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REFLECTION SPECTRA FROM MONOLAYERS OF AMPHOTERICIN B AND AMPHOTERICIN B–CHOLESTEROL SPREAD ON WATER

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SUMMARY

Polarized specular reflection spectra have been obtained of the antibiotic polyene, amphotericin B, in its monolayers and in those of cholesterol in its expanded–liquid phase. An analysis of the reflectivity of the long wavelength member of the 3 band group observed in these spectra gives both its extinction coefficient and the orientation of its transition moment. In the case of the amphotericin B monolayers, an increase in surface pressure from 9.4 to 15.6 dynes/cm produces a decrease in the molar extinction coefficient from $1.20 \cdot 10^5$ to $0.65 \cdot 10^5$ and possibly a decrease in the angle which the heptaene transition moment makes with the surface. Accompanying these changes is a red-shift of all three bands which shifts the long wavelength band from 4072 to 4150 Å. These changes in the molar extinction and wavelength with compression appear to be due to the formation of polyene–polyene complexes. The effect of an expanded–liquid monolayer of cholesterol on the surface interactions of amphotericin B is to increase the polyene orientation from 6° to 35° and shift the 4150 Å band to 4190 Å without further affecting the extinction coefficient of $0.65 \cdot 10^5$. The values of the wavelengths, extinction coefficients and orientations obtained from the reflection spectra of the two polyene monolayers were in agreement with those obtained from the transmission spectra of similar systems.

INTRODUCTION

This paper will demonstrate how polarized specular reflection spectra can be utilized to obtain the orientation and extinction coefficient of a strong chromophore in a monolayer spread on water. Although for some of the systems discussed, this information can be more easily obtained by means of absorption spectra, there are cases where the latter method may be impractical. Examples of these are: (1) surfaces of biological tissues, (2) monolayers and bilayers interacting with water-soluble chromophores whose aqueous absorption cannot be separated from the spectra of the bound molecules, (3) determination of the orientations of vibrational modes in wet surfaces where the infrared absorption of water would obscure the desired absorption spectra.

Previous examples of the use of polarized specular reflection spectra of monolayers to obtain the orientations of transition moments were those of Francis and Ellison [1] and Kolb and McIntyre [2]. In the former, the infrared reflection spectra of both monolayers and multilayers of zinc, barium and calcium stearate on steel and silver surfaces were used to identify the vibrational modes which are parallel and perpendicular to the surface. The latter publication gives the ultraviolet–visible reflection spectra of adsorbed oxygen on gold and showed the transition moments of the strong electronic bands of the Au_2O_3 molecule to be parallel to the surface. The somewhat related technique of attenuated total reflection has been used to obtain the polarized infrared spectra of built-up monolayers of stearic acid on a germanium surface [3]. These spectra gave the orientation of the molecular axis with respect to the surface as well as the absorption coefficients of some of the transitions. Also reported recently were the first reflection spectra from a black lipid bilayer, namely. those of chlorophyll *a* and *b* in lecithin–chlorophyll bilayers [4].

The chromophore in this study is that of amphotericin B, whose interaction with lipid monolayers [5, 6], bilayers [6], dispersions [6] and biological membranes [7] has been extensively studied. The systems consist of monolayers of amphotericin B at two different surface pressures and an expanded–liquid monolayer* of cholesterol interacting with the polyene injected underneath it. Because of its amphipathic structure [5], amphotericin B forms monolayers when either injected into or spread on water.

In order to verify the analysis of the weak reflection spectra which, in this study, ranged between reflectivities of 0.0008 and 0.0043, the polarized absorption spectra were also obtained for similar systems. The latter spectra were used to determine the orientation of the chromophore and its extinction coefficient for comparison with the results from the reflection spectra for the two amphotericin B monolayer systems.

METHODS

Materials

Amphotericin B was a gift from 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. Cholesterol was obtained from Eastman Kodak Co. Stock solutions of 0.001 M of cholesterol were stored at −60 °C until needed.

