the concentration of tetramethylammonium ions on the outside of the vesicles to an undetectable level. The solution was diluted and antibiotic added. Periodically, 5 μl of 1.0 M $K_3Fe(CN)_6$ solution in ^2H_2O was added to a 0.5 ml aliquot of the solution to differentiate inside and remaining outside tetramethylammonium ions, and the spectrum was taken immediately. Ultraviolet studies indicated that ferricyanide oxidized the polyene antibiotics in solution (but not antibiotic bound to the vesicles), so a fresh aliquot was taken for each determination. Mn^{2+} was also used to check the permeability of the vesicles to ions. In this case 5 μl of a 0.5 M solution of $MnCl_2$ in ^2H_2O was added just before taking the spectrum. Broadening of the internal choline methyl resonance indicated the extent of leakage. An H_2O capillary lock was used in these Mn^{2+} experiments.

RESULTS AND DISCUSSION

Gel permeation chromatography

The binding of the polyene antibiotics to vesicles is most easily characterized by Sepharose column chromatography. In order to test the binding properties of the polyene antibiotics, mixtures of antibiotic and phosphatidylcholine:cholesterol vesicles are eluted through the column. The extent to which the antibiotic peak trails the vesicle peak gives a rough indication of the strength of binding.

In the absence of antibiotic, vesicle preparations of composition 2:1 in phosphatidylcholine:cholesterol elute from the column as indicated in Fig. 1A. The concentration profile for the lipids is easily detected as a change in the refractive index of the eluant. These refractive index values are proportional to the amount of lipid and are independent of the particle size. V_t and V_v indicate the elution volumes for very small and very large particles, respectively. The position of the refractive index maximum for the vesicles in Fig. 1A occurs at a point characteristic of particles 320 Å in diameter. The absence of a large void volume peak indicates that more than 90 % of the lipids are in vesicle form. Shifts of the vesicle elution volume to smaller values on addition of the antibiotics would indicate a larger effective vesicle size.

The presence of polyene antibiotics bound to the vesicles can be detected by monitoring simultaneously the refractive index and polyene absorbance at wavelengths greater than 300 nm and then testing for overlap of the peaks in the two profiles.

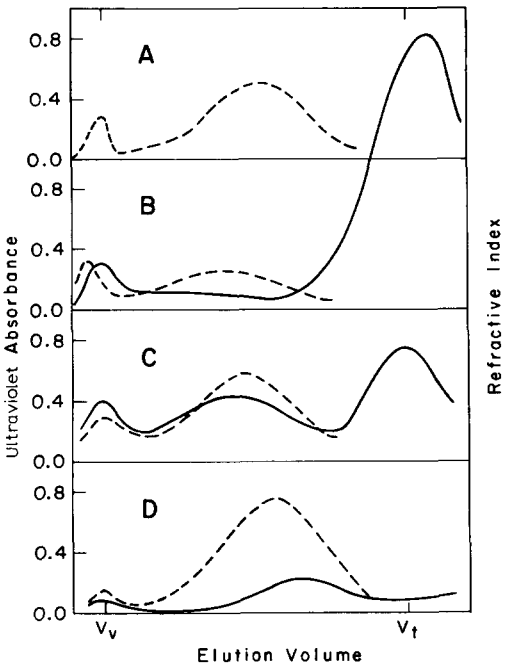

Fig. 1. Refractive index and ultraviolet absorption elution profiles from Sepharose 2B chromatography. (A) 2 : 1 phosphatidylcholine : cholesterol, no antibiotic; (B) 2 : 1 phosphatidylcholine: cholesterol, $1 \cdot 10^{-3}$ M cholesterol, $5 \cdot 10^{-4}$ M nystatin; (C) 2 : 1 phosphatidylcholine : cholesterol, $1 \cdot 10^{-3}$ M cholesterol, $5 \cdot 10^{-4}$ M filipin complex; (D) 4 : 1 phosphatidylcholine : cholesterol, $1 \cdot 10^{-3}$ M cholesterol, $5 \cdot 10^{-4}$ M amphotericin B.

Such simultaneous ultraviolet absorbance and refractive index profiles for vesicles treated with each of the three polyene antibiotics are shown in Figs. 1B–1D.

