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VIBRATIONAL RAMAN SPECTRA OF LIPID SYSTEMS CONTAINING AMPHOTERICIN B

MARGARET R. BUNOW and IRA W. LEVIN

Laboratory of Chemical Physics, National Institute of Arthritis, Metabolism and Digestive Diseases, National Institutes of Health, Bethesda, Md. 20014 (U.S.A.)

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Summary

Resonance-enhanced and normal vibrational Raman spectra were observed for both multilamellar and single-wall vesicle assemblies of dimyristoyl phosphatidylcholine containing amphotericin B, a channel-forming polyene antibiotic, and cholesterol. The decrease in the frequency of the polyene antibiotic C = C stretching mode at 1556 cm^{-1} and the increase in intensity of the C-C-H in-plane deformation mode at 1002 cm^{-1} indicate that amphotericin B is ordered in a lipid-cholesterol medium similarly to the solid, but is surrounded by a slightly more polar environment. The intensity of the C = C stretching mode I_{1556} decreases 4-fold during the broadened gel to liquid crystalline phase transition (16—32°C) of dimyristoyl lecithin-cholesterol (4 : 1) multilayers. Other resonance-enhanced vibrations of amphotericin B exhibit similar behavior. For amphotericin B in pure dimyristoyl lecithin multilayer or vesicle systems, however, the vibrational intensity associated with the C = C stretching mode remains constant during the melting of lipid hydrocarbon chains. In addition, a third effect occurs in liquid crystalline egg lecithin-cholesterol (4 : 1, mol ratio) multilayers in which I_{1556} first increases by 25% between 3 and 25°C, in parallel with the loss of active channels, and then remains constant as the temperature increases from 25 to 42°C. This latter intensity pattern is masked in the dimyristoyl lecithin-cholesterol system by the overwhelming effect upon the C = C mode from changes in the lipid chain packing characteristics which occur during the phase transition.

The broadened phase transition in 4 : 1 dimyristoyl lecithin-cholesterol multilayers (16—32°C), as followed by the ratio of intensities at 2880 and 2850 cm^{-1} (asymmetric and symmetric methylene C-H stretching modes, respectively) is slightly narrowed by the addition of amphotericin B, and effect from which a binding stoichiometry at 24° of 1 : 1 amphotericin B : cholesterol is estimated. This stoichiometry was confirmed by differential calorimetric scans, which also show the presence of a peak proportional to cholesterol content.

Raman $I_{2880/2850}$ peak height ratios in pure dimyristoyl lecithin bilayers were increased over the 14—38°C range by amphotericin B, a spectral effect which suggests an ordering of the lipid matrix perhaps as a consequence of the polyene binding to the bilayer surface. For bilayers containing cholesterol, the ratios of intensities of the 2935 cm^{-1} feature, composed mainly of acyl chain terminal methyl and underlying methylene C-H stretching modes, to the 2850 cm^{-1} feature are significantly increased by amphotericin B. This effect indicates that the antibiotic penetrates the bilayer in the lipid-sterol system.

Introduction

The polyene antibiotic amphotericin B is thought to form channels through lipid bilayers and black lipid films containing either cholesterol or certain other sterols. This behavior has been deduced from ion and non-electrolyte permeability studies (for reviews see refs. 1 and 2) and from the apperance of conductance steps of up to $2 \cdot 10^{-10}\ \Omega^{-1}$ in black lipid films containing cholesterol and bathed in a 0.1 M NaCl aqueous phase (see Fig. 1) [3]. In addition, the polyene antibiotic nystatin, which is similar to amphotericin B in structure and behavior, has recently been reported to exhibit channels that appear to be approximately the size of those of amphotericin B [4].

A model of the channels has been suggested on the basis of both amphotericin B cooperativity and the arrangement of the hydrophobic and hydrophilic structural features of the polyene (see Fig. 2) [5]. In a channel amphotericin B molecules are probably oriented with the ionizable mycosamine and carboxylic functions at the lipid-water interface and are aligned with the polyene axis normal to the plane of the bilayer. Characteristic changes in the ultraviolet spectrum of amphotericin B in the presence of certain sterols imply sterol association [6]. Analysis of the absorption of polarized light by ampho-

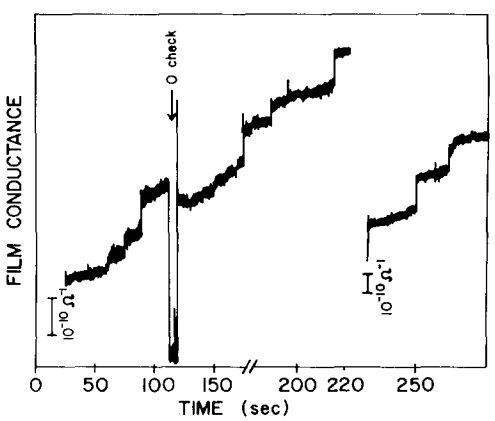

Fig. 1. Discrete positive conductance jumps in the record of conductance vs. time for a phosphatidylserine-cholesterol black film (molar ratio 1 : 1 in the film-forming decane solution) to which lipid vesicles containing 4 mol % amphotericin were added to one side with fusing agent (see ref. 3). Bathing solution was 0.1 M NaCl, 10 mM Tris · HCl, 10 mM $CaCl_2$, pH 7.5. The conductance steps lie between about $2 \cdot 10^{-11}$ and $2 \cdot 10^{-10}\ \Omega^{-1}$. The conductance steps rarely fall.