Preparation of monolayers

Monolayers were prepared by spreading the polyene or cholesterol on unbuffered and distilled water which was filtered and deionized. The trough was made of Delrin, a very inert material, had an area of 34 cm^2 (6.6×5.1 cm) and a depth varying from 0.2 to 0.7 cm. The volume of water in the trough varied between 16 and 20 ml. Prior to spreading, the water surface was cleaned with a Pasteur pipette. The surface tensions were measured by the Wilhelmy balance method 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.

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* Monolayers in the expanded–liquid phase are more dense than those in the gaseous phase but have surface pressures smaller than 1 dyne/cm.

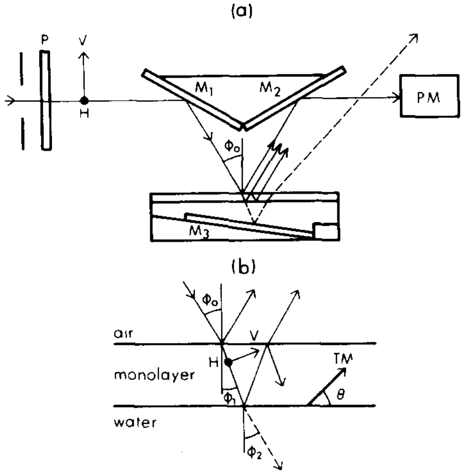

Fig. 1. (a) Optics for the sample beam. P, polaroid film polarizer; V, H, polarization directions; M_1, M_2, M_3, mirrors; PM, photomultiplier; ϕ_0, incidence angle of 30°; (b) Enlarged view of optical paths in monolayer. TM, transition moment of a chromophore. Multiple reflections from monolayer schematically indicated by the solid line rays, multiple refractions by the dashed ray.

Spectroscopic measurements

Reflection spectra of the monolayers were obtained using a Perkin Elmer 350 double beam spectrometer. The trough containing the monolayer systems was placed in the sample compartment. The sample optics is illustrated in Fig. 1a. The mirror M_1 deflects the light from the monochromator onto the monolayer surface at an incident angle, φ_0, of 30°. The reflected rays are deflected into the photomultiplier by mirror M_2. Mirror M_3, immersed in the subphase, deflects all of the light transmitted by the monolayer away from the photomultiplier preventing the superposition of absorption spectra on the reflection spectra. Without M_3 there is sufficient diffuse reflection from the trough bottom to overwhelm the reflection spectra by that of absorption. Both the sample and reference beams were polarized with Polaroid HNP' B polarizer sheets.

The binding of the polyene to the films (in moles) is given by the difference between the total number of moles spread or injected and that found in the subphase. 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 7 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 gives the amount of amphotericin B in the latter.

RESULTS

The reflection spectra obtained in this study are displayed in Fig. 2. These curves have been obtained by expanding the original tracings by factors varying from 1 to 3.4 to normalize them to the same instrumental gain. The modified curves have been smoothed, however, the signal-to-noise for the reflection bands is about 3/1 for the vertical spectra and 7/1 for the horizontal. The absolute reflectivities were obtained by recording the polarized reflection curves for water with each experiment. The