The profile due to 2:1 phosphatidylcholine:cholesterol vesicles mixed with nystatin 15 min before adding the sample to the column is shown in Fig. 1B. The ultraviolet absorbance profile shows only small amounts of absorbance coincident with the vesicle refractive index peak and some slight tailing to V_t where the bulk of the nystatin appears to elute. This behavior is characteristic of weak binding of nystatin to the vesicle. In the absence of vesicles or in the presence of pure phosphatidylcholine vesicles all of the nystatin elutes at or after V_t. These results indicate that cholesterol is necessary for nystatin binding.

In the profile for the sample containing amphotericin B, Fig. 1D, an absorbance peak trails slightly behind the vesicle refractive index peak. There is virtually no V_t absorbance peak. These facts suggest that amphotericin B binds very strongly, but reversibly, to cholesterol-containing vesicles. No amphotericin B binds to pure phosphatidylcholine vesicles, but unlike nystatin it does not appear in the total volume either. Together with the presence of a low background ultraviolet absorbance due to the polyene long after the total column volume is eluted, this suggests that the antibiotic is bound to the column and is only slowly leached out. This makes it difficult to quantitatively evaluate binding to the cholesterol-containing vesicles but it is still possible to say that the binding is strong.

The profiles resulting from elution of phosphatidylcholine:cholesterol 2:1 vesicles mixed with filipin complex, Fig. 1C, shows a substantial amount of filipin retained in the vesicle fraction with the ultraviolet absorbance profile exactly overlapping the refractive index profile for the vesicles. This suggests very strong binding. In apparent contradiction to the strong binding to the vesicles, the absorbance profile also shows a large amount of filipin eluting at V_t, with some trailing to even larger volumes. The explanation lies in the fact that the filipin complex is a mixture of at least four molecular species having nearly identical absorbance spectra [17]. Thin-layer chromatographic examination of the vesicle and V_t fractions show that the components migrating most like filipins II and III are bound to the vesicles with none of these components found in the V_t fraction. Rechromatography of the vesicle fraction through the Sepharose column shows an absorbance peak overlapping the refractive index peak with no absorbance in the V_t fraction, suggesting that the active components bind to the Sepharose like amphotericin B.

The fact that there is little variation in the intensity of vesicle refractive index profiles indicates that few vesicles are destroyed in the presence of filipin or the other antibiotics. This contrasts with filipin's disruptive effect on extended bilayers. The phenomenon was investigated more thoroughly by varying the filipin concentration in the region $5 \cdot 10^{-4}$–$2 \cdot 10^{-3}$ M while keeping the filipin:cholesterol ratio fixed at 1:2 using 4:1 phosphatidylcholine:cholesterol vesicles. Except for the expected decreases in the profile intensities with lipid concentration, no other changes are noted. The lack of absolute concentration dependence at low antibiotic:cholesterol ratios in column elution behavior rules out many potentially destructive effects such as micelle formation or detergent action.

When the antibiotic:cholesterol ratio is increased up to and beyond 1:1 a dramatic and disproportionate decrease in the refractive index intensity in the vesicle peak occurs. No increase in the intensity of either the void volume or the total volume peaks is seen, so the vesicles must decay or aggregate to structures which either adhere strongly to the column or are too large to pass through. Thin-layer chromatographic examination of the remaining vesicles shows the initial phosphatidylcholine, cholesterol and antibiotic composition, so the resulting structures must simply be due to a transformation of the vesicle structure. Experiments with 4:1 phosphatidylcholine:cholesterol vesicles and experiments with different total cholesterol concentrations show similar abrupt changes near the 1:1 filipin:cholesterol ratio. Thus it is the stoichiometry and not the absolute filipin concentration that is the crucial factor.

