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PRE-RESONANCE RAMAN SPECTRA AND CONFORMATIONS OF NYSTATIN IN POWDER, SOLUTION AND PHOSPHOLIPID-CHOLESTEROL MULTILAYERS

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Summary

A Raman scattering study of the channel-forming polyene antibiotic nystatin, is reported in the solid state, in organic and aqueous solutions as well as in phospholipid and phospholipid-cholesterol multilayers. Measurements of the solid and solution spectra as a function of excitation wavelengths in the 459.7—514.5 nm range, and the phospholipid spectra as a function of temperature in the 10—60°C range, have also been made. The spectral features indicate a pre-resonance-enhanced Raman spectrum in which the C=C and C-C stretching modes of the polyene segment of nystatin are dominant. However, in contrast to previously published results on the nearly isostructural polyene antibiotic amphotericin B, a line at 1610 cm^{-1} assignable to the C=O stretching mode is also observed to be strongly resonance enhanced. This is explained by a postulated ground-state conformation model in which a twisting of the molecule is facilitated by the break in the polyene chain. This allows the C=O group at one end of the molecule to be aligned along the polyene unit at the other end, and the C=C stretching vibration, which is strongly modulated by the polyene $\pi \rightarrow \pi^*$ excited state, to mix with the C=O stretching vibration. The peak frequencies and intensities of the C=C and C-C stretching modes in nystatin, however, remain essentially unchanged compared with amphotericin B, indicating that the polyene units in nystatin remain planar and *trans* both in the ground and excited states.

The intensity of the C=O mode with respect to the C=C stretching mode was observed to vary appreciably with nystatin environment, indicating a

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Abbreviation: Me_2SO, dimethylsulphoxide.

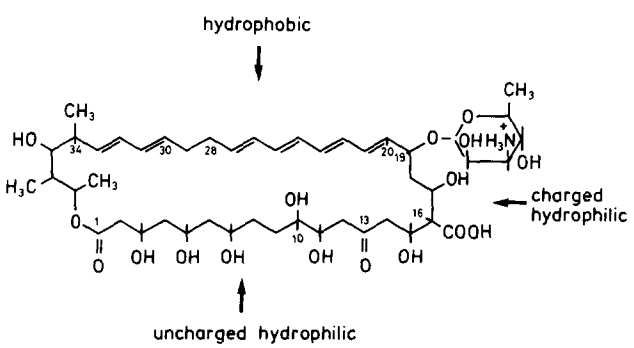
change from the postulated twisted conformation, present in the solid and solutions, to a largely planar conformation in phospholipid-cholesterol multilayers, pure phospholipid multilayers in the gel phase and in aqueous solutions containing cholesterol.

In contrast to amphotericin B, the methanol solution spectra of nystatin are not sensitive to pH in the range 1.0—9.0, and the relative Raman spectral intensities of the C=O, C=C and C-C stretching modes do not change when dimystristoyl and dipalmitoyl phosphatidylcholine-cholesterol multilayers containing nystatin (molar ratio 24 : 6 : 1) transform from the gel to the liquid-crystal phase. The relative stability of nystatin compared with amphotericin B, indicated by these studies, is consistent with the postulated conformational flexibility of the nystatin molecule.

Introduction

Ion and non-electrolyte permeability measurements and the appearance of conductance steps in black film studies indicate that the polyene antibiotic nystatin forms channels through lipid bilayers containing either cholesterol or certain other sterols [1—4]. The behaviour of nystatin parallels that of the polyene antibiotic amphotericin B, except that the pore formed in the nystatin-containing system is somewhat smaller as indicated by the permeability measurements. Nystatin-containing membranes are nearly impermeable to glucose, which has a Stokes-Einstein radius of 0.4 nm. The expected nystatin-cholesterol pore is, therefore, expected to have a diameter in the range 0.4—0.7 nm. The polyene-cholesterol pores were found to be anion selective, with discrimination occurring via anionic size [2—4].

