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INTERACTION OF AMPHOTERICIN B WITH MONOLAYERS OF EGG LECITHIN AND CHOLESTEROL: POLARIZED ABSORPTION SPECTRA

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SUMMARY

Polarized absorption spectra have been obtained of the antibiotic polyene, amphotericin B, interacting with monolayers of egg lecithin, cholesterol and equimolar egg lecithin–cholesterol at low and high surface pressures. An expression is derived which enables the determination from the polarization data of the orientations of the transition moments of the polyene absorption bands at 4077 and 3645 Å. For some of the systems the 3645-Å band is replaced by a previously unreported band appearing between 3610 and 3632 Å. The orientation of the 4077-Å transition moment (parallel to the long molecular axis) is found to vary from an angle of 64 ° with the surface for the low-pressure monolayers of cholesterol to 21 ° for the high-pressure films of egg lecithin–cholesterol. For the band between 3610 Å and 3645 Å, the angle varies from 90 ° for cholesterol to 18 ° for the high-pressure mixed-lipid film. It is found that a large increase in surface pressure of the cholesterol and egg lecithin–cholesterol monolayers causes a decrease in the angle of the 4077-Å moment for both films and that of the higher energy moment for the mixed film. Increasing the content of cholesterol in these monolayers rotates the orientation of the transition moments for both bands toward the surface normal, the change being greatest for the low-pressure films. The effectiveness of amphotericin B in lowering the surface tension of these lipid monolayers is related to its binding, orientation and extent of penetration. For low-pressure cholesterol films where the surface interaction with the polyene is greatest, the binding and penetration are large and the polyene molecule is oriented with its long dimension nearly perpendicular to the surface.

INTRODUCTION

There has been an increasing application of optical spectroscopy to the study of both model membranes and biological membranes. These techniques can provide important information on the structure of membranes as well as their interaction with electric fields and functionally important ions and molecules.

Examples of such studies on model membrane systems employing absorption spectra are the determination of the orientation of chlorophyll molecules in black

lipid membranes [1] and the study of the binding of antibiotic polyenes to dispersions of phospholipids and sterols [2, 3]. The study of chlorophyll molecules in black lipid membranes has also been recently approached by means of its reflection spectrum [4]. Raman spectroscopy has been employed in the study of the interaction of cholesterol with egg lecithin [5] and dipalmitoyl lecithin dispersions [6]. Infrared spectra have been used to interpret the effects of hydration and temperature [7] and electric fields [8] on the structure of phospholipids. Fluorescent probes have been used to study the structure of spherical [9] and planar black lipid membranes [10], the effect of electric fields on the latter [11] and the structural changes arising from the gel–liquid crystal phase transition in phospholipid dispersions [12].

Example of the application of spectroscopic methods to biological membranes is the study of the ultraviolet spectra of polyenes interacting with erythrocyte ghost and *Acholeplasma laidlawii* membranes [2], and observing the effect of action potentials on the infrared spectra of conducting nerve axons [13]. There have also been studies of the effect of lipid phase transitions in *Escherichia coli* [14] and that of action potentials [15] and voltage clamping [16] in squid axon membranes on the fluorescence of probe molecules.

This paper will be concerned with the application of polarized absorption spectroscopy to the study of the interaction of the polyene antibiotic, amphotericin B, with monolayers of egg lecithin, cholesterol and equimolar egg lecithin–cholesterol.

The investigation of the effects of polyene antibiotic molecules on lipids has been an area of considerable interest. This is primarily due to the ability of these molecules to mediate large changes in the ionic and nonelectrolyte permeabilities of both biological membranes such as erythrocytes [17] and mycoplasma [18], and of model lipid membrane systems such as liposomes [19] and single bilayers [20]. Paralleling these interactions in membranes is the effect of polyenes in lowering the surface tension of lipid monolayers [21]. In almost all of the systems studied, it has been found that sterols greatly enhance the polyene–lipid interaction [22]. However, there has been considerable disagreement over the role played by sterols. The earliest and still most widely held view is that the polyenes form a hydrophobic complex with the sterols in the membrane which then modifies its structure. The structural requirements of the sterol for optimal interaction is a 3β-OH on the steroid nucleus, an intact side chain at C-17 and a planar sterol nucleus. In equimolar egg lecithin–cholesterol liposomes, the order of effectiveness of the polyene–sterol interaction is filipin, amphotericin B, etruscomycin and pimarcin [2]. However, recently it has been suggested that the sterols affect the polyene–lipid interaction in membranes by altering the order of the lipid hydrocarbon chains with an increase in order leading to an enhanced interaction [3, 23].

It is the purpose of this paper to further the understanding of polyene–lecithin–sterol systems by presenting determinations of the binding and orientation of amphotericin B after it interacts with monolayers of egg lecithin, cholesterol and equimolar mixtures of the two. Amphotericin B was chosen because of its strong interaction with lipids and its large molar extinction coefficient ($1.82 \cdot 10^5$ in methanol for the 4046-Å band). The strong chromophore enables one to observe the monolayer absorption spectra of surface bindings as small as one polyene per 50 lipid molecules with the instrumentation used in this research.

METHODS

Materials

Amphotericin B was donated by the Squibb Institute for Medical Research, New Brunswick, N. J. Stock solutions of concentration of about 300 μg/ml were made in doubly distilled methanol. Egg lecithin from Sylvana Co. was purified by silica gel chromatography. Thin-layer chromatograms showed no foreign bands. Cholesterol was obtained from Eastman Kodak Co. Stock solutions of 0.001 M of egg lecithin, cholesterol and equimolar mixtures of the two in chloroform–methanol (2:1, by vol.) were stored at -60 °C until needed. They were discarded when the monolayers failed to give the correct isotherms.

Preparation of monolayers

Monolayers were prepared by spreading the lipids on unbuffered and distilled water which was filtered and deionized. The trough was made of Delrin, a very inert material, had an area of 32 cm^2 (6 cm $\times$ 5.3 cm), and a depth varying from 0.2 to 0.9 cm. The volume of water in the through varied between 16 and 20 ml. Prior to spreading the lipid, the surface was cleaned with a Pasteur pipette. The surface tensions were measured by the Wilhelmy balance method [24] using a sandblasted platinum blade suspended from a torsion balance. Measurements were made before and after both the recording of the spectra as well as the sampling of the subphase with a syringe. The initial surface pressures were established by the amount of lipid spread. Amphotericin B was then injected beneath the lipid monolayer, the resultant pressure change generally stabilized within 5 min.

Subphase exchange

Since the contribution to the optical density of the polyene band from the subphase ranged from 2 to 10 times that of the monolayer, it was found useful to obtain spectra with the concentration of amphotericin B is the subphase reduced by about a factor of 10. This was accomplished by exchanging the subphase with about 40 ml of water without disruption of the monolayer. The exchange was done through the monolayers using two No. 18 syringe needles for the input and output of a Buchler Instruments Polystaltic pump.