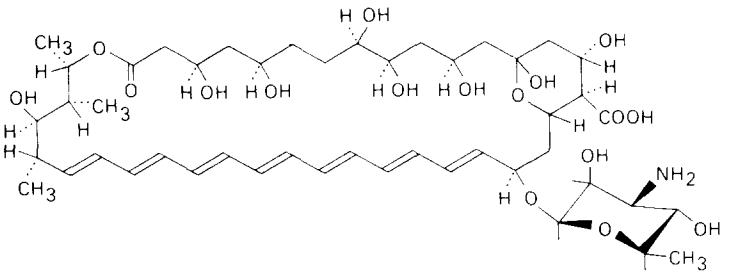

Fig. 2. Structure of amphotericin B. The X-ray structure determined for the crystalline *N*-iodoacetyl derivative shows the polyene chain to be all-*trans* [5]. Note that one side of the long axis of amphotericin B is hydroxylic in character and the other side polyenic; presumably the hydroxylic sides of an array of molecules, aligned along their long axes, line the interior of a channel in a lipid bilayer.

tericin B adsorbed onto low pressure monolayers of cholesterol also supports the view that the polyene axis is directed normal to the plane of the bilayer [7].

The electronic structure of the polyene backbone of amphotericin B leads to a resonance-enhanced Raman spectrum that allows the molecule to be observed at low concentrations in lipid assemblies. In order to further probe intermolecular interactions of amphotericin B with either itself, sterol, or lipid, we present in this study the vibrational Raman behavior of amphotericin B in multilayers of dimyristoyl lecithin with cholesterol as a function of temperature. Specific spectral features of the lipid are also monitored. In addition, spectra of amphotericin B in egg lecithin-cholesterol bilayers, in pure dimyristoyl lecithin bilayers, and in solvents are investigated and discussed.

Materials and Methods

Reagents. Dimyristoyl lecithin was obtained from Calbiochem, egg lecithin from Sigma, dioleyl lecithin from Miles Laboratories, and cholesterol from Nutritional Biochemicals. Amphotericin B (United States Food and Drug Administration standard) was a gift from Dr. I.M. Asher; amphotericin B, type A, was a gift from the Squibb Institute of Medical Research, courtesy of Dr. S.T. Lucania. Spectral grade solvents were used.

Lipid preparations. Multilayer samples were prepared by first dissolving components in chloroform/methanol solutions, drying under N_2 and then in vacuo. Samples were hydrated in 50 mM phosphate buffer to 30% total lipid by weight by vortex shaking for 15 min at 30° C under N_2. Sonicated samples were prepared similarly, except that 15 min of sonication (using a Branson sonifier with a standard microtip probe) followed a brief vortex shaking. Both types of sample were centrifuged at either 5000 × *g* or 50 000 × *g* at 5° C in order to remove unincorporated amphotericin B or sonifier tip debris. The upper half of the centrifugate was employed for spectral scans. Sonicated samples were suspended in low-ionic strength (5 mM) buffer in order to prevent fusion. All ratios of bilayer components that are given in the text refer to molar ratios.

Raman spectroscopy. The Raman instrumentation, including a modified Cary 81 with an external sampling compartment, has been previously described

[8]. Samples were illuminated using one of the excitation lines available from an argon laser (Coherent Radiation Model 52G) or from a Coherent Radiation Model 490 dye laser using rhodamine 6G as the dye element. Scattered radiation was collected at 90° C to the incident radiation. Typical laser power employed was 400 mW. Fluorescence of samples containing amphotericin B was quenched through long exposures in the laser beam. The ultraviolet spectrum of amphotericin B was checked before and after laser beam exposure in order to confirm its molecular integrity. The integrity of amphotericin B was also inferred from the features of the Raman spectrum itself. Scanning rates were generally 0.25 $cm^{-1} \cdot s^{-1}$. Spectral frequencies, calibrated with atomic argon emission lines, are reported to ± 2 cm^{-1}.

Solid spectra were recorded with a rotating sample holder. Amphotericin B powder was ground with KBr and packed into a circular groove in the rotating disk. Samples containing lipid were placed in a 3 mm × 3 mm cuvette seated in a coppper block whose temperature was controlled by water circulation. The temperature of the sample in the laser beam was monitored by a copper-constantan thermocouple. Additional standardization of the temperature reported by the thermocouple, which is critically dependent on its position with respect to the laser beam, was supplied by a probe inserted into the copper block and a thermometer in the constant temperature water bath. The temperature during a spectral scan was constant to ± 0.1° C. Samples were allowed 20 min to equilibrate to new temperatures, which were usually within 2° C of the previous measurement. All comparisons of band intensities, unless explicitly stated to be otherwise, refer to illumination with the 514.5 nm argon laser line.

Differential scanning calorimetry was performed with a Perkin-Elmer DSC-2. Samples of 1—2 mg total weight, 50% buffer, were read in tared aluminum planchettes. Scan rates were 2.5—5 degrees/min; cooling rates were sometimes faster. After scanning, the planchette covers were punctured with a needle. The samples were dried in a 70° C oven and weighed on a microbalance. Wet and dry weights of sample were then computed.