The molecular structure of nystatin has been proposed [5,6] and is shown in Fig. 1. The structure differs from that of amphotericin B in two important respects: (i) in the conjugated polyene chain, in which there is a break between positions 28 and 30 as indicated in Fig. 1, and (ii) in the ordering of the OH groups around the hydrophilic sites of the molecule. Both nystatin and



Nystatin
$C_{47}H_{75}O_{17}N$

Fig. 1. Proposed molecular structure of nystatin (after Refs. 5 and 6).

amphotericin B contain a carboxyl group and an amino sugar (mycosamine) residue, which render the antibiotics amphoteric. In a lipid membrane environment, nystatin and amphotericin B are probably oriented with the ionizable mycosamine and carboxylic groups at the lipid-water interface and the polyene segment normal to the lipid bilayer plane [7]. Characteristic changes in the visible-ultraviolet absorption spectra of nystatin and amphotericin B in the presence of cholesterol, imply antibiotic-sterol association [8]. Based on stereochemical considerations a cylindrical channel containing a (cholesterol-polyene)$_8$ complex has been proposed [2,9]. The inside of the channel is lined with the OH groups and is therefore hydrophilic in character, whereas the outside consisting of the polyene segment is hydrophobic.

The localized electronic transitions associated with the conjugated polyene chains of nystatin and amphotericin B lead to pre-resonance Raman spectra with excitation in the visible region, thus allowing the biologically important polyene segment of the molecules and its interactions to be sampled at low concentration. A study of amphotericin B using this technique has been recently reported by Bunow and Levin [10]. In this paper we report on a pre-resonance Raman study of nystatin in phospholipid-cholesterol multilayers as well as in solutions and in the solid state. The results obtained indicate appreciable differences in conformational flexibility and stability between nystatin and amphotericin B, in spite of their overall similarity in biological activity and chemical structure.

Materials and Methods

Materials. Dimyristoyl phosphatidylcholine and dipalmitoyl phosphatidylcholine were obtained from Fluka; and cholesterol from Sigma Chemical Corp. Nystatin was purchased from the Squibb Institute. Solvents used were of spectral grade.

Multilayers samples were prepared by first dissolving components in methanol solution, drying under N_2 and then in vacuo. The samples were hydrated in 0.1 M KCl solution, and sonicated for 15 min in a sonification bath at 25°C. Vesicles were prepared similarly except that the sample was sonified for 15 min using a Branson sonifier with a standard microtip.

Intrumentation and technical details. The Raman instrumentation consisted of a Spex 1401 double monochromator with holographic gratings and pulse-counting detection. The spectra were excited with one of the lines of an argon ion (Spectra Physics 171) laser. The solution and lipid suspension sampling techniques used are the same as described by Weidekamm et al. [11]. Fluorescence of samples containing nystatin was quenched through half an hour exposure in the laser beam. The solid state spectra of nystatin placed in a capillary tube were recorded with approx. 20 mW laser power.

The Raman frequencies were calibrated to an accuracy of ± 1.0 cm^{-1}. The infrared and visible-ultraviolet spectra were measured using Perkin Elmer 621 and Cary 18 spectrometers, respectively, with standard sampling techniques. Differential scanning calorimetry was performed using a Perkin Elmer DSC-2, and fluorescence spectra were recorded with a Perkin Elmer MPF-4 spectrofluorimeter.

Results and Spectral assignments

Electronic spectrum

A survey ultraviolet-visible spectrum of nystatin in methanol is shown in Fig. 2. Similar spectra were obtained in dimethylsulphoxide (Me_2SO) and in a Tris/acetate buffer (pH ≈ 7.0) solution. The important feature of the spectrum is a series of sharp optical transitions located at 281, 291, 318 and 304 nm (quoted in order of increasing intensity). The intensity pattern and peak positons remain fairly constant when the solvent is changed. Our data in the above spectral range are in good agreement with that published by Norman et al. [8] in Tris/acetate buffer. These authors also observed that the optical spectrum of nystatin is independent of the solvent pH, a result which is in contrast to their observations on amphotericin B. In addition, we detected a second and much weaker series of lines (located at 361, 381 and 405 cm^{-1} in methanol) which shift to lower energies with increasing solvent polarity.