Spectroscopic measurements

Absorption spectra of the monolayer–subphase systems were obtained using a Perkin Elmer 350 double-beam spectrometer. The trough containing the monolayer–polyene system was placed in the sample compartment while an identical trough containing water was inserted into the reference compartment. This gave a flatter spectral baseline than would be the case if the sample beam optics wouldn't have been used in the reference beam. The optical system is illustrated in Fig. 1. Three mirrors were used in each beam to deflect the light from the monochromator into the troughs and then into the photomultipliers. The first and third mirrors were cemented onto aluminum blocks at appropriate angles for the incident light to enter the film or water at a 45 ° angle and the emergent light to be directed into the photomultipliers. Each mirror assembly was mounted on a shaft to allow rotation about an axis parallel to the water surface for alignment of the reflected beams on the detector slits. To prevent light

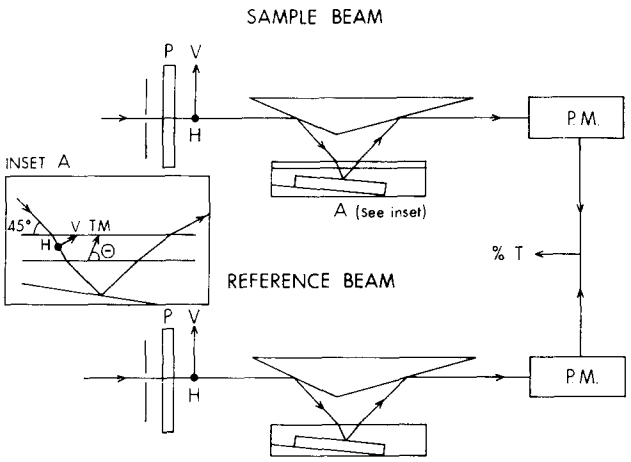


Fig. 1. Optics for the sample and reference beams. P, polaroid film polarizer; V, H, polarization directions; TM, transition moment of an absorption band making an angle θ with horizontal.

reflected from the monolayer or water surface from entering the detectors, the immersed second mirrors were oriented at an appropriate angle to direct only the transmitted beams into the photomultipliers. Both the sample and reference beams were polarized with Polaroid HNP'B polarizer sheets.

The binding of the polyene to the films (in moles) prior to exchanging the subphase is given by the difference between the total number of moles injected and that found in the subphase. (An insignificant amount of polyene adheres to the surfaces of the trough). The amount of polyene in the subphase was obtained by removing 1-ml samples with a syringe through the monolayer. These samples were then diluted in 3 ml of methanol and a measurement of the absorbance of the 4046-Å band gave their content of polyene. The product of this quantity with the subphase volume gave the amount of amphotericin B in the latter. For the exchanged subphase systems the amount of polyene in the subphase was similarly obtained but the binding was determined from an analysis to be discussed in a subsequent section.

RESULTS

The isotherms for the lipid monolayers are presented in Fig. 2a while the effect of amphotericin B on lowering the surface tensions of the monolayers is shown in Fig. 2b. The decrease in surface tension which is equivalent to the increase in surface pressure will be denoted by the symbol, π_A. It should be pointed out that Fig. 2b is not comparable with previously published curves [21] for the following reason. In the previous studies, the lipid pressure was increased by mechanically compressing the monolayers thereby maintaining a constant ratio of polyene to lipid. In this study since it was not feasible to compress the monolayers in this manner, the surface pressure was raised by increasing the amount of spread lipid thereby decreasing the ratio of polyene to lipid with increasing pressure. All of the spectra to be discussed in this paper were obtained for initial pressures of about 5 dynes/cm and 25–30 dynes/cm.

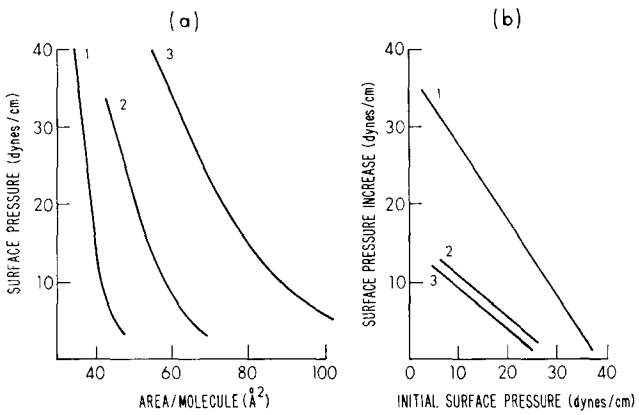


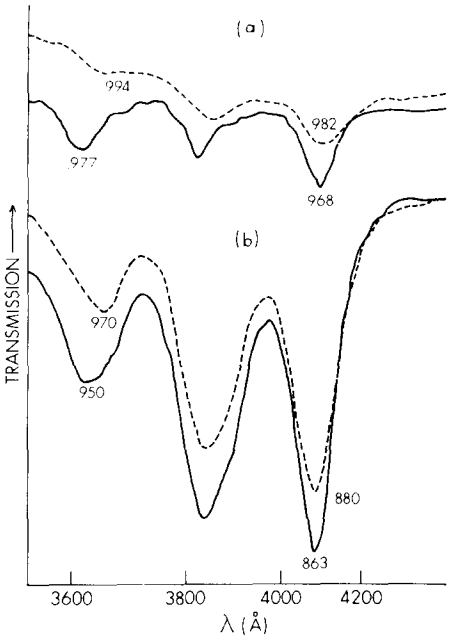
Fig. 2. (a) Pressure–area curves for egg lecithin–cholesterol monolayers. 1, cholesterol; 2, equimolar mixture of egg lecithin and cholesterol; 3, egg lecithin. (b) Interaction of amphotericin B with egg lecithin–cholesterol monolayers. Numbers refer to same lipids as in (a). After sufficient lipid was spread to achieve the desired pressure, a quantity of amphotericin B varying from 0.011 to 0.014 μmoles was injected underneath the monolayer and the decrease in surface tension (equal to the increase in surface pressure, π_A) was measured. The curves approximate a best fit to the measured points.

These correspond to the conditions for large and small decreases in surface tension, respectively, resulting from the addition of amphotericin B to the subphases [21].

Representative spectra are shown in Figs 3–6. These spectra show the three strongest bands of the $\pi \rightarrow \pi^*$ vibronic transition of the amphotericin B chromophore characteristic of a linear array of seven conjugated carbon–carbon bonds (heptaene). The transition moments of these bands under normal circumstances have been shown to be parallel to the polyene chain direction [25]. (See Fig. 7 for a schematic of amphotericin B based on the recent determination of its structure [26].)

In Fig. 3 is shown the spectra of amphotericin B interacting with a monolayer of cholesterol at low pressure. Of all the systems studied, this one gave spectra showing the most striking differences from that of this polyene in water without a monolayer. The effect of the bound polyene can readily be seen in Fig. 3b against the much greater absorption of the subphase. The spectrum for the vertically polarized incident light shows increased absorption as well as the appearance of a new band near 3600 Å. These changes become more distinctive in Fig. 3a where the subphase concentration of the chromophore has been reduced by a factor of 6. The horizontally polarized spectrum in this case is characteristic of the polyene in water and is due solely to absorption by the subphase. The vertical spectrum shows enhanced absorption for all of the bands and in particular for the one at 3610 Å. In addition, the bands at 3610 and 3825 Å have undergone marked blue shifts with respect to the horizontal spectrum. As will be discussed in a following section, these spectra are indicative of two transition moments of which the one at 3610 Å is oriented perpendicular to the surface and that at 4077 Å being nearly normal to it.