Results and Discussion

(A) Vibrational Raman spectra of amphotericin B

The three major resonance-enhanced bands of amphotericin B occur, in the solid, at 1560, 1156, and 1002 cm^{-1}, with relative peak heights of 1.0, 0.45, and 0.08, respectively. These features are polarized in solution. In linear *trans*-polyenes, the strongest band, here at 1560 cm^{-1}, has been assigned to the C = C stretching transition, while the 1156 and 1002 cm^{-1} bands have been assigned, respectively, to a C-C stretch coupled with the in-plane C-C-H bend, and to the C-C-H in-plane bending deformation coupled with the C = C-C bending distortion [9,10]. Both the latter bands have contributions from the C = C stretching vibration. A compilation of the bands is given in Table I. Fig. 3 displays a survey Raman spectrum of solid amphotericin B. The dependence of the main band intensities on the wavelength of the laser exciting line is given in Fig. 4. By choosing suitable laser excitation wavelengths, one may monitor amphotericin B-lipid mixtures for primarily either the resonance-enhanced spectra of the polyene component or the normal Raman spectra of the lipid constituent.

TABLE I

FREQUENCIES OF RAMAN TRANSITIONS FOR AMPHOTERICIN B IN THE SOLID PHASE, IN DIMETHYLSULFOXIDE SOLUTION, IN METHANOL SOLUTION AND IN DIMYRISTOYL LECITHIN-CHOLESTEROL MULTILAYERS

Solid [a] (cm^{-1})	Relative intensity	Amphotericin B		Dimyristoyl lecithin-cholesterol [b] (cm^{-1})	*Trans*-β-carotene carbon tetrachloride soln. (cm^{-1})
		Dimethylsulfoxide soln. (cm^{-1})	Methanol soln. (cm^{-1})		
986	0.01	— [c]	— [c]	985	
1002	0.08	— [c]	— [c]	1002	963
1010 sh	0.05			1008	
					1005 m
					1024 sh
1136 sh	0.13	1137	1137	1136	
1156	0.45	1157	1157	1154	1158 s
					1176 sh
1188	<0.01				
1201	0.02	1201	1198	1198	1193 m
					1213 w
1294	0.04	1297	1298	1295	1270 w
					1448 w
1560	1.00	1559	1559	1556	1523 vs
1603	0.02	—	1602	1602	
1636	0.020	1638	1640	1631	
1644	0 015	—	—	1638	

[a] sh, w, m, s and vs represents shoulder, weak, medium, strong and very strong, respectively.
[b] Dimyristoyl lecithin-cholesterol (mol ratio 4 : 1) multilayers were recorded at 25° C.
[c] Weak features under solvent peaks.

The C = C stretching frequency decreases from 1560 cm^{-1} in the solid to 1559 cm^{-1} in dimethylsulfoxide or methanol, to 1557 cm^{-1} in a buffered suspension (pH 7) with or without suspended cholesterol and to 1556 cm^{-1} at room temperature in dimyristoyl lecithin-cholesterol liposomes of varying composition (0—20 mol % cholesterol). The 1156 cm^{-1} peak, at 1157 cm^{-1} in solvents, decreases to 1154 cm^{-1} in the lipid-cholesterol environment. The shift in the ethylenic C = C transition to lower frequencies in solvents of increasing polarity is consistent with the shift to longer wavelengths of the ultraviolet absorption spectrum of amphotericin B and implies a slight delocalization of the polyene π electron system.

The spectrum of amphotericin B in acidic or basic methanol (pH 1 or 10) is markedly altered. A shoulder at 1540 cm^{-1} appears on the 1557 cm^{-1} C = C peak, and the 1157 cm^{-1} peak with its accompanying 1136 cm^{-1} shoulder is transformed into a doublet at 1164 and 1147 cm^{-1}. In addition, the weak 1198 and 1290 cm^{-1} transitions are replaced by transitions at 1218 (medium intensity) and 1230 cm^{-1} (weak), and in the case of pH 1, also 1270 cm^{-1} (weak). These changes are similar to those reported for ethanolic solutions of *trans*-retinal upon acidification, particularly with respect to the appearance of a low-frequency shoulder on the C = C transition [11]. The splitting of both the C = C and C-C modes is also characteristic of retinal *cis*-isomer formation [12],

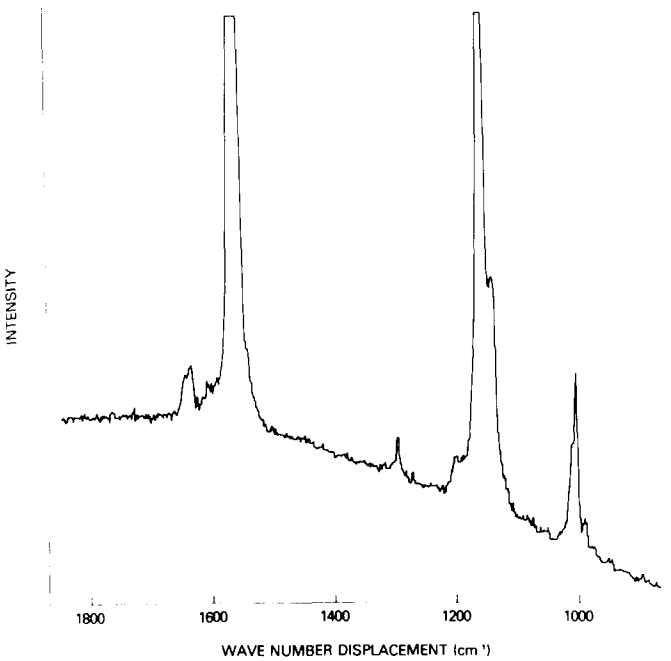

Fig. 3. Survey Raman spectrum of solid amphotericin B mixed with KBr. 514.5 nm laser excitation at 120 mW. 3 cm^{-1} spectral resolution.