The first series of lines in nystatin can be assigned to the strongly dipole-allowed $^1A_g \rightarrow {}^1B_u$ (in the point-group C_{2h}) transition of an extended *trans*-polyene chain, predicted by molecular orbital theories [12]. The lower energy series of lines could be associated with one of the following: (a) impurity fraction in nystatin; (b) charge transfer, or (c) a $\pi \rightarrow \pi^* {}^1A_g - {}^1A_g$ dipole-forbidden transition to an excited state involving a doubly excited configuration, as suggested by Hudson and Kohler [12]. The likelihood of a low energy optical transition in nystatin is also supported by the fact that the fluorescence spectrum of nystatin in methanol excited at 331 nm shows maxima at 398 nm,

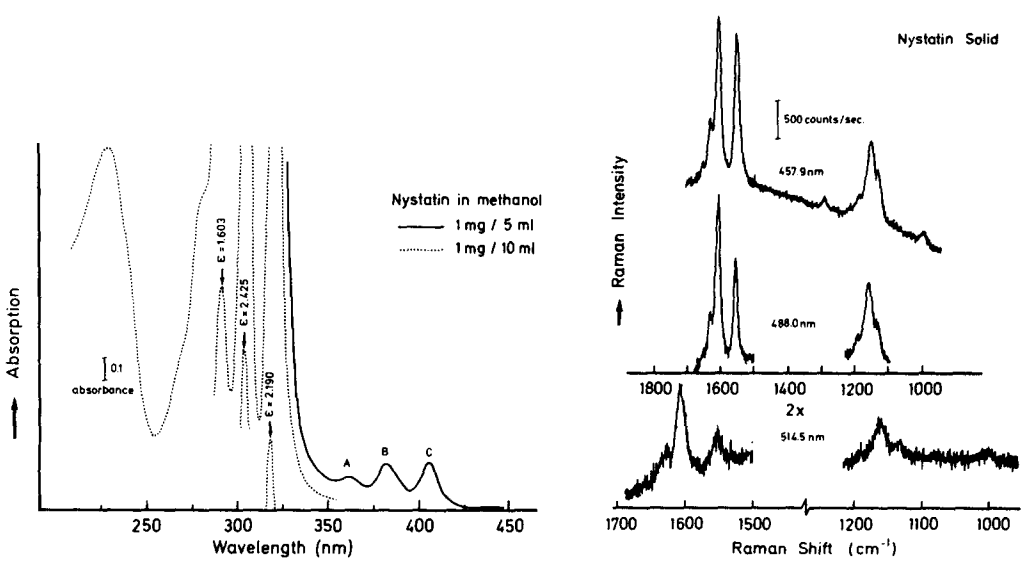


Fig. 2. Survey ultraviolet-visible spectrum of nystatin in methanol. Concentrations used are indicated in the figure.

Fig. 3. Raman spectra of nystatin powder using 457.9, 488.0 and 514.5 nm excitation wavelengths. 10 mW laser power and 3 cm^{-1} instrumental resolution were used.

425 nm and a shoulder at 380 nm, which do not coincide with the observed $\pi \rightarrow \pi^*$ dipole allowed $^1A_g \rightarrow {}^1B_u$ transition bands. Similar lack of coincidence of the fluorescence emission and optical absorption spectra has been observed in a number of *trans*-polyene systems [12,13].

Raman spectra

In solid state. The Raman spectra of solid nystatin excited with Argon laser-exciting lines at 457.9, 488.0 and 514.5 nm are shown in Fig. 3. These spectra show the relative unnormalized peak intensities. The frequencies and peak intensities for the solid state spectrum excited with 457.9 nm radiation are listed in Table I. The Raman peak frequencies remain essentially independent of excitation frequency and are nearly the same as that reported for amphotericin B [10].

The relative simplicity of the Raman spectrum of nystatin and the intensities of the lines near the expected polyene C=C and C-C stretching frequency regions around 1550 and 1150 cm^{-1} suggest that a pre-resonance Raman spectrum via the polyene $^1A_g \rightarrow {}^1B_u$ electronic transition, is being observed (Fig. 3). However, the remarkable feature of the pre-resonance Raman spectrum of nystatin is that the lines in the 1600–1650 cm^{-1} region, which are assignable to largely C=O motions associated with the two carbonyl groups in the nystatin molecule (Fig. 1), are strongly enhanced. This is in marked contrast to the behaviour of the related amphotericin B molecule, in which the C=O modes are only weakly resonance enhanced [10]. The data shown in Fig. 3 also indicate qualitatively that the intensities of the C=O and C-C modes behave differently with exciting wavelengths compared with the C=C mode

TABLE I

FREQUENCIES OF PRE-RESONANCE-ENHANCED RAMAN PEAKS OF NYSTATIN IN SOLID, METHANOL SOLUTION AND DIMYRISTOYL PHOSPHATIDYLCHOLINE-CHOLESTEROL MULTILAYERS, TOGETHER WITH RELATIVE INTENSITIES AND PROBABLE ASSIGNMENTS

Excitation was at 457.9 nm.