Experiments with varying amphotericin B:cholesterol and nystatin:cholesterol ratios produce similar results with respect to the stoichiometry of binding, but comparisons are difficult to make since decreases in the absorbance and refractive index profiles of the vesicle peak are not as abrupt. These comparisons are best left to NMR experiments which will be described shortly. Qualitatively, however, gel chromatography shows that at levels below 1:1 antibiotic:cholesterol ratios all three antibiotics bind to cholesterol-containing vesicles. The strength of binding as determined by the exactness of the overlap of the vesicle refractive index and the polyene absorbance profiles is in the order filipin > amphotericin B > nystatin. For all three antibiotics the initial binding is relatively nondestructive. Only at concentrations approaching 1:1 antibiotic:cholesterol does vesicle breakdown become apparent.

Proton magnetic resonance studies

Quantitative evaluation of the extent of the bilayer structural transformation that occurs at antibiotic : cholesterol stoichiometries of 1 : 1 is easily accomplished by examining ^{1}H NMR spectra. NMR spectra of pure phosphatidylcholine vesicles in the absence of antibiotics give well resolved resonances. At 100 MHz and 27 °C the largest resonances are a choline methyl peak at 3.2 ppm of 5 Hz line width, a hydrocarbon chain methylene peak at 1.3 ppm of 20 Hz line width and a chain terminal methyl peak at 0.9 ppm of 10 Hz line width. Vesicles containing cholesterol give resonances that are substantially broader but they are still well resolved (Fig. 2). The peaks at 2.9 and 2.5 ppm from Me_4Si are due to tetramethylammonium ions and Me_2SO, respectively.

Adding polyene antibiotics to a vesicle preparation containing cholesterol results in an apparent loss of intensity of the choline resonance (Fig. 2). This is especially pronounced when the antibiotic concentration is greater than that of cholesterol. The spectral changes are reminiscent of the alterations that occur when vesicles break down to extended bilayer or multilayer structures. This transformation broadens the resonances so much (approx. 150 Hz for the choline methyl) they are not easily detected on narrow sweep ranges and the effect is an apparent loss in intensity of the resolvable peaks. The exact origin of the broadening is in dispute. Both an increase in the ordering of the phosphatidylcholine molecules and an increase in particle size have been suggested as the primary cause [19, 20]. Because of this, a definitive correlation with a morphological transformation cannot be made from the NMR data alone. In filipin-treated vesicles, however, recent electron microscopic studies confirm a transformation to multilayer structures [21]. The Sepharose chromatography of samples containing this and the other antibiotics is consistent with the production of large structures. Given this correlation the NMR data provide a useful means of quantitating the extent of vesicle collapse at various times and under various antibiotic concentrations. The choline peak is most easily monitored, since the antibiotic

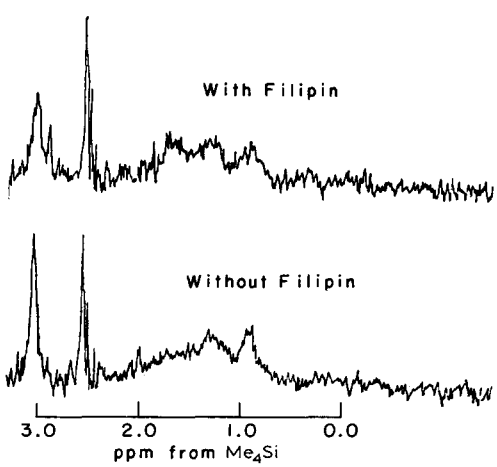

Fig. 2. 100 MHz ^{1}H NMR spectra of 2 : 1 phosphatidylcholine : cholesterol vesicles at $2 \cdot 10^{-3}$ M cholesterol concentration; with and without $2 \cdot 10^{-3}$ M filipin complex.