The spectra for the polyene interacting with egg lecithin monolayers are shown in Fig. 4. In contrast to the cholesterol system, horizontally polarized incident light is more strongly absorbed and the band maxima appear at essentially the same wave-



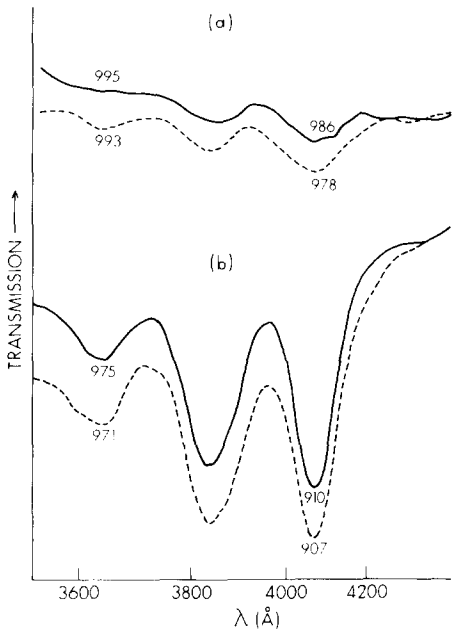


Fig. 3. Spectra of amphotericin B interacting with a monolayer of cholesterol. The initial surface pressure was 4.5 dynes/cm and the total polyene injected was 0.012 μmole. - - -, incident light polarized parallel to film (horizontal polarization); –, light polarized perpendicular to film (vertical polarization). The numbers refer to percent transmission of the corresponding bands. (a) With subphase exchanged giving a concentration of polyene of $7.4 \cdot 10^{-8}$ M. $\pi_A = 18$ dynes/cm. (b) Before exchange, concentration of polyene in subphase is $4.5 \cdot 10^{-7}$ M. $\pi_A = 32$ dynes/cm. (These spectra and those shown in the following figures were recorded using an expanded scale of the spectrometer giving a 5-fold magnification.)

Fig. 4. Spectra of amphotericin B interacting with a monolayer of egg lecithin. The initial surface pressure was 5.5 dynes/cm and the total injected polyene was 0.014 μmole. - - -, incident light horizontally polarized; –, light vertically polarized. The numbers refer to percent transmission of the corresponding bands. (a) With subphase exchanged giving a concentration of $5.6 \cdot 10^{-5}$ M. $\pi_A = 9.6$ dynes/cm. (b) Before exchange, concentration of polyene in subphase is $6.2 \cdot 10^{-7}$ M. $\pi_A = 11$ dynes/cm.

lengths for the two polarizations. Reducing the subphase concentrations by a factor of 11 (Fig. 4a) heightens these effects. These spectra will be shown to be characteristic of a transition moment making a small angle with the monolayer surface.

The spectra shown in Fig. 5 provide a comparison for the interaction of amphotericin B with low-pressure monolayers of egg lecithin, cholesterol and equimolar egg lecithin–cholesterol. Since the subphase concentrations of the polyene have been greatly reduced for these absorption curves, they reflect the binding of the chromophore to the monolayers. In the case of horizontally polarized incident light (Fig. 5b), the absorption decreases in going from egg lecithin to cholesterol. In fact, a calculation of the transmission of the subphase in the cholesterol system agrees with the observed value indicating that, in this case, the bound polyene doesn't absorb horizontally polarized light. For vertically polarized light the opposite behavior occurs. In addition, there is a blue wavelength shift for the two shorter wavelength

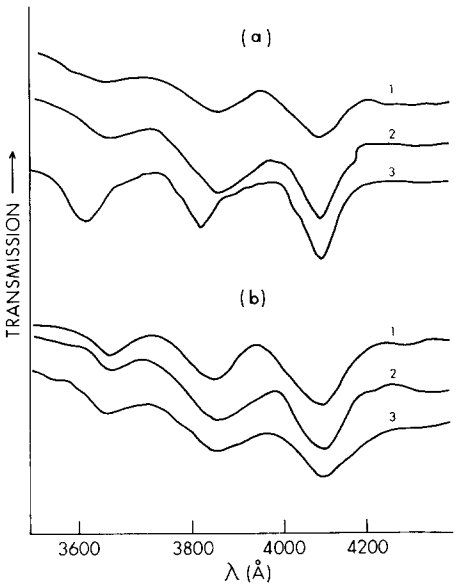


Fig. 5. Spectra of amphotericin B interacting with monolayers of egg lecithin (1), equimolar egg lecithin–cholesterol (2), and cholesterol (3) after subphases were exchanged. The initial surface pressures were 5.5, 7.5 and 4.5 dynes/cm respectively. The amounts of injected polyene were 0.014 μmole, 0.014 μmole and 0.012 μmole and the subphase concentrations were $5.6 \cdot 10^{-8}$ M, $6.2 \cdot 10^{-8}$ M and $7.4 \cdot 10^{-8}$ M for the egg lecithin, egg lecithin–cholesterol and cholesterol films respectively. The corresponding values of π_A were 9.6, 7.6 and 18 dynes/cm. (a) For vertically polarized incident light. (b) For horizontally polarized light.

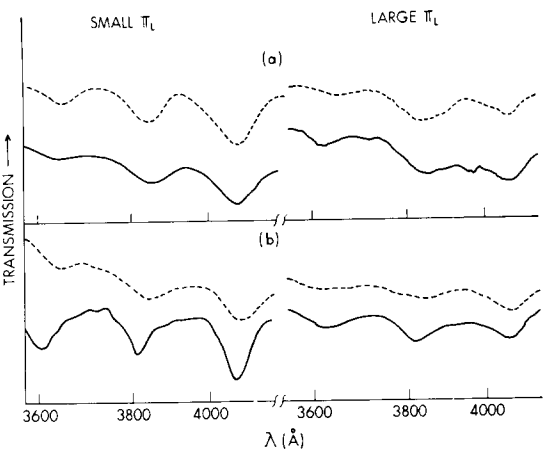


Fig. 6. Spectra of amphotericin B interacting with monolayers of egg lecithin and cholesterol after subphases were exchanged. - - -, incident light polarized horizontally; –, light polarized vertically. (a) Egg lecithin films. The initial surface pressures are 5.5 and 26 dynes/cm, the amounts of injected polyene are 0.014 μmole in both cases. The concentrations of polyene in the subphases are $5.6 \cdot 10^{-8}$ M and $1.2 \cdot 10^{-7}$ M and the values of π_A are 9.6 and 4.5 dynes/cm for the small- (low)- and large- (high)- pressure films respectively. (b) Cholesterol films. The initial surface pressures are 4.5 and 31 dynes/cm, the corresponding amounts of injected polyene are 0.012 μmole and 0.011 μmole. The concentrations of polyene in the subphases are $7.4 \cdot 10^{-8}$ M and $5.3 \cdot 10^{-8}$ M and the values of π_A are 18 and 1 dyne/cm for the small- and large-pressure films respectively.