Solid (cm^{-1})	Rel. intensity	Methanol solution (cm^{-1})	Rel. intensity	Dimyristoyl phosphatidyl-choline-cholesterol multilayers (cm^{-1})	Rel. intensity	Assignments [14]
1000 (998) **	0.06	— *				C-C-H in-plane bend + C = C-C
1140	0.30	1140	0.12	1140	0.24	C-C stretch + C-C-H bend in-plane
1159 (1158)	0.48	1159	0.36	1158	0.4	
1190	0.12	—				
1295	0.06					
1557 (1556)	0.9	1559	1.0	1554	1.0	C = C stretch
1610 (1612)	1.0	1610	0.9	1609	0.4	C = O mixed with C = C
1633 (1635)	0.4	1634	0.18	1636	0.17	
1652	0.14	—				

* Solvent peak interference.

** Infrared peak frequencies measured in KBr matrix.

intensity. This behaviour will be considered further in the next subsection on the basis of normalized solution data.

The infrared lines in solid nystatin in a KBr matrix were also recorded and the frequencies corresponding to the Raman lines are listed in Table I. It was observed that these lines coincide quite closely with those observed in the pre-resonance Raman spectrum. Infrared lines associated with C-H stretching (approx. 2900 cm^{-1}), -COOH (1700 cm^{-1}), $-CH_3$ and $-CH_2$ group deformations (approx. 1370 cm^{-1}) and C-OH bending (1060 cm^{-1}) modes, were observed in the infrared but not in the pre-resonance Raman spectrum, presumably due to lack of coupling of these modes with the polyene backbone vibrations. The vibrational assignments of the observed Raman lines are indicated in Table I on the basis of the data and calculations of Rimai et al. [14] on model polyene systems. It must be noted here that the modes observed in the pre-resonance Raman spectrum must be admixed to some degree with the polyene C=C stretching eigenvector.

In solutions. The Raman spectra of nystatin were recorded in methanol, Me_2SO and in an aqueous buffer (pH $\approx$ 7.0) solution. The major pre-resonance-enhanced peaks were found to be polarized. The spectrum in methanol in the C-C, C=C and C=O stretching regions (Table I) and in a Tris/acetate buffer solution, in the C=C and C=O regions, are shown in Figs. 4 and 6, respectively. The spectrum in Me_2SO corresponds closely to that in methanol and is not reproduced here. In contrast to the results for amphotericin B [10], the spectrum of nystatin in methanol does not change in the pH range 1.1—9.0.

The C-C and C=O frequencies in solution do not shift detectably in comparison with the values observed in the solid state spectrum (Table I). However, the C=C frequency shifts from 1557 ± 1 cm^{-1} in the solid to 1559 ± 1 cm^{-1} in both methanol and Me_2SO.

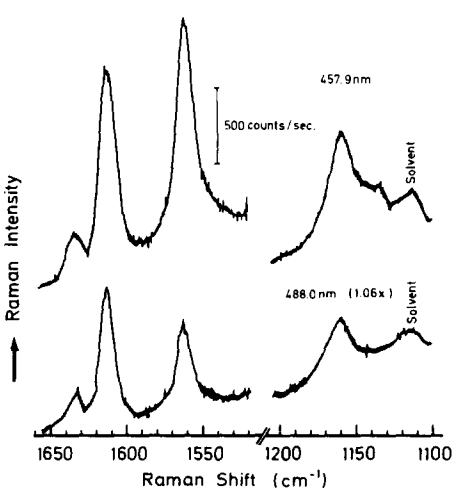


Fig. 4. Raman spectrum of nystatin in methanol (1 mg/5 ml) using 457.9 and 488.0 nm excitation wavelength. 200 mW laser power and 3 cm^{-1} instrumental resolution were used.