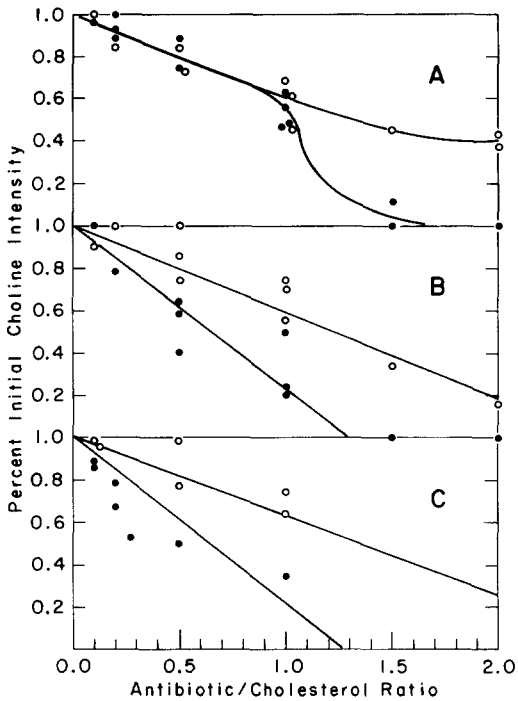

Fig. 3. ^{1}H NMR choline resonance intensity vs. the mol ratio of antibiotic to cholesterol for vesicles mixed with (A) filipin complex, (B) nystatin and (C) amphotericin B. ○, 4 : 1 phosphatidylcholine: cholesterol; ●, 2 : 1 phosphatidylcholine : cholesterol.

methyl resonances tend to obscure changes in the phosphatidylcholine methylene and chain terminal methyl peaks at high antibiotic concentrations.

Fig. 3A presents the intensity of the choline peak as a function of the filipin : cholesterol ratio. The concentration of cholesterol is $2 \cdot 10^{-3}$ M. For a 2 : 1 lecithin : cholesterol vesicle dispersion, little breakdown is seen as long as the amount of filipin added is less than the amount of cholesterol. When the amount of filipin is greater than the amount of cholesterol, nearly complete vesicle break down is observed. The intensity of the resolved choline peak drops to less than 20 % of its initial value.

The above results for the interaction of filipin with vesicles also hold for a 1 : 1 lecithin : cholesterol ratio. However, when the concentration of the cholesterol in the lipid bilayer is reduced to 4 : 1 lecithin : cholesterol, the amount of choline intensity remaining when the filipin : cholesterol ratio exceeds 1 : 1 can be as high as 40 %.

Since filipin binds strongly to the vesicles, these results confirm the fact that at low antibiotic : cholesterol ratios the complex formed is not destructive to the vesicle. It appears, however, that a destructive interaction becomes important when the cholesterol available for the initial binding becomes saturated. This second mode may be the rather nonspecific detergent-like interaction suggested by others [6] or it may be due to formation of the circular filipin : cholesterol aggregates seen in electron microscopic studies [7, 8]. Experiments on pure phosphatidylcholine vesicles where nonspecific interactions should dominate do in fact show some vesicle destruction.

However, the fact that 2 : 1 phosphatidylcholine : cholesterol vesicles are more easily destroyed than 4 : 1 vesicles at high filipin concentrations suggests that it is the concentration of filipin : cholesterol complex in the membrane rather than the concentration of filipin alone that is important. This would be consistent with aggregate formation causing vesicle break down.

Nystatin interacts with vesicles in a more complex manner than filipin. If vesicle spectra are examined shortly after the addition of nystatin, especially for 4 : 1 phosphatidylcholine : cholesterol vesicles, the results are reminiscent of the effects of filipin. Small changes in intensity of the choline peak are observed at antibiotic : cholesterol ratios of less than one, but at higher concentrations the intensity disappears, indicating extensive vesicle disruption. The drop is not as pronounced as with filipin and it does not extrapolate to complete vesicle destruction until a nystatin : cholesterol ratio of 2 : 1 is reached. The higher apparent stoichiometry may be the result of the lower binding constant for nystatin.

If the spectra are examined after a longer period of time has elapsed (of the order of 1 day, Fig. 3B) it is apparent that vesicle break down occurs at all antibiotic : cholesterol ratios but at rates much slower than when excess antibiotic is added. This slow behavior could be the result of detergent action of the unbound nystatin in equilibrium with the bound form or it could be that the nystatin : cholesterol complex induces a small degree of instability in the vesicle.

Amphotericin B, despite its higher binding constant, shows behavior similar to that of nystatin (Fig. 3C). The long time scale behavior is approached much more rapidly, however. Assuming similar detergent activities for nystatin and amphotericin B, this result suggests that vesicle instability is the more appropriate explanation for the long time scale break down.

The slow destruction of the vesicles at low nystatin and amphotericin B concentrations does not reach completion but stops after several days. Given the length of time involved, any number of transformations, including chemical degradation, may deactivate the antibiotics.