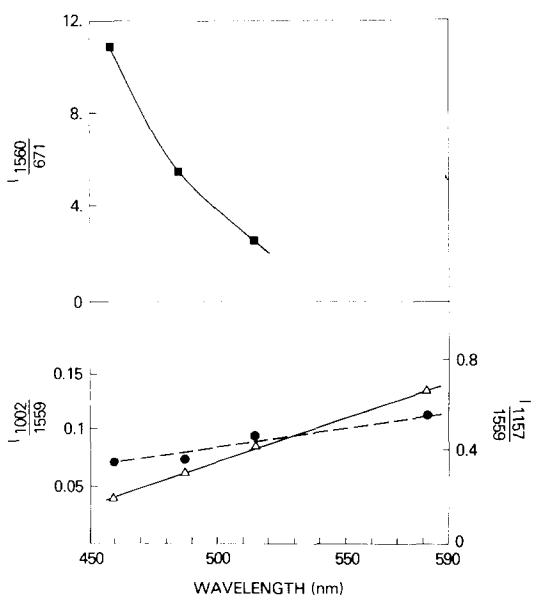

Fig. 4. Dependence of amphotericin B vibrational intensities on the wavelength of the incident laser line. Top: Amphotericin B in dimethylsulfoxide. ■, ratio of the ethylenic C = C peak height at 1560 cm^{-1} to the 671 cm^{-1} peak of dimethylsulfoxide. Bottom: Solid amphotericin B mixed with KBr. ●, represents $I_{1157/1559}$ ratios; △, represents $I_{1002/1559}$ ratios. Laser lines used were 457.9, 488.0, 514.5 and 582.2 nm.

a structural modification which cannot be excluded in the case of amphotericin B. These spectral alterations, which may be associated in part with the isomerization of amphotericin B following the cleavage of the macrolactone ring, serve as convenient indicators of the integrity of the amphotericin B structure. No such changes were observed in spectra under any other of the conditions reported here. In this sense, amphotericin B has proved more stable to laser beam exposure than rhodopsin [13] or, in our studies, *trans*-β-carotene.

The 1002 cm^{-1} C-C-H in-plane bending mode falls in intensity relative to the 1556 cm^{-1} C = C stretch (I_{1556}) from 0.08 in the solid to 0.01 in methanol or dimethylsulfoxide. The feature is restored to 0.08—0.10 relative intensity in pure lipid or lipid-cholesterol systems. Apparently, the solid amphotericin B or lipid-cholesterol matrix maintains the rigid extended conformation of the amphotericin B molecule. Thus, the C-C-H group maintains sufficient planarity with the polyene chain such that the distortion along the C-C-H deformation normal coordinate contributes to the overall geometrical changes involved in the $\pi^* \leftarrow \pi$ electronic transition [10,14]. As the C-C-H moiety loses planarity with the polyene skeleton, the C-C-H bending distortion leads to carbon atom displacements that are somewhat skew to the direction of structural change, an effect which would decrease the magnitude of resonance enhancement [10, 14]. Thus, the motional freedom of the polyene in solvents reduces the intensity enhancement.

(B) Temperature dependence of amphotericin B spectra

In multilayers of dimyristoyl lecithin-cholesterol-amphotericin B (24 : 6 : 1 and 150 : 3 : 1), the 1556 cm^{-1} C = C vibration shifts upwards by 3 cm^{-1} upon lowering the temperature from 38 to 7° C through the liquid crystalline to gel phase transition (which is centered at 24° C). About the same shift occurs with the minor bands at 1200 and 1600 cm^{-1}. The 1631 cm^{-1} band appears to shift by 6 cm^{-1} from 1628 to 1634 cm^{-1}. The general trend is consistent with the average environment of amphotericin B becoming less polar at reduced temperatures.

The most dramatic temperature effect arises in the intensities of the resonance enhanced spectrum. Specifically, the intensity of the C = C stretching vibration of amphotericin B, relative to the moderately constant 1440 cm^{-1} acyl chain CH_2 deformation of the lipid, decreases by 4-fold over the gel to liquid crystalline phase transition of dimyristoyl lecithin, in both low cholesterol sonicated vesicles and high cholesterol multilayers (dimyristoyl lecithin : cholesterol : amphotericin mol ratios 150 : 3 : 1 and 24 : 6 : 1, respectively). Thus, the double bond intensity acts as a probe of the hydrocarbon region of the lipid. In contrast, for sonicated dimyristoyl lecithin single-shell vesicles lacking cholesterol, the intensity of the double bond transitions of amphotericin B is constant between 14 and 38° C. The latter behavior probably reflects the low penetration of amphotericin B into the interior of the cholesterol-free lipid bilayer from the lipid surface and aqueous phase, a conclusion which is consistent with the vibrational behavior of the dimyristoyl lecithin in the 2800 cm^{-1} region discussed below. The C-C stretching intensity follows the C = C intensity changes within 10%, remaining 0.40 of the C = C stretching intensity. The weaker resonance enhanced peaks at 1001, 1205, and 1630 cm^{-1}

also follow the 1556 cm^{-1} peak in relative intensity, being about 0.09, 0.02, and 0.02, respectively.