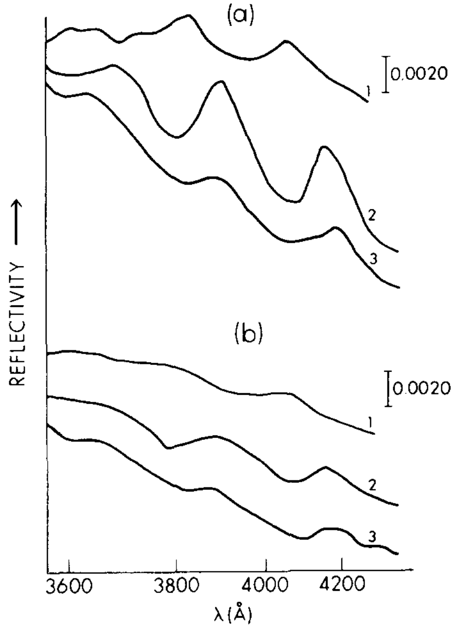

Fig. 2. Reflection spectra of monolayers containing amphotericin B. (a) For horizontally polarized light, (b) for vertically polarized light. Films 1 and 2 contained $0.23 \cdot 10^{-8}$ and $0.42 \cdot 10^{-8}$ moles of polyene respectively. Film 3 had $0.50 \cdot 10^{-8}$ moles of polyene bound to $0.44 \cdot 10^{-8}$ moles of cholesterol. The amounts of spread or injected amphotericin B were $0.7 \cdot 10^{-8}$, $2.3 \cdot 10^{-8}$ and $2.2 \cdot 10^{-8}$ moles respectively. The corresponding values of π were 9.4, 15.6, and 16.6 dynes/cm. These spectra, recorded at different instrumental gains, have been normalized to Spectrum (b) 3. The redrawn curves have been smoothed out, the actual signal-to-noise ratios are about 7/1 for (a) and 3/1 for (b).

calculated reflectivities of water at 4000 Å, using the refractive index 1.34 [8] are 0.0124 and 0.0318 for the vertical and horizontal polarizations respectively. These measurements consequently calibrate the reflectivity for each monolayer experiment.

Inspection of Fig. 2 shows the reflection spectra to consist of three bands. These are the strongest vibronic components of the π–$\pi^{\star}$ transition for the heptaene chromophore which has previously been observed in the absorption spectra of amphotericin B [5, 7]. The corresponding transition moments have been shown to be oriented along the polyene chain direction (long molecular dimension) [9]. The major contribution to the sloping baselines appears to be the increasing reflectivities due to the increasing index of refraction of the polyene films with decreasing wavelength. Increasing the amount of amphotericin B in its monolayer compresses it, thereby increasing the reflectivities for both polarizations (Table I) and appreciably shifting the wavelengths of the three peaks toward the red (compare Curves 1 and 2). A comparison of Spectra 2 and 3 shows that the effect of an expanded–liquid monolayer of cholesterol is to cause a large decrease in the reflectivities from the bound amphotericin B. In addition, the long wavelength band undergoes a further red-shift while the short wavelength band is blue-shifted. In fact, a doubling of the amount of cholesterol in the monolayer, which is still insufficient to give sterol film pressures of 1 dyne/cm, sufficiently reduces the reflection bands so that they are no longer visible.

TABLE I

RESULTS OF THE SURFACE AND REFLECTION SPECTRA MEASUREMENTS

N_A, moles of amphotericin B either spread on surface or injected under cholesterol monolayers; π, surface pressure of the films; N^b, moles of polyene in monolayer; λ^h, λ^v, maxima of the long wavelength reflection bands for horizontally and vertically polarized light respectively; ΔR^h, ΔR^v, intensities of the corresponding reflection bands; ε, θ, extinction coefficient and polyene orientation calculated for the long wavelength band.

	Amphotericin B	Cholesterol	
N_A ($\times 10^8$ moles)	0.70	2.30	2.23
π(dynes/cm)	9.4	15.6	16.6
N^b ($\times 10^8$ moles)	0.23 ± 0.05	0.42 ± 0.07	0.50 ± 0.07
λ^h (Å)	4072 ± 10	4150 ± 10	4190 ± 10
ΔR^h	0.0021 ± 0.0003	0.0043 ± 0.0008	0.0014 ± 0.0003
λ^v (Å)	4072 ± 15	4168 ± 10	4180 ± 50
ΔR^v	0.0009 ± 0.0002	0.0012 ± 0.0002	0.0008 ± 0.0002
ε ($\times 10^{-5}$)	1.20 ± 0.35	0.65 ± 0.15	0.65 ± 0.15
θ	$19 \pm 14°$	$6 \pm 6°$	$35 \pm 15°$

Appearing in Table I are the wavelengths and the reflectivities of the long wavelength member of each triad, the number of moles of the polyene in the films and the increase in film pressures due to amphotericin B.