Changes in vesicle properties that occur at low antibiotic concentrations and are not associated with vesicle destruction can also be monitored by magnetic resonance techniques. Changes in permeability, for example, can be monitored by measuring the rate of leakage of tetramethylammonium ion from the inner compartment of the vesicle. If most of the outside ions are initially removed, as described in the methods section, and $K_3Fe(CN)_6$ is added to the solution to shift the outside ions upfield, then the resonance intensity of the inside versus the outside ions can be easily compared. Addition of any one of the antibiotics to a concentration in excess of its apparent interaction stoichiometry, followed by examination in the above manner, shows an immediate loss of intensity of the internal tetramethylammonium peak. This is consistent with breakage of the vesicles prior to reaggregation into extended bilayer structures. It also confirms our ability to monitor permeability changes.

At low concentrations of nystatin, a 0.1 : 1 nystatin : cholesterol ratio in a 1 : 1phosphatidylcholine : cholesterol vesicle system, a 30 % leakage of ions occurs immediately and then slow leakage continues to completion in a day (Fig. 4). Despite this dramatic effect for nystatin-treated vesicles, the initial rapid leakage, which appears to parallel vesicle destruction, makes analysis difficult.

For 4 : 1 phosphatidylcholine : cholesterol vesicles at low antibiotic concentra-

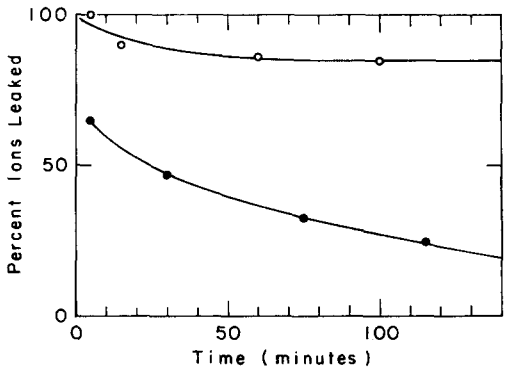

Fig. 4. Amount of tetramethylammonium ions leaked vs. time after mixing with antibiotic. In both cases the ratio of antibiotic to cholesterol is 0.1 in 1 : 1 phosphatidylcholine : cholesterol vesicles. ●, nystatin-treated vesicles; ○, filipin-treated vesicles.

tions there is no substantial vesicle destruction when the antibiotic is added initially. The results for nystatin and amphotericin B still show complete leakage of the trapped ions in 1 day. These results are consistent with pore formation causing the permeability change.

Similar low concentrations of filipin in either 2 : 1 or 4 : 1 phosphatidylcholine : cholesterol vesicle dispersions causes much less leakage. In fact, for low ratios it is less than or equal to the 20 % vesicle destruction that occurs immediately after addition of filipin.

The pores which form in the nystatin or amphotericin B cases must either form slowly or be of a transient nature, since the existence of a single pore even for 1 ms would be sufficient to equilibrate the internal and external contents of a typical vesicle. An experiment using paramagnetic Mn^{2+} to broaden the choline methyls of phosphatidylcholine suggests that the former may be the case. Fig. 6D shows the choline region for an amphotericin B-treated 4 : 1 lecithin : cholesterol vesicle to

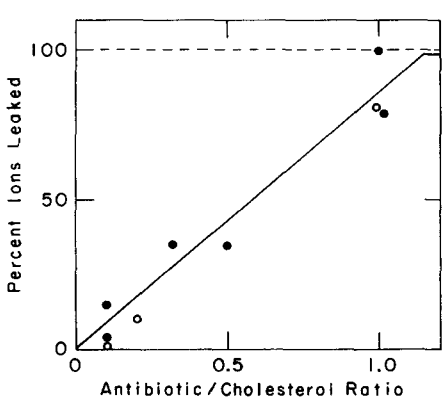

Fig. 5. Amount of tetramethylammonium ions leaked 1 day after adding antibiotic to the vesicles. (- - -) Effect of nystatin and amphotericin; (—) effect of filipin on (○) 4 : 1 and (●) 2 : 1 phosphatidylcholine : cholesterol vesicles.