In egg lecithin-cholesterol-amphotericin (36 : 9 : 1) multilayers, the intensity of the C = C mode of amphotericin B does not decrease on raising the temperature. Instead, the feature undergoes a reversible 25% gain in intensity, beginning at approx. 25° C as the temperature is increased from 3 to 42° C, where the temperature range lies within the liquid crystalline phase of egg lecithin/cholesterol mixtures. Also, the frequency of the mode centered at 1557 cm^{-1} remains invariant over this temperature range. (The C = C vibration also occurs at 1557 cm^{-1} in dioleyl lecithin-cholesterol-amphotericin B (36 : 9 : 1) multilayers and remains invariant in frequency from 3 to 30° C.) We conclude that the dramatic decrease with increasing temperatures in I_{1556} in dimyristoyl lecithin-cholesterol multilayers reflects the changes in hydrocarbon chain packing as the system passes through the gel to liquid crystalline phase transition. Similarly, the 3 cm^{-1} shift to lower frequencies in the amphotericin B C = C peak position with higher temperatures, which is seen only in the dimyristoyl lecithin multilayers, may reflect an increase in dielectric constant of the environment of the polyene produced by a thinning of the bilayer as the system enters the liquid crystalline phase. Since these trends are absent in the egg lecithin-cholesterol system, it appears that they do not reflect a temperature-dependent amphotericin B-cholesterol interaction. Rather, the observed 25% increase in I_{1556} in the latter system may be correlated with the dissociation of active amphotericin complexes with increasing temperature and a lessened interaction with cholesterol; however, the configurational changes which are involved are not easily interpreted from these observations. The steep temperature dependence of amphotericin activity occurs in other membrane systems in the same 3—25° C range (see, for example, ref. 15). Also, the I_{1556} increase correlates with the increase in the intensity of the longest-wavelength (414 nm) feature in the ultraviolet absorption spectrum of amphotericin B, which is taken as an indication of loss of interaction with sterol in a variety of systems [15].

The fluorescence of amphotericin B appeared to vary inversely with availability of water to the molecule. The only systems examined here in which fluorescence was low were amphotericin in buffer with and without cholesterol, and in pure dimyristoyl lecithin where the fluorescence was somewhat higher. The latter observation is consistent with amphotericin B being surface bound in dimyristoyl lecithin multilayers.

(C) Raman spectra of the lipid multilayer matrix

Since the vibrational behavior of amphotericin B responds to the state of the lipid-cholesterol matrix, the lipid spectrum may in turn be examined for perturbations by amphotericin B. The weak cholesterol signals representative of the mol fractions used in the lipid mixtures posed no complicating effects [8, 16].

In the lipid, both the hydrocarbon chain skeletal C-C vibrational modes in the 1100 cm^{-1} region and the C-H stretching modes, in the 2800—3100 cm^{-1} region have been shown to reflect the packing of lipid multilayers (see, for example, refs. 16—20 for scans of representative spectra in these regions). For the lipid-amphotericin systems described here, the skeletal vibrational region is

shared by the resonance-enhanced 1154 cm^{-1} C-C band of amphotericin B; hence, the more intense and less distorted 2800 cm^{-1} spectral region for the acyl chains was examined. The features at approx. 2850, 2880, and 2935 cm^{-1} have been assigned, respectively, to methylene C-H symmetric stretching, methylene C-H asymmetric streching, and acyl chain terminal methyl C-H symmetric stretching modes [8]. The 2935 cm^{-1} region also includes additional chain methylene modes which are state dependent. We introduce this point below and further analyze this band in a forthcoming publication. Both the peak frequencies and the ratios of the peak intensities reflect the gel liquid crystalline phase transition [18].

The ratio of peak heights for symmetric and asymmetric methylene C-H stretching modes, $I_{2880/2850}$, plotted in Fig. 5a for 4 : 1 dimyristoyl lecithin-cholesterol, shows a transition width of about 16° C. For reference, the transition monitored by Raman spectroscopic changes in the C-C chain skeletal modes for 3 : 1 dipalmitoyl lecithin-cholesterol multilayers was found to be about 24° C wide [17]. This broadening of the phase transition by cholesterol