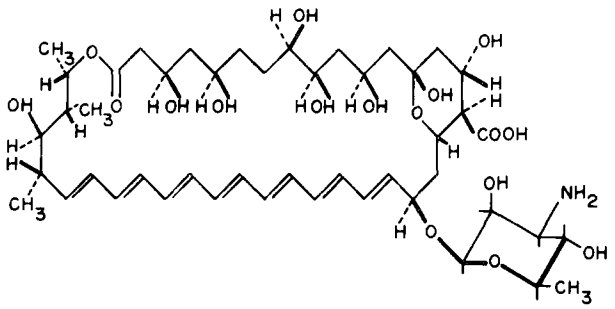


Fig. 7. Schematic representation of the structure of amphotericin B .The transition moment for the π–π^* vibronic band is parallel to the polyene chain.

bands in cholesterol relative to the other lipids. These spectra also indicate that the orientation of the transition moment associated with the 4077-Å maximum for the equimolar mixture of the lipids lies between that for the egg lecithin and cholesterol monolayers in the case of low lipid pressures.

The effect of surface pressure on the spectra of amphotericin B in monolayers of egg lecithin and cholesterol is shown in Fig. 6. The decrease in absorption with increasing pressure for both systems is indicative of the reduction in binding. The change in dichroism with increasing pressure indicates a reorientation of the transition moments for both the low- and high-energy bands of amphotericin B for the cholesterol and egg lecithin monolayers. Corresponding changes in the wavelengths particularly for the highest-energy bands are seen.

The binding of amphotericin B to the monolayers was determined for the three lipid systems both before and after the subphases were exchanged. Tabulated in Table I are the values for the binding in moles as well as the binding of polyene per mole of lipid, (N^b/N_l). The addition of cholesterol to egg lecithin appears to reduce the binding with the equimolar mixture showing the smallest values of the three systems. However, with further addition of cholesterol the binding increases again. The increase of the initial lipid pressures as well as the reduction of the subphase concentration of polyene both appreciably reduce the binding for all three lipid systems.

DISCUSSION

Analysis of polarized spectra

The derivation of the equations relating the percentage transmission of a monolayer–subphase system for both polarizations of incident light, to the absorption coefficients of a chromophore present in both the subphase and monolayer is given in the Appendix. The results are

$$T^v = e^{-k_s t_s}(1 - 0.78\, k_v t - 0.83\, k_h t) \tag{1}$$

$$T^h = e^{-k_s t_s}(1 - 1.21\, k_h t) \tag{2}$$

T^v, T^h, are the percent transmissions for vertically and horizontally polarized incident light respectively; k_s, t_s, is the absorption coefficient and path length of the subphase; k_v, k_h, are the absorption coefficients of the monolayer for light traversing the film for

TABLE I

SURFACE PRESSURES AND THE BINDING AND ORIENTATION OF THE TRANSITION MOMENTS OF AMPHOTERICIN B

A quantity of amphotericin B, varying from 0.011 moles to 0.014 moles, was injected beneath the monolayers. The systems were allowed to stabilize, as measured by the absorption of the 4077-Å band, for about 45 min before the spectra were recorded. π_L, surface pressure of the lipid films; π_A, increase in surface pressure due to addition of polyene; N^b, moles of polyene bound to film; N^b/N_l, ratio of moles of bound polyene to that of lipid in film; ε, molar extinction coefficient ($l \cdot mole^{-1} \cdot cm^{-1}$); θ, angle transition moment makes with surface, the subscripts 1, 2 refer to the 4077-Å and (3600-Å) bands respectively.

	Cholesterol		Cholesterol–egg lecithin		Egg lecithin	
π_L (dynes/cm)	4.2	32	7.5	24	5.1	26
π_A (dynes/cm)	32	5.2	11	2.3	11	2.7
	(18)★	(1)	(7.6)	(1.6)	(9.6)	(4.5)
N^b ($\times 10^8$ moles)	0.37 ± 0.06	0.18 ± 0.07	0.27 ± 0.07	0.06 ± 0.08	0.34 ± 0.07	0.14 ± 0.06
	(0.21 ± 0.07)	(0.07 ± 0.06)	(0.13 ± 0.06)	(0.15 ± 0.20)	(0.07 ± 0.06)	(0.03 ± 0.06)
N^b/N_l	0.29	0.12	0.27	0.06	0.56	0.18
	(0.15)	(0.05)	(0.13)	(0.15)	(0.12)	(0.04)
4077-Å band						
ε_1 ($\times 10^{-5}$)	1.5 ± 0.3	1.3 ± 0.3	1.5 ± 0.3	1.3 ± 0.3	1.3 ± 0.3	1.3 ± 0.4
θ_1	$64 \pm 12°$	$42 \pm 20°$	$50 \pm 14°$	$21 \pm 21°$	$34 \pm 18°$	$29 \pm 20°$
	$(90 \pm 20°)$	$(0 \pm 20°)$	$(50 \pm 20°)$	$(22 \pm 22°)$	$(23 \pm 23°)$	$(0 \pm 20°)$
(3600-Å) band						
ε_2 ($\times 10^{-5}$)	1.2 ± 0.3	1.2 ± 0.3	0.7 ± 0.2	0.5 ± 0.2	0.4 ± 0.2	0.8 ± 0.2
θ_2	—	$82 \pm 15°$	$70 \pm 20°$	$18 \pm 18°$	$31 \pm 23°$	$62 \pm 15°$
	$(90 \pm 20°)$	$(75 \pm 15°)$	$(50 \pm 20°)$	$(67 \pm 23°)$	$(16 \pm 16°)$	$(63 \pm 28°)$

★ Quantities in parentheses refer to exchanged-subphase systems.

the vertical and horizontal polarizations, respectively, and t is the monolayer thickness. We can transform these equations to a more useful form by using the relation, $k = 2.31\,\varepsilon c$; where ε is the molar extinction coefficient, and c is the molar concentration of polyene. Substituting this into Eqns 1 and 2 and writing $c = N/At \times 10^3$ where A is the monolayer area (32 cm^2) one obtains

$$T^{v} = e^{-2.31\,\varepsilon_s c_s t_s}(1 - 55\varepsilon N_v - 59\varepsilon N_h) \tag{3}$$

$$T^{h} = e^{-2.31\,\varepsilon_s c_s t_s}(1 - 86\varepsilon N_h) \tag{4}$$

It should be noted that this substitution has essentially transformed the anisotropy of the absorption coefficient to the equivalent representation of two independent groups of absorbing molecules, those with transition moments perpendicular to the surface (N_v) and those with transition moments parallel to the surface (N_h). In obtaining Eqns 3 and 4, the assumption has been made that $\varepsilon_v = \varepsilon_h = \varepsilon$. The error in this assumption is certainly smaller than the uncertainties in the calculated values of the extinction coefficients which will be discussed later in this section.