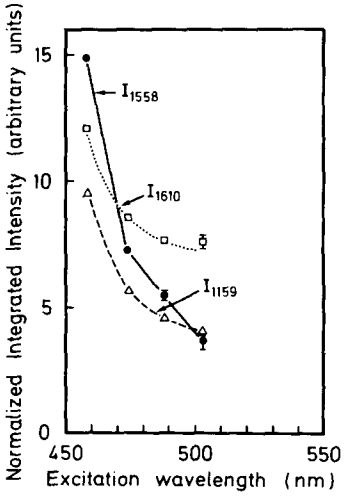


Fig. 5. Pre-resonance Raman excitation profiles of the normalized integrated intensities of the C=C, C-C and C=O peaks of nystatin in methanol (1 mg/5 ml).

In the buffer solution the C=C frequency is located at the same value as in the solid, but, compared with the solid state spectrum (Fig. 3) the relative intensity of the second C=O peak at 1633 cm^{-1} increases relative to the strong C=O peak at 1610 cm^{-1} (Fig. 6a).

Careful measurements of the integrated intensities of the 1610, 1560 and 1159 cm^{-1} peaks in methanol solution were made at different exciting frequencies. The intensities were then normalized against the solvent peak at 1032 cm^{-1} and these values were plotted as a function of exciting wavelengths to give the excitation profiles for the 1610 (C=O), 1560 (C=C) and 1159 (C-C) cm^{-1} lines (Fig. 5).

The excitation profiles (Fig. 5) show a sharper increase in intensity below 480 nm of the C=C and C-C lines compared with the C=O line. Qualitatively this is also reflected in the Raman spectra show in Figs. 3 and 4, where it is evident, that the drop in intensity of the C=C line with increasing excitation wavelength from 457.9 to 488.0 nm is greater compared with that for the C=O stretching line.

Raman spectra of nystatin in phospholipid-cholesterol environments

The Raman spectra of nystatin in dimyristoyl phosphatidylcholine multilayers (molar ratio 1 : 24), in dimyristoyl phosphatidylcholine-cholesterol multilayers and vesicles (molar ratio 1 : 24 : 6), in dipalmitoyl phosphatidylcholine-cholesterol multilayers and vesicles (molar ratio 1 : 36 : 9) and in a