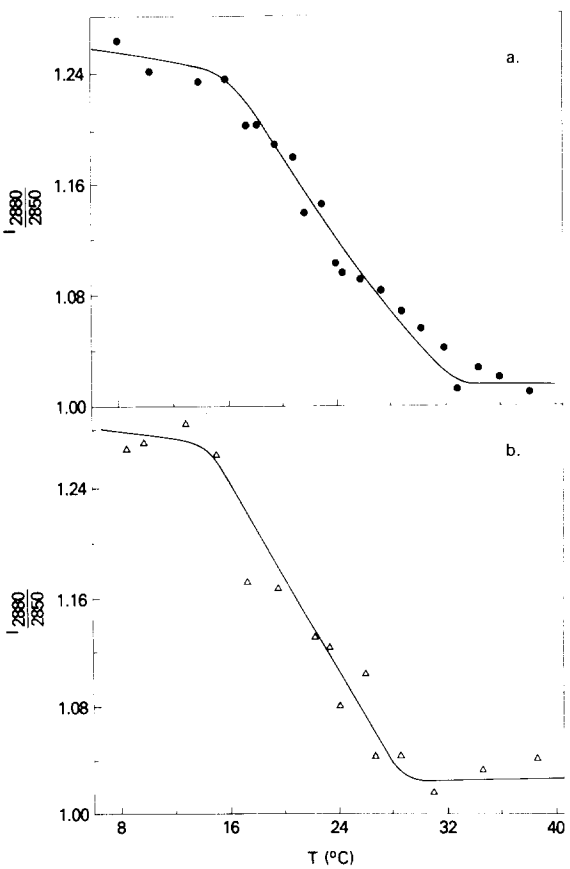

Fig. 5. The ratio of peak heights for lipid chain methylene asymmetric and symmetric C-H stretching modes, $I_{2880/2850}$, as a function of temperature. (a) Dimyristoyl lecithin-cholesterol (4 : 1) multilayers. (b) Dimyristoyl lecithin-cholesterol-amphotericin (24 : 6 : 1) multilayers.

has been recorded by differential thermal analysis and a variety of spectral methods and is interpreted as a lowering of the cooperativity of the phase transition by the incorporated cholesterol, or possibly, as formation of a second cholesterol-lecithin complexed phase dispersed within the remaining pure lecithin [21—23]. Amphotericin B appears on several grounds to compete with lecithin for association with cholesterol. In addition to the requirement for sterol for its action in lipid membranes, amphotericin B has, for example, been shown by differential scanning calorimetry to reverse completely the reduction in the heat of transition caused by 33 mol % cholesterol in dielaidoyl lecithin [6]. This experiment also constituted a straightforward determination of the stoichiometry of cholesterol bound by amphotericin B, which was found to be 4 to 1 in the dielaidoyl lecithin-cholesterol system at the transition temperature of about 10° C [1].

The effect of amphotericin B on the phase transition in 4 : 1 dimyristoyl lecithin-cholesterol multilayers, as monitored by $I_{2880/2850}$, appears less pronounced (see Fig. 5b) than that reported for the dielaidoyl lecithin system [6]. At most, the transition in dimyristoyl lecithin-cholesterol has been narrowed by 4° C. From this narrowing, an estimate of the stoichiometry of binding is made as follows.

The transition width for the dimyristoyl lecithin-cholesterol system, as found by Hinz and Sturtevant by differential thermal scanning (see Fig. 3 in ref. 21), can be approximated by a linear function of mol percent cholesterol between 0 and 20 mol % cholesterol. Similarly, we assume that the dependence of the transition width derived using the Raman spectral $I_{2880/2850}$ parameter can also be approximated as linear through this region, taking as endpoints a 1° C width at 0 mol % cholesterol and a 16° C width at 20 mol %. In this case, the narrowing of the transition width from 16 to 12° C by amphotericin B represents an apparent lowering of the cholesterol concentration from 20 to 15%. In other words, 25% of the cholesterol present was annexed by amphotericin B. The mol ratio of cholesterol to amphotericin B present in the system was 6 to 1, so each molecule of amphotericin was associated with about 1.5 of cholesterol. This number represents an upper bound to the amount of cholesterol actually sequestered by amphotericin B since the dependence on cholesterol content of the phase transition width becomes steeper near 20 mol % cholesterol. Hence, a ratio of amphotericin B to cholesterol content in the neighborhood of 1 : 1 is estimated from the Raman data. This result for the dimyristoyl lecithin system may be more typical of lipid systems at 25° C than that obtained by Norman et al. [6] for the dielaidoyl lecithin system at 10° C. Since membrane permeability to ions induced by amphotericin B rises steeply with lowered temperatures, the amphotericin B-cholesterol channels may evolve in size and/or stability as the temperature is lowered with additional molecules of cholesterol becoming attached to the multimeric structures.

The effect of amphotericin B on 4 : 1 dimyristoyl lecithin-cholesterol multilayers was verified by differential scanning calorimetry. For the 4 : 1 dimyristoyl lecithin-cholesterol multilayers, the heat absorbed under the very broad main transition did not change significantly with incorporation of amphotericin B (see Figs 6c and 6d). Hence, a system with lower cholesterol and a high amphotericin B cholesterol ratio (1 to 3 rather than 1 to 6) was surveyed (see

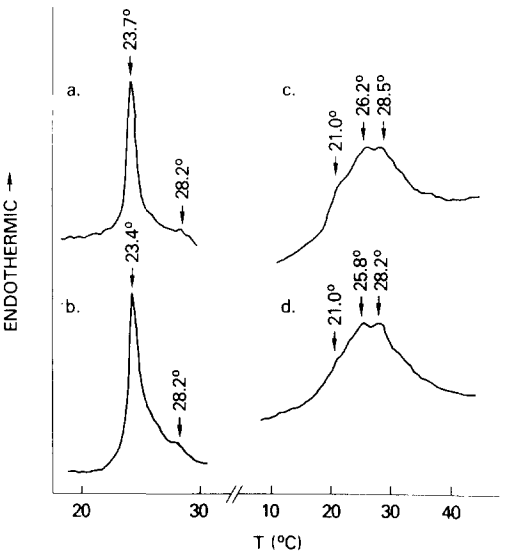

Fig. 6. Effect of amphotericin B on heating curves for dimyristoyl lecithin-cholesterol multilayers. (a) Dimyristoyl lecithin-cholesterol (8 : 1). (b) Dimyristoyl lecithin-cholesterol (8 : 1) plus amphotericin B (cholesterol : amphotericin B, 3 : 1). (c) Dimyristoyl lecithin-cholesterol (4 : 1). (d) Dimyristoyl lecithin-cholesterol (4 : 1) plus amphotericin B (cholesterol : amphotericin, 6 : 1). The ordinate in scans a and b is, on the basis of dry weight of dimyristoyl lecithin, about four times that in c and d. For reference, the main transition for pure dimyristoyl lecithin occurs at 24.0° C and the pre-transition occurs at 16.0° C. The main transition peak half widths were 1.5 and 1.2° C for a and b, respectively; 12° C for c and d; and 0.9° C for pure dimyristoyl lecithin. (Transition temperatures are averages of two or more scans.)