Eqns 3 and 4 together with the relation $N_v + N_h = N^b$, where N^b is the total number of moles of amphotericin B bound to the monolayer, constitute a system of three equations in the three unknowns, ε, N_v and N_h. The percentage transmissions T^v and T^h are obtained from the spectra. The path length of the subphase, t_s, as a function of its volume was obtained from the measurement of the absorbance of a dye solution with a known absorption coefficient in the sample tray. The concentration of polyene in the subphase, c_s, is obtained from sampling the subphase as previously described. The extinction coefficient, ε_s, is obtained from a calibration curve prepared by measuring the absorbances of known amounts of amphotericin B in water. Due to the extensive formation by the polyene of micelles in water, ε_s varies considerably with c_s.

It will be convenient to solve Eqns 3 and 4 for ε and N_h. The results of these calculations are

$$\varepsilon = \frac{1 - T^v/T_s - 0.046(1 - T^h/T_s)}{55N^b} \tag{5}$$

$$N_h = \frac{R - 1 + 55\varepsilon N^b}{(86R - 4)\varepsilon} \tag{6}$$

$T_s = e^{-2.31\varepsilon_s c_s t_s}$ and $R = T^v/T^h$. Eqns 5 and 6 are used to obtain ε, N_h and $N_v = N^b - N_h$ for the two bands at 4077 Å and (3600 Å)*. In the experiments where the subphases were diluted by an exchange with water, since N^b could not be determined directly (because of losses to the pumping system) the pre-exchange values of ε were used which together with Eqns 4 and 6 give N_h and N^b. The angles θ which the transition moments make with the surface are given by the following equation

$$t_g^2\theta = N_v/N_h^{**} \tag{7}$$

The results of these calculations together with the corresponding surface pressures are tabulated in Table I. The major sources of errors were in the measurements of T^v, T^h

* (3600 Å) refers to the high-energy band appearing between 3610 Å and 3650 Å.

** In the following discussion, θ_1 and θ_2 will refer to the 4077-Å and (3600-Å) bands respectively.

($\approx 0.6\%$) and T_s ($\approx 0.3\%$) due to the difficulty in estimating the baselines (100% transmission points) from the spectra. The effect of these errors on the values of N^b, ε, θ_1, and θ_2 are given in Table I. For the egg lecithin and the mixed-lipid systems the θ values are insensitive to the variations in ε.

Interpretation of the anomalous behavior of the (3600-Å) band

Inspection of Fig. 3 and especially Fig. 3a indicates that for the two higher-energy bands and most strikingly for the (3600-Å) band, the vertically polarized maxima are shifted toward the blue relative to those horizontally polarized. In addition, the intensities of these bands relative to the 4077-Å band are also enhanced in the vertical spectrum. Similar effects, somewhat less pronounced, are also seen in the spectra for the low-pressure monolayers of the mixed lipids prior to subphase exchange* and in the spectra of the high-pressure films of cholesterol (Fig. 6b), egg lecithin (Fig. 6a) and equimolar cholesterol–egg lecithin after subphase exchange. In addition to the unusual nature of these spectra, the calculated orientations of the transition moments of the 4077-Å bands differ significantly from those of the (3600-Å) bands for several cases. These are for the high surface pressure monolayers for the initial egg lecithin system and all three systems following subphase exchange (see Table II). Finally in those systems for which the vertical spectra are appreciably blue-shifted, the ratios of the optical density of the (3600-Å) band to that of the 4077-Å band are greater for the vertical spectra than for the horizontal spectra (see Table II). An increase in this ratio has recently been used as a criterion for a strong polyene–cholesterol interaction [2].

The only explanation that appears to be consistent with these changes in wavelength, intensity and orientation of the transition moment of the (3600-Å) band relative to the 4077-Å band is the following one. A new band appears between 3610 Å and 3632 Å for all of these cases except for the high-pressure films of egg lecithin where it is at 3646 Å. Its transition moment appears to be localized on the opposite end of the heptaene from the sugar (see Fig. 7). This band appears to be induced in amphotericin B by its lipid neighbors under those conditions in which at least part of the polyene penetrates into the monolayer so that $\theta_2 > 63\,^\circ$. In these cases the intermolecular distances between the chromophore and the non-polar structures of its lipid neighbors are relatively small providing for greater interaction. Summarizing this discussion, the arguments for the existence of this new localized band are: (1) A new absorption peak appears at wavelengths between 3610 Å and 3632 Å under conditions in which the peak of the lowest-energy vibronic band remains unchanged at 4077 Å. If the entire chromophore would be subjected to a similar change of environment, all three vibronic components would undergo similar intensity changes and wavelength shifts .(For instance, each of the three bands is blue-shifted by about 25 Å in methanol solution compared to water solution). (2) The wavelengths** of the (3600-

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* The following discussion will refer to the non-exchanged systems unless subphase exchange is specified.

** In the case of the high-pressure egg lecithin monolayers, this band appears at 3646 Å, characteristic of a hydrophilic surrounding. However, since $\theta_2 > \theta_1$, this band must also be associated with a different transition than for the one at 4077 Å. The unshifted wavelength would indicate very little penetration of the chromophore into the monolayer (see Fig. 8).

TABLE II

SPECTROSCOPIC DATA AND TRANSITION MOMENT ORIENTATIONS FOR THE (3600-Å) BAND

The parameter, R, refers to the absorbance ratio of the (3600-Å) to the 4077-Å peaks for the polarization indicated by the superscript. The quantities θ and π_L are defined in Table I.

	Cholesterol		Cholesterol–egg lecithin		Egg lecithin	
π_L (dynes/cm)	4.2	32	7.5	24	5.1	26
λ^v (Å)	3610, 3650 (3610)*	3621 (3610)	3632 (3646)	3639 (3629)	3640 (3640)	3646 (3610)
λ^h (Å)	3650	3650	3645	3644	3646	3650
R^v	0.45 (0.72)	0.41 (0.77)	0.31 (0.26)	0.24 (0.26)	0.29 (0.36)	0.30 (0.37)
R^h	0.28 (0.33)	0.27 (0.33)	0.27 (0.23)	0.25 (0.18)	0.30 (0.30)	0.27 (0.24)
θ_1	64°, (90°)	42°, (0°)	50°, (50°)	21°, (22°)	34°, (23°)	29°, (0°)
θ_2	90°, (90°)	82°, (75°)	70°, (50°)	18°, (67°)	31°, (16°)	62°, (63°)

* Quantities in parentheses refer to exchanged-subphase systems.

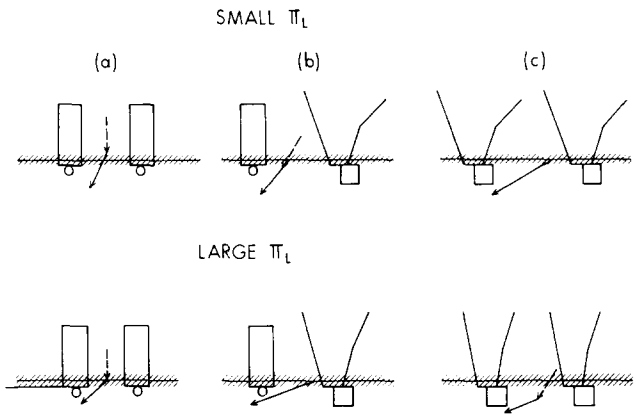

Fig. 8. Schematic representation of the disposition of the transition moments for the two transitions of amphotericin B in monolayers of cholesterol (a), equimolar egg lecithin–cholesterol (b), and egg lecithin (c) prior to subphase exchange. π_L refers to the initial film pressures. The spacing between monolayer neighbors is proportional to the (molecular areas)$^{1}/^{2}$. ←, represents transition moment of the 4077-Å band; <- - -, represents transition moment of the (3600-Å) band. The depth of penetration of the latter, maximum for cholesterol, is presumed to be correlated with its blue shift. For small π_L (c) and large π_L (b) this transition doesn't appear.

