Determination of Molecular Order in Supported Lipid Membranes by Internal Reflection Fourier Transform Infrared Spectroscopy

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ABSTRACT When polarized internal reflection infrared spectroscopy is used to determine molecular order in supported lipid membranes, the results are critically dependent on the accuracy of assumptions made about the evanescent electric field amplitudes in the membrane. In this work, we examine several expressions used for calculating evanescent electric field amplitudes in supported lipid monolayers and bilayers, and test their validity by measuring the infrared dichroism of poly-γ-benzyl-L-glutamate and poly-β-benzyl-L-aspartate under conditions in which their molecular order is known. Our results indicate that treating such systems as a simple single interface between two semi-infinite bulk phases is more accurate than the commonly employed thin-film approximation. This implies that earlier conclusions about molecular order in supported lipid membranes may require substantial revision.

INTRODUCTION

Polarized attenuated total internal reflection–Fourier transform infrared (PATIR-FTIR) spectroscopy is being used with increasing frequency to determine the molecular order of polypeptides and lipids in various types of supported oriented membrane preparations. These preparations are stratified three-phase systems composed of a flat IR-transparent internal reflection element as the first or "support" phase, a lipid membrane as the second or "intermediate" phase, and either humidified air or an aqueous buffer as the third or "bulk" phase (Fig. 1). The lipid membranes are variously prepared as multibilayers formed by evaporating aqueous liposome suspensions (Lüneberg et al., 1995), multibilayers that self-assemble upon evaporation of an organic solvent (Brauner et al., 1987), single supported bilayers formed by the spontaneous adsorption and fusion of liposomes onto a hydrophilic surface (Kalb et al., 1992; Wenzl et al., 1994), or monolayers applied to an alkylsilane-treated surface by the Langmuir-Blodgett technique (Axelsen et al., 1995a,b).

In studies of this type, molecular order is calculated in three steps (Fringeli and Günthard, 1981; Axelsen et al., 1995b). First, the dichroic ratio for a selected vibrational mode (or band) is determined by calculating the ratio of measured absorption intensities for light polarized parallel versus perpendicular to the plane of incidence. Second, an order parameter, S, is calculated for the transition moment of the selected mode based upon the relationship

$$S(R_z) = \frac{\langle E_x^2\rangle - \langle E_y^2\rangle R_z + \langle E_z^2\rangle}{\langle E_x^2\rangle - \langle E_y^2\rangle R_z - 2\langle E_z^2\rangle}, \quad (1)$$

where R_z represents the dichroic ratio, and $\langle E_x^2\rangle$, $\langle E_y^2\rangle$, and $\langle E_z^2\rangle$ are the mean square electric field amplitudes of the evanescent polarized radiation in the membrane (Fraser and McRae, 1973). Third, the order parameter for the transition moment is scaled to yield a molecular order parameter, S_m, according to

$$S_m = \langle P_2(\cos\gamma)\rangle = S(R_z)/\langle P_2(\cos\theta)\rangle, \quad (2)$$

where θ is the angle between the transition moment and a conveniently defined molecular axis, $P_2(x) = (3x^2 - 1)/2$, and γ is the angle between the molecular axis and the z axis. By convention, $S = 1.0$ implies perfect order perpendicular to the membrane surface, $S = -0.5$ implies perfect order parallel to the membrane surface, and $S = 0.0$ implies an isotropic distribution or perfect disorder.

Most investigators using PATIR-FTIR spectroscopy employ Eq. 1, in one form or another, to relate infrared absorption dichroism and molecular order. However, there are significant differences in the manner by which different investigators estimate electric field amplitudes when applying Eq. 1 to the study of supported lipid membranes. For example, some investigators ignore the effects of the membrane on field amplitudes within the membrane and assume instead that the electric field amplitudes within the membrane are equivalent to the amplitudes that would be expected at the interface of the support and bulk phases (Higashiyama and Takenaka, 1974; Cropek and Bohn, 1990; Jang and Miller, 1995). We refer to this approach below as the "two-phase" approximation. In contrast, other investigators attempt to calculate precise values for the electric field amplitudes within a membrane (Harrick, 1967; Fringeli and Günthard, 1981; Mirabella and Harrick, 1985), using expressions for the x and y components of the fields