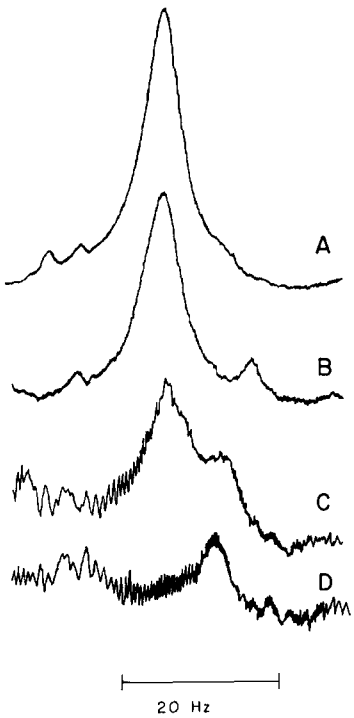

Fig. 6. ^{1}H NMR spectra of the choline and tetramethylammonium resonances. Each sample is $2 \cdot 10^{-3}$ M cholesterol in 4 : 1 phosphatidylcholine : cholesterol vesicles, with 0.5 M tetramethylammonium originally trapped inside the vesicles. (A) No antibiotic, 0.01 M $K_3Fe(CN)_6$; (B) $1 \cdot 10^{-3}$ M amphotericin B, 0.01 M $K_3Fe(CN)_6$; (C) no antibiotic, 0.005 M $MnCl_2$; (D) $1 \cdot 10^{-3}$ M amphotericin B, 0.005 M $MnCl_2$.

which Mn^{2+} is added 1 day after the antibiotic addition. An immediate loss of all the resolvable choline intensity occurs. This is compared to the two thirds loss of intensity (external cholines only) that occurs when Mn^{2+} is added to a vesicle preparation without amphotericin B. This shows that there are sufficient pores in the amphotericin B-treated vesicle after 1 day to make all the choline accessible to Mn^{2+} within a few minutes. Similar results are seen with nystatin. Thus the slow initial leakage of ions must be due to the slow formation of pores, which may be due to several processes such as the necessary transport of the antibiotic across the lipid bilayer membrane. The time scale of the process, approx. 1 h, appears slow compared to the rapid leakage observed in liposomes or whole cells [11]. The difference probably arises because a single pore releasing the entire contents of a cell or vesicle will release a much smaller fraction of the total trapped population in the case of the vesicle because of the smaller internal volume.

The pores in the vesicle system, although numerous enough to completely leak Mn^{2+}, do not seem to cause complete vesicle destruction. Only at high concentrations of both cholesterol and antibiotic does this become important. The fact that a high membrane concentration of pores can cause vesicle destruction is consistent with the fact that at intermediate antibiotic concentrations the vesicle destruction is twice as great in the 2 : 1 as in the 4 : 1 phosphatidylcholine : cholesterol vesicle system. It is

also consistent with the fact that the vesicles continue to collapse at longer times, as if the pore concentration continued to increase long after reaching a sufficient level to leak the vesicle contents.

The existence of large numbers of pores in nystatin-treated vesicles or extensive regions of aggregated filipin : cholesterol complex in filipin-treated vesicles could produce noticeable changes in the phosphatidylcholine NMR resonances. Our recent work with cholesterol-containing vesicles shows the choline methyl resonance to broaden by more than 50 % when comparing cholesterol- and non-cholesterol-containing vesicles of comparable size [22]. A reversal of this broadening should occur if most of the cholesterol is removed from contact with the phosphatidylcholine by aggregation with antibiotic. Larger changes may occur for the phosphatidylcholine methyl and methylene resonances, but because of spectral overlap with polyene resonances these would be difficult to detect.

Examination of the choline resonance for vesicles with various phosphatidylcholine : cholesterol ratios a day or more after treatment with either filipin or amphotericin B showed no change in the linewidth greater than the experimental error. Nystatin, on the other hand, causes substantial broadening of the choline peak. For filipin and amphotericin B this suggests that the antibiotic : phosphatidylcholine interactions compensate for the removal of cholesterol or that sequestration of cholesterol is not extensive enough to remove it from the normal phosphatidylcholine contacts. For nystatin the broadening of the choline resonance indicates a structural change. This change must be more complex than simple cholesterol sequestration and may well involve specific interactions with phosphatidylcholine as well.