Å) band in the anomalous cases are characteristic of a hydrophobic environment. (In a methanol solution this band is at 3626 Å while in water it is at 3650 Å). In contrast, the lowest-energy band at 4077 Å lies close to its wavelength in water (4080 Å). Hence its transition moment appears to be localized in the hydrophilic part of the chromophore, namely toward the sugar end. (3) Correlated with the blue-shift of the (3600-Å) band are increases in both the intensities of the vertical spectra (R_v in Table II) and of the transition moment orientation, θ_2, of this band relative to the one at 4077 Å. (4) In the system of maximum polyene–lipid surface interaction occurring with the low-pressure cholesterol monolayer, the new band has its greatest intensity, is located at the shortest wavelength and has its transition moment oriented parallel to the long dimension of its sterol neighbors which are oriented normal to the surface. One cannot as yet say whether the transition moment of the new band has a fixed orientation relative to the molecular geometry of amphotericin B as is the the case for the 4077-Å band. Its angle with the long molecular axis of the polyene may vary depending on the orientation of the neighboring lipid molecules. Consequently, for those cases where $\theta_2 \neq \theta_1$, one doesn't know whether the polyene–lipid interaction produces a bending of the heptaene chain about an axis in the molecular plane or that it induces a rotation of the transition moment of the new band relative to the chain direction.

A remark should be made here concerning the assumption made in the preceding discussion that the polyene–lipid interaction is responsible for the appearance of the new band. Another possible explanation would depend on a polyene–polyene interaction mediated by an appropriate lipid environment. It seems highly unlikely that this interaction has any relevance in these experiments for the following three reasons: (a) the slopes of the graphs of log (N^b/N_1) vs the log of the subphase concentration of polyene are approximately linear indicating a lack of cooperativity among

the polyenes; (b) the new band appears in the high-surface pressure monolayer systems where the binding of amphotericin B is considerably reduced; (c) spectra of compressed monolayers of amphotericin B obtained in this research but not included in this paper show new bands arising on the long wavelength side of the uncompressed bands; these presumably arise from polyene–polyene interactions.

Effect of initial lipid pressure

For all three lipid systems, an increase in the initial surface pressure from about 5 dynes/cm to 30 dynes/cm produces large reductions in π_A and binding. In the case of egg lecithin there is essentially no change in θ_1, while for the other two systems there is a decrease in this parameter indicating a rotation of the 4077-Å transition moment to a more horizontal orientation (see Fig. 8). The increased pressure in the egg lecithin films induces a new transition moment at 3646 Å with a more vertical orientation than that of the 4077-Å band. For the mixed-lipid system the new transition at 3632 Å disappears. These results seem to indicate that compressing the cholesterol and cholesterol–egg lecithin monolayers causes a decrease in the penetration of amphotericin B into the hydrophobic regions with the accompanying loss of vertical orientation of the chromophore chain. The reduction in intermolecular spacings probably plays the leading role in these effects. It is difficult to understand how compression of the egg lecithin film decreases the binding of amphotericin B on the one hand and induces a new band at 3646 Å with a fairly vertical orientation of its transition moment on the other hand. In fact, the water-like wavelength of this band indicates very little penetration into the monolayer, a condition which has been found necessary for inducing the new (3600-Å) band in the cholesterol and the equimolar egg lecithin–cholesterol systems. This difficulty can be resolved if it is assumed that the choline groups are oriented normal to the surface in the compressed egg lecithin monolayers as has been proposed by Shah and Shulman [28]. These polar groups could then be responsible for inducing the observed transition without any need for polyene penetration into the hydrophobic region.

Effect of cholesterol

The interaction of amphotericin B with equimolar egg lecithin–cholesterol monolayers when compared to those of egg lecithin indicates that for low values of the initial surface pressures the presence of cholesterol does not affect π_A but produces a decrease in binding, an increased vertical orientation of the 4077-Å transition moment and the appearance of a new band slightly more vertically oriented than the original one. At high initial lipid pressures, cholesterol does not affect π_A, brings about a considerable decrease in binding, does not affect the orientation of the moment of the 4077-Å band and eliminates the anomalous (3600-Å) band. The further addition of cholesterol in going from the mixed equimolar monolayers to pure cholesterol produces increases in both π_A and polyene binding for both low and high surface pressures. In addition, θ_1, is increased and the new band which appeared at 3632 Å for the low pressure case and at 3629 Å for the high-pressure exchanged case becomes much stronger, shifts to 3610 Å and its transition becomes essentially perpendicular to the surface. The major effects of cholesterol on the interaction of amphotericin B with egg lecithin monolayers can be summarized as follows: (a) In going from egg lecithin to equimolar egg lecithin–cholesterol films, there is a decrease in binding and an increase

in the vertical orientation and penetration of the polyene at low initial film pressures, and a decrease in binding at high pressures; (b) in going from the egg lecithin–cholesterol films to those of cholesterol, there is an increase in binding, penetration and vertical orientation of the polyene for both low and high initial film pressures. Two explanations have been previously given for the role of cholesterol in these interactions, namely, the formation of a complex with the polyenes [2] and effecting an increase in the order of the nonpolar chainsof the lipids [27]. This ordering presumably mediates an increase in polyene binding and penetration by aligning the lecithin chains in a more parallel array so as to facilitate the penetration by the polyene molecules [3, 23]. Both explanations could account for all of the observed effects of cholesterol reported in this paper except for the decrease in polyene binding in going from egg lecithin to egg lecithin–cholesterol films. However, the complexing theory cannot account for the binding to the egg lecithin monolayers or to synthetic phospholipids not containing sterol [3, 23].

Effect of subphase exchange

As previously mentioned, the spectra of the monolayer systems were also obtained after the concentration of amphotericin B in the subphase was appreciably reduced. This was particularly useful in delineating the behavior of the new 3610-Å band in the low-pressure cholesterol system. However, as can be seen from Tables I and II, the exchange of the subphases, with some exceptions, brings about changes in π_A, N^b, θ_1 and θ_2. Since the surface tensions of the pure lipid systems were found to be unaffected by the exchange procedure it appears that the changes observed in π_A are caused by a combination of the washing-off of the weakly bound polyenes as well as changes in their orientations and degree of penetration. This study was not extensive enough to explain the effect on π_A in terms of these molecular parameters.