DISCUSSION

Analysis of the spectra: determination of the orientation and extinction coefficient of the chromophore

If we assume homogeneous monolayers characterized by the complex index of refraction, $n_1 - iK$, that are spread on a nonabsorbing* aqueous medium with refractivity n, then from Vašiček (Eqn 5.941, in ref. 10) one obtains the following expression for the reflectivity of the film.

$$R(K) = \frac{a^2+b^2+(c^2+d^2)e^{-2x'}+2[(a^2+b^2)(c^2+d^2)]^{\frac{1}{2}}e^{-x'}\cos(x+\delta_1-\delta_2)}{1+(a^2+b^2)(c^2+d^2)e^{-2x'}+2[(a^2+b^2)(c^2+d^2)]^{\frac{1}{2}}e^{-x'}\cos(x+\delta_1-\delta_2)} \qquad (1)$$

a, c are the real components of the reflectivity at the air–film and film–water interface respectively while b, d are the imaginary components of the respective reflectivities (the interface reflectivities are given by the Fresnel equations [10]); δ_1, δ_2, x and x' are phase parameters given by $\delta_1 = tg^{-1}(b/a)$, $\delta_2 = tg^{-1}(d/c)$ and

$$x = (4\pi/\lambda)n_1 t \cos\varphi_1; \quad x' = (4\pi/\lambda)Kt\cos\varphi_1 \qquad (2)$$

where φ_1 is the angle of refraction at the air–film interface and t is the thickness of the chromophore given by $t = 10$ Å$(\cos\theta + 2\sin\theta)$. This expression is reasonable for the dimensions of the heptaene group where θ is the angle between the long molecular axis and the surface. Since we are interested only in the contribution of the monolayer

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* Although the subphase is not strictly nonabsorbing, the concentration of polyene in it is so small, $\approx 10^{-7}$ M, that the imaginary term in the refraction index is negligible.

absorption to $R(K)$, we need to substract from Eqn 1 the contribution from the non-absorbing reflectivity, namely,

$$R(0) = \frac{(a')^2+(c')^2+2a'c' \cos x}{1+(a')^2(c')^2+2a'c' \cos x} \tag{3}$$

where a', c', are the reflectivities from the air–film, film–water interfaces respectively, when $K = 0$. Hence the magnitude of the reflection bands is given by

$$\Delta R(K) = R(K)-R(0) \tag{4}$$

Eqns 1, 3 and 4 have to be considered separately for each polarization.

The relation between the macroscopic, imaginary part of the index of refraction, K, and the molar extinction coefficient, ε, is given by $K = 2.31\, \epsilon c\lambda/4\pi$. The quantity c is the molar concentration of chromophore and is given by $N^b/At \times 10^{-3}$, where N^b is the moles of polyene bound to the film, A is the film area of 34 cm^2 and t is the chromophore thickness. It is shown in the Appendix that incident light polarized perpendicularly to the incident plane interacts with the horizontal component of a vector representation of the transition moment given by $K^h = (K/2)\cos^2 \theta$. Consequently, for perpendicularly polarized light (designated as horizontally polarized), the appropriate expression for K to be used in calculating the reflectivity is given by

$$K^h = \frac{2.7 \cdot 10^7 \lambda \varepsilon N^b \cos^2 \theta}{\cos \theta + 2 \sin \theta} \tag{5}$$

The corresponding reflectivity is given by Eqn A.1, namely

$$\Delta R^h = R_s(K^h)-R_s(0) \tag{6}$$

For incident light polarized in the plane of incidence (called vertically polarized), the appropiate K is $K^v = K \sin^2 \theta$ where

$$K^v = \frac{5.4 \cdot 10^7 \lambda \varepsilon N^b \sin^2 \theta}{\cos \theta + 2 \sin \theta} \tag{7}$$

The corresponding reflectivity is given by Eqn A.3, namely

$$\Delta R^v = \cos^2 \varphi_1\, \Delta R_p(K^h)+\sin^2 \varphi_1\, \Delta R_p(K^v) \tag{8}$$

Since the quantities a, b, c and d in Eqn 1 are functions of K, inspection of Eqns 5–8 indicates that for an angle of incidence, φ_0, and for measured values of n_1 and N^b, the magnitude of a reflection band is a function of ε and θ for each incident polarization. Consequently, the measurement of ΔR^h and ΔR^v for each system should enable the determination of ε and θ. An outline of a graphical solution to this problem follows.