Figs. 6a and 6b). In this case, the heat of transition in the 8 : 1 dimyristoyl lecithin-cholesterol system (Fig. 6a) was found to be only two-thirds of that in the 24 : 3 : 1 dimyristoyl lecithin-cholesterol-amphotericin B system (Fig. 6b). Hinz and Sturtevant [21] noted that the heat of transition in dimyristoyl lecithin is linearly dependent on the mol fraction of cholesterol, falling to zero at 35 mol % cholesterol. On this basis, it is deduced that one amphotericin B molecule displaced one out of three of the cholesterol molecules from interaction with the lecithin; or the stoichiometry of amphotericin B to cholesterol is about 1 : 1, in rough agreement with the results from the Raman data.

It is noteworthy that the differential scanning calorimeter scans for cholesterol-lecithin systems show a second peak at 28° C (Fig. 6), which grows from a shoulder at 11 mol % cholesterol to a visible peak at 20 mol %. This peak is apparently associated with the phase of the cholesterol-lecithin complex. A remark on its existence has been made by Mabrey and Sturtevant [24]. The apparent upward shift in the main transition temperature ($\approx$23.5° C at 11 mol % cholesterol to $\approx$26° C at 20 mol %) may be partially due to the contribution of the 28° C peak. The addition of amphotericin B apparently does not affect the position of the transition, which is consistent with its being shielded from the lipid by cholesterol.

The values of $I_{2880/2850}$ away from the phase transition in 24 : 6 : 1 dimyristoyl lecithin-cholesterol-amphotericin B multilayers are equivalent to those for the same multilayers without amphotericin B. In contrast, the ratio $I_{2935/2850}$, taken to indicate in part an effect upon the chain terminal methyl

group, is elevated by amphotericin B throughout the 6—38° C range. This effect is displayed in Fig. 7. Similar increases for this intensity ratio have been reported by Larsson [19] as occurring on the change from L_2 to cubic phases in glyceryl mono-2-bromoundecanoate and as resulting from the accessibility of polar molecules including water and polypeptides to acyl chains in several model systems [25]. From these model studies, two types of effects are tentatively assumed to contribute toward an intensification of the 2935 cm^{-1} feature: extensive disorder in packing of the acyl chains, and increased polarity of the environment of the chain extremities [25]. Since these effects are inseparable, our interpretations are generally consistent with those of Larsson and Rand [25]. Thus, in the presence of cholesterol, amphotericin B forms hydroxylic channels which penetrate the lipid bilayer. The introduction of the channels into the hydrocarbon region alters the symmetry of the acyl chains such that the CH_2 asymmetric stretching mode $\approx$2920 cm^{-1} which is Raman inactive under local C_{2h} symmetry for the all-trans conformation, becomes Raman allowed. The increased population of these CH_2 asymmetric modes, which lie under the 2935 cm^{-1} methyl feature, also results in an apparent increase of the methyl stretching vibration. The intensity comparison of $I_{2935/2850}$ with and without amphotericin B is more marked at lower temperatures (Fig. 7), in agreement with the increased activity of amphotericin B with reduced temperature.

(D) 2800 cm^{-1} region of dimyristoyl lecithin-amphotericin B single-shell vesicles

Within the temperature range 14—38° C, 2% amphotericin B in sonicated dimyristoyl lecithin vesicles increases $I_{2880/2850}$ above that for either pure dimy-

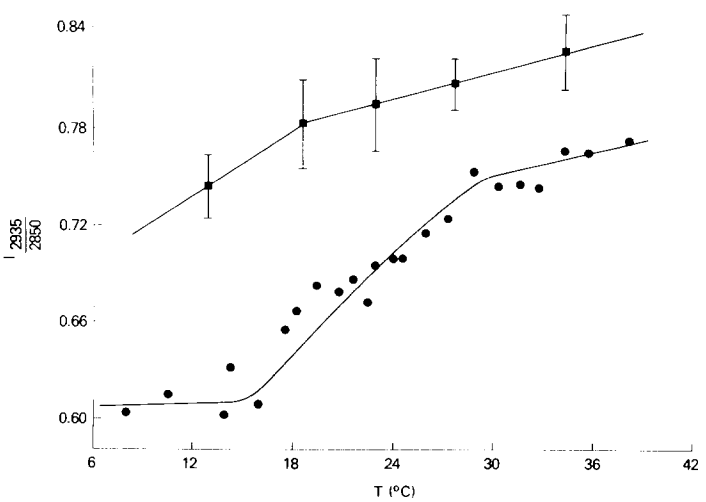

Fig. 7. $I_{2935/2850}$ ratios for multilayers of dimyristoyl lecithin-cholesterol (4 : 1) (●) and dimyristoyl lecithin-cholesterol (4 : 1) plus amphotericin B (cholesterol : amphotericin, 6 : 1) (■). Because of the fluorescence background in the amphotericin B-containing system, points for this system have been averaged within the five intervals between 6, 16, 21, 25, 30 and 38° C. Bars indicate the maximum errors in the ratios.