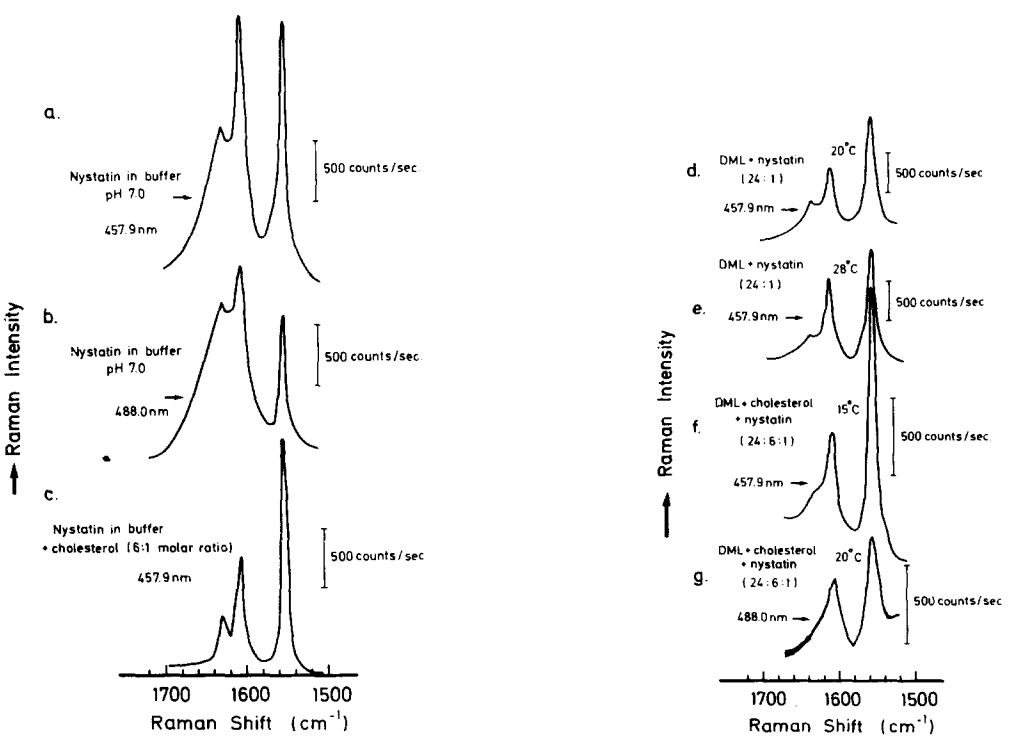


Fig. 6. Raman spectra in the C=C and C=O regions of nystatin in the various environments, temperatures and excitation frequencies indicated on the figure. Instrumental details are the same as in Fig. 4. DML, dimyristoyl phosphatidylcholine multilayers.

cholesterol/buffer suspension (molar ratio 1 : 6), were investigated. The temperature dependence of the spectra of the C-C, C=C and C=O modes of nystatin and the C-H modes of the phospholipids were monitored in order to study the effect of nystatin on the gel to liquid-crystal phase transitions in the phospholipid systems. Some representative spectra depicting the C=C and C=O regions of nystatin in these environments are displayed in Fig. 6a–g, and the mode frequencies in dimyristoyl phosphatidylcholine-cholesterol multilayers are listed in Table I.

From Fig. 6c it is evident that the C=O stretching mode at 1610 cm^{-1} excited to 457.9 nm shows an appreciable drop in intensity relative to the C=C mode, in a nystatin-cholesterol suspension in Tris/acetate buffer. The C=O mode is also weaker relative to the C=C mode in a nystatin-dimyristoyl phosphatidylcholine multilayer at 20°C (Fig. 6d). However, above the gel to liquid-crystal transition at 24°C, the intensity of the C=O line increases as shown in Fig. 6e. In nystatin-cholesterol-dimyristoyl phosphatidylcholine or dipalmitoyl phosphatidylcholine multilayers the C=O line intensity is the weakest among the systems investigated. The intensity ratios discussed are listed in Table II, together with the values in the solid state and methanol solution. In all these environments, the pre-resonance Raman frequencies are nearly the same as in solid nystatin (Table I) and amphotericin B [10].

As a function of temperature in dimyristoyl phosphatidylcholine-cholesterol and dipalmitoyl phosphatidylcholine-cholesterol, the nystatin lines remain constant in intensity with respect to the C-H deformation lines of the liposomes in the 1440 cm^{-1} region. This behaviour is in contrast to that of amphotericin B in which a drop in relative intensity of the C=C line is reported to occur above the gel to liquid-crystal phase transition [9].

The effect of nystatin on the cooperativity of the phase transition in the liposomes was also investigated by studying the temperature dependence of the C-H stretching lines of the phospholipid chains and also by monitoring the phase transition in the same samples by means of differential thermal calorim-

TABLE II

RATIO OF RAMAN INTENSITIES OF NYSTATIN C = C (1557 cm^{-1}) TO C = O (1610 cm^{-1}) PEAKS IN VARIOUS ENVIRONMENTS

DML, dimyristoyl phosphatidylcholine multilayers, 457.9 nm laser excitation was used.

Nystatin environment	Temperature (°C) and state	Ratio (I_{1557}/I_{1610})
Solid	22°C	0.9
Methanol	22°C, 1 mg/5 ml solution	1.09
In buffer, pH 7.0	22°C, 1 mg/5 ml solution	1.00
Cholesterol in buffer	22°C, 1 : 6 molar ratio, suspension	2.08
DML in 0.1 M KCl	20°C, 1 : 24 molar ratio multilayer, gel phase	1.71
	28°C, 1 : 24 molar ratio multilayer, liquid-crystal phase	1.39
Cholesterol + DML in 0.1 M KCl	15°C, 1 : 6 : 24 molar ratio multilayer	2.52

etry. Nystatin (molar ratio nystatin : dimyristoyl phosphatidylcholine = 1 : 24) does not appear to affect the width of the transition region measured by the intensity ratio of the CH lines at 2883 and 2845 cm^{-1} and differential thermal calorimetry. However, the transition temperature of dimyristoyl phosphatidylcholine multilayers as measured by differential thermal calorimetry shows a small drop (approx. 1.3 K), in the presence of nystatin (molar ratio nystatin : dimyristoyl phosphatidylcholine = 1 : 24). Nystatin does not have a measurable effect on the broadened phase transition of phospholipid multilayers in the presence of cholesterol, as monitored both by Raman spectroscopy and differential thermal calorimetry.