Firstly, the family of curves ΔR^h as a function of K^h are calculated from Eqns 1–4 for several values of θ. The intersections of the measured ΔR^h with these curves give values of K^h, θ which determine a new curve, $K^h(\theta)$. Then, Eqn 5 is used to generate a series of curves, $\underline{K}^h(\theta)$, for several values of ε; each value of ε gives a different curve. The values of ε, θ at the intersections of $\underline{K}^h$ with K^h are plotted as the function $\varepsilon^h(\theta)$. Next, the family of curves of ΔR^v as a function of θ are calculated from Eqns 1–8 for the same values of ε used previously; each value of ε generating a different curve. Finally, the intersection of the measured ΔR^v with these curves, $\Delta R^v(\theta)$, give values of ε, θ which are plotted as the function, $\varepsilon^v(\theta)$. The intersection of $\varepsilon^h(\theta)$ with $\varepsilon^v(\theta)$ is the solution, ε, θ to the problem.

Discussion of results

The errors in the calculations are principally due to the uncertainties in the measurement of both the reflectivities and the number of polyene molecules in the films. These are indicated in Table I. In addition, the lack of a measured index of refraction, n_1, is also a contributing factor. The calculations were done for several values of n_1 varying between 1.34 and 1.70. An approximate determination was made of the effect of these 3 sources of error on the curves $\varepsilon(\theta)$ and their intersection. The results of this treatment are given in Table I as the most reasonable solutions to the analytical problem. Absorption measurements were made on similar monolayer systems in order to obtain independently, values of ε and θ for verification of the reflectivity method. The absorption spectra could only be analyzed for the two amphotericin B films because for the cholesterol system the spectra of the bound polyene could not be separated from that of the aqueous molecules. The absorption spectra and their analyses are not included in this paper but the methodology is described in a previous publication [5]. The values of ε and θ obtained from the absorption experiments are $1.20 \pm 0.35 \cdot 10^5$, $0 \pm 10^\circ$; and $0.60 \pm 0.20 \cdot 10^5$, $0 \pm 7^\circ$ for the lower and higher pressure polyene films respectively. Within the large uncertainties, it is seen that the results for the two methods are in agreement.

A few remarks will be made concerning the reflection spectra and the values of ε and θ obtained from them. Increasing the amount of amphotericin B in the monolayers by about a factor of 2 causes all three bands to be red-shifted and halves the extinction coefficient of the longest wavelength band. This would seem to indicate the formation of polyene complexes in which the heptaene groups are interacting with each other. In a recent paper [11] this interaction has been suggested to explain the effect of sterol on the absorption spectra of several polyenes including amphotericin B. In the forementioned study it was proposed that the polyene–polyene interaction as mediated by the sterol increases the ratio of the absorbance of the short wavelength band to that of the long wavelength band in the triad. This absorbance change was not accompanied by any wavelength shifts. In the present study, the ratio of reflectivities of these two bands is increased from 0.36 to 0.59 in going from the less dense to the more dense polyene monolayers and from 0.59 to 0.87 on adding cholesterol to the dense polyene system. (These ratios are the average for the 2 polarizations). This behavior is in accord with this recent report. However, in contrast to the absorption studies, the increased interaction produces wavelength shifts in the reflection spectra. The explanation for this difference is not known at present. For both polyene monolayers the orientation of the chromophore appears to be nearly horizontal. The effect of introducing an expanded–liquid cholesterol monolayer is to greatly reduce the intensities of the reflection bands, and to effect a small additional red-shift of the long wavelength band, a small blue-shift of the middle band and an appreciable blue-shift of the short wavelength band. There is no change in ε but the chromophore is tilted from about 6° to 35°. The wavelength and extinction coefficient for the long wavelength band in the cholesterol system is still characteristic of a polyene complex.

The effect of cholesterol in producing a change in orientation of the polyene toward the normal as well as a blue-shift of the short wavelength band has been previously reported for cholesterol monolayers at initial surface pressures of 5 dynes/cm [5]. This orientation change appears to be responsible for the decreased intensity of the bands and for their complete disappearance upon a doubling of the cholesterol content. A larger angle of incidence would allow the observation of more vertically oriented chromophores.