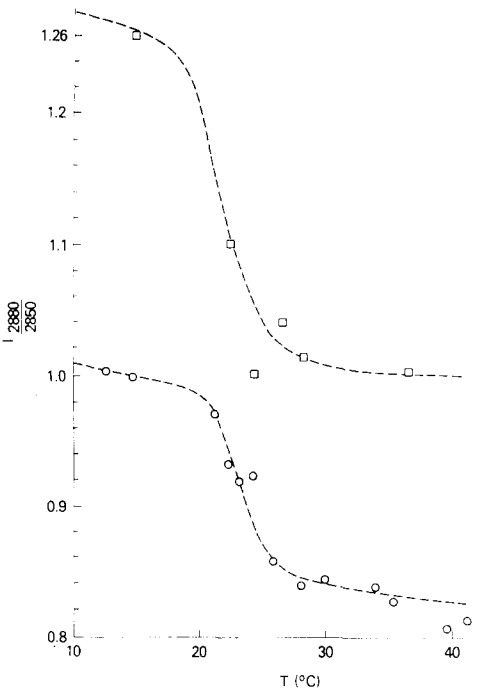

Fig. 8. $I_{2880/2850}$ ratios for single-shell vesicles. ○, dimyristoyl lecithin sonicate; □, dimyristoyl lecithin sonicated with 2 mol % amphotericin B.

ristoyl lecithin vesicles or multilayers as shown in Fig. 8, while the ratio $I_{2935/2850}$ is not significantly higher. Since a high $I_{2880/2850}$ ratio is characteristic of the lipid chain methylene C-H stretching modes in the gel phase and in anhydrous polycrystalline lipid, the intensity trend observed for the dimyristoyl lecithin sonicate suggests less *trans-gauche* rotational isomerization and a tighter packing of the lipid chains, a constraint probably mediated by the polar head group interface. The absence of an effect of amphotericin B on the $I_{2935/2850}$ ratio is consistent with the antibiotic being bound at the lipid interface and thus being incapable of distorting the vibrational characteristics of chain methylene stretching modes in the interior of the lipid bilayer.

Very little effect of amphotericin B on pure dimyristoyl lecithin is detected in the co-sonicate by differential scanning calorimetry. The pre-transition feature at 16° C is not reduced by 4 mol % amphotericin, while the position of the main transition at 24° C is raised by 1° C.

Conclusion

For use as a structural probe, the vibrational Raman intensity of the amphotericin B polyene C = C stretching mode is highly sensitive to the molecular changes which occur during the phase transition in dimyristoyl lecithin-cholesterol multilayers containing, in our experiments, up to 20 mol % cholesterol. From this spectral parameter and from $I_{2880/2850}$ plots for the lipid component, we confirm that the large changes in the packing of the lipid chains which

ordinarily occur during the gel to liquid crystalline phase transition are now spread over a large temperature interval, rather than being suppressed, by the addition of cholesterol. This result is consistent with that of spin label studies [26], in which a 15% change in molecular surface area is sensed by the spin probe through the phase transition (over a 20°C interval) in 4 : 1 dipalmitoyl lecithin-cholesterol multilayers.

A sensitivity of the ethylenic stretching intensity to the phase transition has also been reported for Raman spectral scans of *trans*-β-carotene in egg lecithin, dimyristoyl lecithin, and dipalmitoyl lecithin liposomes [27] and in erythrocyte ghosts [28]. In our studies, however, we have not observed any significant variation in the intensity ratio $I_{1154/1556}$ analogous to that reported for β-carotene in egg lecithin liposomes [27].

The response of the amphotericin B C = C stretching intensity to the state of the phospholipid chains, in addition to the apparent effect upon the terminal methyl groups of the acyl chains as inferred from $I_{2935/2850}$ ratios, implies that the amphotericin B molecules penetrate the hydrocarbon interior of the dimyristoyl lecithin bilayer in the presence of cholesterol over the temperature range 14—28°C. An alternative interpretation is that changes in lipid chain packing are transmitted to amphotericin B bound at the polar interface of the lipid and hence affect I_{1556}. However, amphotericin B adsorbed at the interface in cholesterol-free lipid does not respond to changes in the lipid state. (We cannot exclude a different mode of adsorption in lipid systems containing cholesterol).

Several polyene antibiotics including amphotericin B have been noted to achieve full activity after being added to model lipid bilayer systems only following an appreciable time lag [15,29,30]. This time delay is attributed either to possible diffusion across or within the plane of the bilayer, or to some configurational rearrangement. The time involved increases at low temperatures. We emphasize that in the present studies with amphotericin B, and in contrast to much of the literature, amphotericin B was initially available to, and equilibrated with, all lipid interfaces while the lipid was in the liquid crystalline phase (at about 30°C).

Since amphotericin B can be induced to move from the polar interface to the interior of the lipid bilayer by the addition of a suitable sterol, amphotericin B can mimic both an extrinsic and an intrinsic membrane component. In this context, the present study provides a survey of the usefulness of both vibrational frequency and intensity parameters toward evaluating structural characteristics of biomembranes by Raman spectroscopy.

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