CONCLUDING REMARKS

The results of this investigation give some insight into the molecular parameters involved in the lowering of the surface tensions of the lipid monolayers in this study by a surfactant molecule like amphotericin B. It appears that the magnitude of π_A depends in some unknown manner on the binding, orientation and degree of penetration of the polyene (a measure of the latter being the magnitude of the blue shift in the (3600-Å) band. For the low-pressure cholesterol monolayers, which gives the maximum surface interaction with amphotericin B in this study, an examination of Tables I and II indicates that this interaction is characterized by maximization of N^b, θ_1, θ_2 and penetration. A reduction in any of these parameters relates to a decreased lowering of the surface tension of the monolayers, π_A, by the addition of the polyene. The largest effect of subphase exchange and increased lipid pressure on reducing π_A appears to be due to the decreased binding. This type of investigation extended to many different systems may be fruitful in developing a molecular theory for surface tension.

This study also gives a better understanding of the role played by cholesterol in increasing the polyene interaction with lipid monolayers. The addition of sufficient cholesterol to egg lecithin films increases the ability of the amphotericin B molecule to penetrate the monolayer which is coupled to orienting the long axis of the polyene to a direction more perpendicular to the surface. This appears to strengthen the

explanation offered recently that cholesterol enhances the interaction of amphotericin B [23] and nystatin [3, 23] with the fluid phases of lipid liposomes by increasing the order of the lipid chains. This increase in order has been established by EPR and NMR studies [27]. These effects of cholesterol on the incorporation of polyenes into lipid monolayers may explain the role of cholesterol in producing highly permeable black lipid membranes when they are modified by polyene antibiotics [20]. The sterol may be responsible for ordering each side of the bilayer so as to allow the polyenes to penetrate with their long axes perpendicular to the surface. This would increase the probability for the polyenes from opposite sides to join to form pores. An illustration of the structural relationships between the polyenes and sterols comprising a bilayer pore is given in a recent article of Finkelstein and Holz [29].

A few comments should be made on the nature of the polyene–sterol and polyene–lipid interaction. The results of this paper emphasizes the structural nature of the monolayer as being most important in the polyene interaction. This neglects the significance of the polyene–sterol complex which Norman and coworkers have shown [2]. An extension of this investigation to monolayers containing other steroids should be helpful in evaluating the relative importance of both points of view. Although the interaction of amphotericin B with egg lecithin is considerably weaker than with cholesterol as far as its effect on the surface tension of these monolayers is concerned, the polyene–lecithin interaction is not non-existent. In fact, measurements on amphotericin B monolayers made in this study but not discussed in this paper show the polyene to be oriented horizontally in these films as compared to an angle of about 30 ° for the low-pressure egg lecithin monolayers.

Although the errors in binding and transition moment orientations are considerable, there is a sufficient consistency in their values especially with regard to the effects of cholesterol and increased film pressure, to give some confidence to the conclusions of this paper. The strong intensity and well defined transition moment orientations of the new (3600-Å) band, especially in the case of cholesterol monolayers, appears to justify its use as a measure of the surface interaction of the polyene even though the origin of this band is unknown.

As a final remark, one of the motivations for this study was to examine the feasibility of obtaining the absorption spectra of amphotericin B interacting with black lipid membranes of equimolar egg lecithin–cholesterol. Extrapolating the binding results of the high-pressure mixed-lipid monolayers to the range of surface pressures expected for bilayers ($>$ 70 dynes/cm) [30], leads to the expectation of much smaller binding for the latter. Experiments on bilayer membranes made from a brushing solution of 2% egg lecithin and 4% cholesterol in *n*-decane did not succeed in yielding any absorption spectra of bound amphotericin B. These results indicate that the ratio of bound polyene to lipid in these membranes is smaller than 0.02 which is the smallest ratio observable in the present apparatus.

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APPENDIX

Derivation of percentage transmission for passage of parallel, linearly polarized light incident at 45 ° through an anisotropic monolayer–subphase system with absorption in both media.

Referring to Fig. 1, we will be concerned with the absorption of light polarized in the plane of the paper (vertical) and perpendicular to this plane (horizontal) as it traverses the film, the subphase and then the film again. The contributions to the transmitted intensity from multiple reflections within the monolayer can be ignored because of the very small reflectivity from the film–subphase interface. We will assume that the transition moments of the absorption bands in the film make an angle, θ, with its surface and are cylindrically symmetric about the normal to the film. In order to resolve the electric vector of the radiation along the transition moment directions, we must first determine the directions of propagation of the light during each of the two passages through the film. Therefore, we need to calculate the angles of refraction at the first air–film interface, φ_1, and at the film–water interface, φ_2, the angle of reflection at the immersed mirror, φ_3, and the angles of incidence, φ_4, and refraction, φ_5, at the water–film interface. From Snell's law, using the refractive indices* of 1.46 for the lipids [31] and 1.34 for water [32] together with the tilt of 5.5 ° of the immersed mirror, one obtains the following values for these angles: $\varphi_1 = 29$ °, $\varphi_2 = 32$ °, $\varphi_3 = 37.5$ °, $\varphi_4 = 43$ ° and $\varphi_5 = 39$ °.

We need to derive an expression for the intensity of light transmitted after one transit of the film. From symmetry considerations as well as by calculation it can be shown that the conical distribution of transition moments assumed here is equivalent to a representation of the transition moments by two vectors of equal magnitude each equal to one half the absorption coefficient, k. In this representation, the first vector, $k^a = k/2$, representing one-half of the absorbing molecules lies in the plane of incidence at an angle θ with the horizontal. The second vector, $k^b = k/2$, lies in the plane perpendicular to the incident plane making a similar angle with the horizontal. Let us consider vertical polarized light of intensity I_{0v} entering the film at the angle of refraction φ. The electric vector of this radiation can be decomposed into a vertical component of intensity $\sin^2 \varphi\ I_{0v}$ and a horizontal component of intensity $\cos^2 \varphi\ I_{0v}$. The vertical component interacts with the vertical components of k^a and k^b defined as k_v^a and k_v^b. The horizontal component interacts only with the horizontal component of k^a defined as k_h^a (since the horizontal component of the light intensity is perpendicular to k^b). Applying Beer's law to these two cases gives for the transmitted intensities through a film of thickness t for incident light vertically polarized

$$I^v = I_{0v} \sin^2 \varphi\, e^{-(k_v^a + k_v^b)t/\cos\varphi} + I_{0v} \cos^2 \varphi\, e^{-k_h^a t/\cos\varphi} \tag{A1}$$

* The effect of polyene absorption on the index has been neglected. In the case of the amphotericin B–cholesterol system an estimate for the 4077-Å band shows that this effect might alter the index by as much as ±0.15. This would affect the calculated orientation of the transition moment by only ±3 °, well within the experimental error.

since $k^a = k^b = k/2$ this becomes

$$I^v = I_{0v}(\sin^2\varphi e^{-k_v t/\cos\varphi} + \cos^2\varphi\ e^{-k_h t/2\cos\varphi}) \tag{A2}$$

For horizontally polarized light of intensity I_{0h}, the only interaction is with $k_h{}^b$ giving

$$I^h = I_{0h} e^{-k_h^b t/\cos\varphi} = I_{0h} e^{-k_h t/2\cos\varphi} \tag{A3}$$

Eqns A2 and A3 will now be applied to our calculation.