APPENDIX

Functional dependence of the intensity of the reflection bands on the imaginary part of the index of refraction of an absorbing film.

Fig. 3 shows the relation between the components of the polarized light and the transition moments of the chromophore for the model of the monolayer used in this paper. This model assumes that the transition moments are distributed uniformly about the vertical (z) axis making an angle θ with the horizontal (xy) plane. It can be shown that this distribution of moments is equivalent to a representation by 2 vectors of equal magnitude each equal to one-half the absorption coefficient and situated in orthogonal planes as shown in Fig. 3. Since the absorption coefficient is proportional to the imaginary part of refractive index, K, each transition moment can be represented by the vector $K/2$.

For horizontally polarized radiation of intensity $I_0{}^h$, the interaction of light responsible for the reflection band is with $(K/2)\cos^2\theta$ defined as K^h. Consequently, the intensity of the reflection band is given by

$$\Delta R^h = R_s(K^h) - R_s(0) \tag{A.1}$$

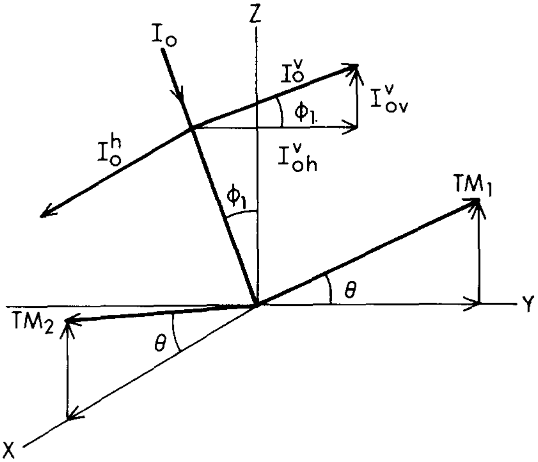

Fig. 3. Geometrical relations between the components of light intensity and the transition moments of a chromophore within a film and uniformly distributed about the normal at an angle θ with the horizontal plane. $I_0{}^h$, $I_0{}^v$ are the light intensities for horizontal and vertical polarization respectively; φ_1 is the angle of refraction at the air–film interface and TM_1, TM_2 designate the transition moment vectors.

where the subscript s refers to using reflectivities perpendicular to the plane of incidence in Eqns 1, 3 and 4.

For vertically polarized radiation of intensity I_0^{v}, there are two interactions to be considered. The horizontal component, I_{0h}^{v} interacts with K^{h} while the vertical component $I_{0\mathrm{v}}^{\mathrm{v}}$ interacts with both vertical components of the transition moment, namely $2 \times (K/2) \sin^2 \theta$ defined as K^{v}. Since $I_{0\mathrm{v}}^{\mathrm{h}} = \cos^2 \varphi_1 I_0^{\mathrm{v}}$ and $I_{0\mathrm{v}}^{\mathrm{v}} = \sin^2 \varphi_1 I_0^{\mathrm{v}}$ we can write the intensity of the reflected band as

$$\Delta R^{\mathrm{v}} = \cos^2 \varphi_1 [R_{\mathrm{p}}(K^{\mathrm{h}}) - R_{\mathrm{p}}(0)] + \sin^2 \varphi_1 [R_{\mathrm{p}}(K^{\mathrm{v}}) - R_{\mathrm{p}}(0)] \tag{A.2}$$

with $K^{\mathrm{h}} = K/2 \cos^2 \theta$, $K^{\mathrm{v}} = K \sin^2 \theta$ and the subscript p referring to using the reflectivities for radiation in the plane of incidence in Eqns 1, 3 and 4. This equation can be more compactly written as

$$\Delta R^{\mathrm{v}} = \cos^2 \varphi_1 \, \Delta R_{\mathrm{p}}(K^{\mathrm{h}}) + \sin^2 \varphi_1 \, \Delta R_{\mathrm{p}}(K^{\mathrm{v}}) \tag{A.3}$$

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