Discussion and Conclusions

The important feature of the pre-resonance Raman spectra of nystatin is the relatively high intensity of the line at 1610 cm^{-1}, which is assignable to a largely C=O stretching mode. Furthermore, the intensity of the 1610 cm^{-1} line with respect to that of the ethylenic C=C stretching mode was found to vary significantly with the environment of the nystatin molecule. These spectral observations are in sharp contrast to the Raman spectral data [10] on the closely related channel-forming polyene amphotericin B. A probable explanation of this behaviour may lie in the break in the conjugated polyene backbone of nystatin (Fig. 1) in contrast to the unbroken backbone of amphotericin B, leading to the possibility of a different ground-state conformation of the nystatin molecule. The C=O stretching mode could be resonance enhanced via coupling with the C=C stretching mode if a conformation is assumed in which the respective eigenvectors are approximately parallel. The polyene units in this conformation should, however, remain *trans* in both the ground and excited states since the polyene backbone stretching line frequencies and intensities do not change noticeably compared with amphotericin B. A space-filling model of nystatin shows that such a conformation can be achieved by a twisting of the nystatin molecule, bringing the C=O group at position 1 nearly parallel to the polyene tetraene unit at the charged hydrophilic end of the molecule (Fig. 1).

These considerations would allow us to conclude that nystatin can exist in two limiting ground-state conformations: (a) a twisted conformation in which the C=O group at position 1 is likely to be aligned along the tetraene unit of the polyene chain, and (b) a planar conformation in which the two units of the polyene chain are approximately in one plane and the overall conformation of the molecule is similar to that in amphotericin B. The twisted conformation appears to exist in the solid state, methanol, Me_2SO and aqueous buffer solution. Small differences between the twisted conformation in the solid and that in methanol are reflected in a small upward shift of the C=C stretching mode frequency (Table I) in methanol. In an aqueous buffer solution (pH 7.0) the weaker C=O component at 1632 cm^{-1} is somewhat more intense than it is in organic solutions and in the solid, also indicating small configurational differences probably involving the C=O groups.

The largely planar conformation exists in the biologically important phospholipid-cholesterol multilayers and in aqueous buffer solutions in the presence

of cholesterol. In these environments, the twisted conformation undergoes a configurational change presumably due to thermal and packing factors coupled with van der Waals interactions. In phospholipid multilayers without cholesterol one can observe a change of conformation from a more planar to a more twisted conformation, by the temperature-induced transformation of the phospholipid multilayers from the gel to the liquid-crystal phase as reflected by the increase in the intensity of the C=O line relative to the C=C stretching line (Fig. 6d and e). This result suggests that in the liquid-crystal phase, phospholipid chain dynamic disorder causes a disruption of the interaction forces which lead to a more planar conformation of the nystatin molecule. It is worth pointing out here that the length of the planar nystatin molecule corresponds to a C_{18} fatty acid all-*trans* chain, while the shorter length of the cholesterol molecule corresponds to the planar span of the polyene segment of nystatin. According to the model of de Kruyff and Demel [9] the nystatin (or amphotericin B)-cholesterol channel could form via an alignment of two cholesterol molecules on either side of the nystatin or amphotericin B ring to form a circular (antibiotic-cholesterol)$_8$ pore. Obviously, a twisted nystatin conformation would impede such a pore-forming process. Therefore, a conformational transformation in nystain is achieved in the biological environment mainly via cholesterol, leading to nearly the same biological activity as in amphotericin B. The more flexible nystatin molecule is more stable to changes in pH of the environment than the more rigid and planar amphotericin B molecule. Unlike nystatin, the hydrophobic polyene segment in amphotericin B is sensitive to the phospholipid chain order in the phospholipid-cholesterol multilayers, as indicated by a large decrease in the intensity of the C=C stretching line on entering the liquid-crystal phase [10].

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