This description contrasts with recent calorimetric results on liposomes which show the polyenes simply to reverse the depression of the gel-liquid crystalline transition enthalpies caused by cholesterol [10]. To determine whether our observations

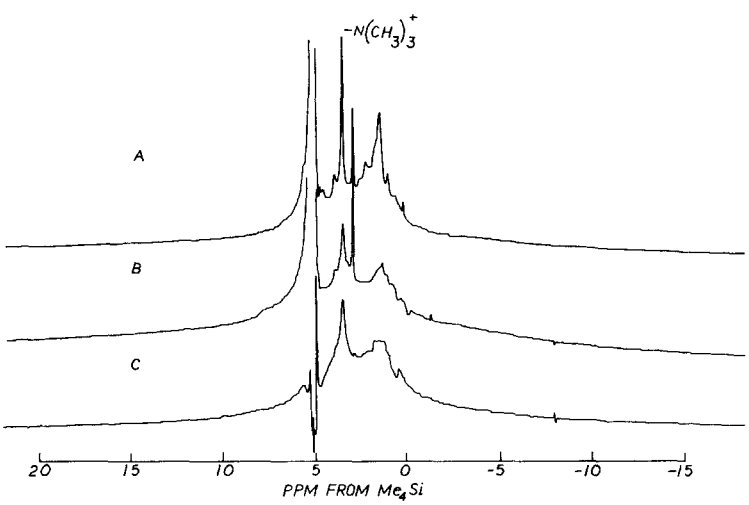

Fig. 7. 270 MHz ^1H NMR spectra of (A) phosphatidylcholine; (B) 1 : 1 cholesterol : phosphatidylcholine. (C) Difference spectrum of 1 : 1.5 : 1 cholesterol : filipin : phosphatidylcholine. All samples are $2.3 \cdot 10^{-3}$ M phosphatidylcholine and are run at 30 °C.

indicate a peculiarity of the vesicle system or indicate that NMR is sensitive to a lipid interaction that is not reversed by polyene complex formation, we present in Fig. 7A comparison of NMR spectra taken approx. 6 h after preparation of liposomes without cholesterol (Fig. 7A) with a 1 : 1 cholesterol : phosphatidylcholine ratio (Fig. 7B) and with a 1 : 1.5 : 1 cholesterol : filipin : phosphatidylcholine ratio (Fig. 7C). The third spectrum has had a spectrum of an equivalent filipin dispersion in buffer subtracted from it to make comparison easier. One can see that here also the very large increase in linewidths observed on addition of cholesterol are not significantly reversed on addition of filipin. Since extensive aggregates are known to be present in liposomes, the restriction of lipid motion reflected in NMR linewidths must be very similar for cholesterol and the polyene-cholesterol complex.

CONCLUSION

It is therefore apparent that the action of the polyene antibiotics on unilamellar vesicle structures containing cholesterol can be investigated in detail by a combination of NMR and gel permeation chromatography. Nystatin and amphotericin B appear to alter the permeability of the vesicle bilayer by forming pores as they do in other membrane systems. At high concentrations filipin appears to disrupt the vesicle structure by some secondary interaction. This is also reminiscent of behavior in other membrane systems. The onset of vesicle destruction for all three antibiotics gives an indication of the stoichiometry of the primary interaction with cholesterol. This interaction is of the order of 1 : 1.

The results contrast with previous studies on liposomal and whole cell systems in that filipin interaction at low concentrations and below a 1 : 1 antibiotic : cholesterol ratio is nondisruptive and induces no permeability changes in vesicles. Release of trapped ions on formation of nystatin and amphotericin B pores in vesicles is also observed on a much slower time scale than other membrane systems. The difference in disruptive behavior of filipin may reflect a fundamental difference between the structures of vesicles and more extended membranes. The difference in rate of pore-induced leakage probably originates with the relatively small fraction of entrapped ions contained in any single vesicle as compared to any single liposome or whole cell.

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