If I_{0v} and I_{0h} are the incident intensities for the two polarizations, and αI_{0v}, αI_{0h} are these intensities after reflection from the first mirror, then since $\varphi = \varphi_1 = 29^\star$ one obtains from Eqns A2 and A3 the following intensities after the first passage through the film

$$I_v^1 = \alpha\gamma I_{0v}(0.24 e^{-1.14 k_v t} + 0.76 e^{-0.57 k_h t}) \tag{A4}$$

$$I_h^1 = \bar{\alpha}\bar{\gamma} I_{0h} e^{-0.57 k_h t} \tag{A5}$$

γ, $\bar{\gamma}$ are the refractivities for the vertical and horizontal polarizations respectively. The light on emerging from the film passes through the subphase with the immersed mirror directing it toward a second transit of the film. The path length through the subphase with absorption coefficient k_s is d_s. The resultant intensities are given by

$$I_v^2 = \gamma'\delta e^{-k_s d_s} I_v^1 \tag{A6}$$

$$I_h^2 = \bar{\gamma}'\bar{\delta} e^{-k_s d_s} I_h^1 \tag{A7}$$

γ', $\bar{\gamma}'$ and δ, $\bar{\delta}$ are the refractivities and reflectivities from the immersed mirror respectively. We obtain the intensities after the second passage through the monolayer by again applying Eqns A2 and A3 with $\varphi = \varphi_5 = 39\ ^\circ\star$. The results are

$$I_v^3 = \gamma''(0.39 e^{-1.29 k_v t} + 0.61 e^{-0.64 k_h t}) I_v^2 \tag{A8}$$

$$I_h^3 = \bar{\gamma}'' e^{-0.64 k_h t} I_h^2 \tag{A9}$$

γ'', $\bar{\gamma}''$ are the refractivities at the water–film interface. The beams emerging from the film are reflected from the third mirror into the photomultiplier with the following intensities

$$I_v^4 = \gamma''' \varepsilon I_v^3 \tag{A10}$$

$$I_h^4 = \bar{\gamma}''' \bar{\varepsilon} I_h^3 \tag{A11}$$

γ''', $\bar{\gamma}'''$ are the refractivities at the film–air interface and ε, $\bar{\varepsilon}$ are the reflectivities of the mirror. Combining Eqns A4–A11 gives

$$\begin{aligned} I_v^4 = I_v = {} & \alpha\delta\varepsilon\gamma\gamma'\gamma''\gamma''' I_{0v} e^{-k_s d_s} \\ & \times (0.24 e^{-1.14 k_v t} + 0.76 e^{-0.57 k_h t}) \\ & \times (0.39 e^{-1.29 k_v t} + 0.61 e^{-0.64 k_h t}) \end{aligned} \tag{A12}$$

$$I_h^4 = I_h = \bar{\alpha}\bar{\delta}\bar{\varepsilon}\bar{\gamma}\bar{\gamma}'\bar{\gamma}''\bar{\gamma}''' I_{0h} e^{-k_s d_s} e^{-1.21 k_h t} \tag{A13}$$

$\star$ If the chromophore is bound to the monolayer but is immersed in the subphase then one must use $\varphi_2 = 32\ ^\circ$ and $\varphi_4 = 43^\circ$ instead of φ_1 and φ_5 respectively.

Now the percentage transmission recorded by the spectrometer is the ratio of the intensities in the sample beam to that of the reference beam. The reference beam intensities are given by

$$I_{\mathrm{v}}^{\mathrm{r}} = \alpha\delta_{\mathrm{r}}\varepsilon_{\mathrm{r}}\beta'\beta''I_{0\mathrm{v}} \tag{A14}$$

$$I_{\mathrm{h}}^{\mathrm{r}} = \bar{\alpha}\bar{\delta}_{\mathrm{r}}\bar{\varepsilon}_{\mathrm{r}}\bar{\beta}''\bar{\beta}''I_{0\mathrm{h}} \tag{A15}$$

β', $\bar{\beta}'$ and β'', $\bar{\beta}''$ are the refractivities for the air–water and water–air interfaces respectively and α, $\bar{\alpha}$; δ_{r}, ε_{r} and $\bar{\delta}_{\mathrm{r}}$, $\bar{\varepsilon}_{\mathrm{r}}$ are the reflectivities for the first, second and third mirrors respectively. Before taking the ratio of the sample to the reference beam intensities, let us simplify Eqns A12 and A13. Since $k_{\mathrm{v,h}}\, t < 1$ for the film ($k_{\mathrm{v,h}} = 0.07$ is the maximum value), we can expand the exponentials in k_{v} and k_{h} and neglect terms quadratic in $k_{\mathrm{v,h}}\, t$ upon taking the products in Eqn A12. The results for the percent transmissions become

$$T^{\mathrm{v}} = \frac{\gamma\gamma'\gamma''\gamma'''}{\beta'\beta''\delta_{\mathrm{r}}\varepsilon_{\mathrm{r}}}\,\delta\varepsilon\,\mathrm{e}^{-k_{\mathrm{s}}d_{\mathrm{s}}}(1-0.78\,k_{\mathrm{v}}t-0.83\,k_{\mathrm{h}}t) \tag{A16}$$

$$T^{\mathrm{h}} = \frac{\bar{\gamma}\bar{\gamma}'\bar{\gamma}''\bar{\gamma}'''}{\bar{\beta}'\bar{\beta}''\bar{\delta}_{\mathrm{r}}\bar{\varepsilon}_{\mathrm{r}}}\,\bar{\delta}\bar{\varepsilon}\,\mathrm{e}^{-k_{\mathrm{s}}d_{\mathrm{s}}}(1-1.21\,k_{\mathrm{h}}t) \tag{A17}$$

The percent transmissions represented by these equations are relative to a flat baseline at 100%. They should also contain scattering factors S^{v}, S^{h}, to represent losses due to scattering by the polyene micelles from the subphases which is mainly responsible for the sloping baseline. In the analysis of a spectrum however, the percent transmission used is the ratio of its value at the band maximum to that of the baseline at the wavelength of the maximum. Consequently, one must divide Eqns A16 and A17 by equations representing the transmission at the baselines. The latter are obtained by equating all of the absorption coefficients in the above equations to zero. Taking the indicated ratios gives the following equations as the final result

$$T^{\mathrm{v}} = \mathrm{e}^{-k_{\mathrm{s}}d_{\mathrm{s}}}(1-0.78\,k_{\mathrm{v}}t-0.83\,k_{\mathrm{h}}t)^{*} \tag{A18}$$

$$T^{\mathrm{h}} = \mathrm{e}^{-k_{\mathrm{s}}d_{\mathrm{s}}}(1-1.21\,k_{\mathrm{h}}t) \tag{A19}$$

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* For bound but immersed chromophores the coefficients of the kt terms are changed from 0.78, 0.83 and 1.21 to 0.96, 0.80 and 1.28 respectively. For the 4077-Å band whose transition moment appears to lie almost entirely in the subphase, these corrections only alter the orientations by 2°–5° from the values given in Table I.

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