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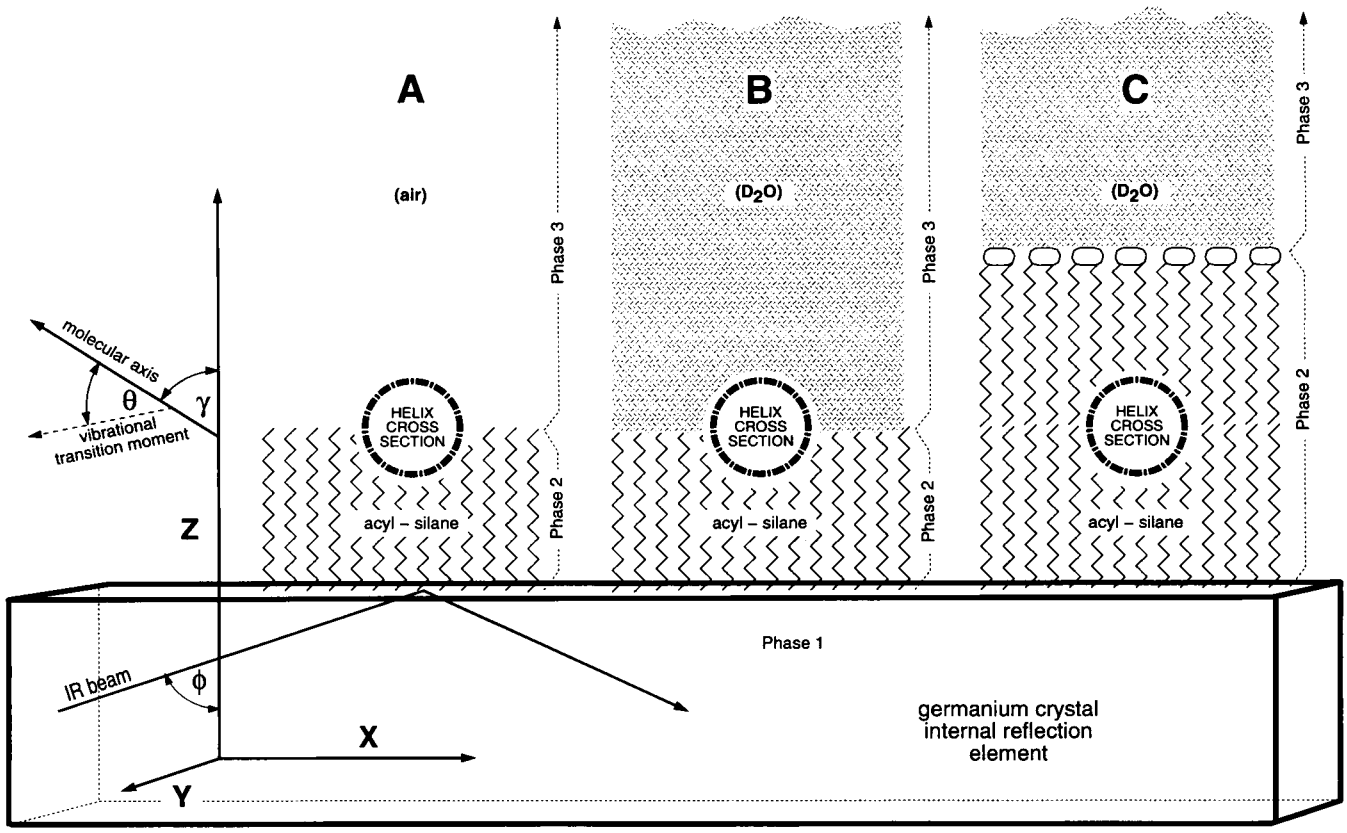

FIGURE 1 Schematic of the angle and axis conventions used in this work, and idealized cross sections of helical polypeptides situated (*A*) on the dry surface of an acyl-silane-treated germanium crystal, (*B*) on the surface of an acyl-silane-treated crystal immersed in D_2O, and (*C*) in between the acyl chains of the silane and a lipid monolayer.

that are identical to those used in the two-phase approximation, but differing significantly in the z component. We refer to this treatment below as the "thin-film" approximation. A priori, it is not clear which of these approaches for the calculation of electric field amplitudes in supported membranes is most appropriate.

Both of these approximations are rooted in expressions derived from a mathematically rigorous treatment of internal reflection and transmission in multiphase stratified systems (Hansen et al., 1966; Hansen, 1968, 1972, 1973). This more rigorous approach treats the intermediate phase thickness and its complex optical properties explicitly. In contrast, the two-phase and thin-film approximations are thickness independent, and they do not treat the refractive index of the intermediate phase as a complex quantity.

To test the accuracy of these approaches, we have examined poly-γ-benzyl-L-glutamate (PBG) and poly-β-benzyl-L-aspartate (PBA) using PATIR-FTIR spectroscopy. These polypeptides form long α-helices when evaporated onto hydrophobic surfaces (Perutz, 1951). Because they are in direct contact with a flat surface, their molecular order is $S_m = -0.5$ with respect to the surface normal. By comparing this known value with the order parameter results obtained using the two-phase and thin-film approximations, we can evaluate the accuracy of these expressions.

These studies also provide a means of verifying the value of θ for an α-helix, i.e., the apparent or effective average orientation of the amide I transition moment with respect to the longitudinal axis of the helix. Earlier IR studies reporting this value used dispersive instruments and transmission techniques, and relied on an assumption about the "oriented fraction" of the polypeptide after manually combing an organic solution as it evaporated (Fraser, 1958; Miyazawa and Blout, 1961; Bradbury et al., 1962; Tsuboi, 1962). In PATIR-FTIR spectroscopy, this assumption is unnecessary because the orientation of the polypeptides perpendicular to the z axis is well defined when each helical molecule lies in direct contact with the support surface.

We have found that attempts to estimate electric field amplitudes within the membrane by means of the thin-film approximation lead to misleading and, in at least one case, plainly unphysical results. In contrast, the two-phase approximation yields results that are both plausible and self-consistent. Furthermore, the two-phase approximation is consistent with the earlier determinations of amide I transition moment orientation in PBA and PBG made by means of transmission mode measurements, as well as the observation that this moment assumes a different orientation in these two polypeptides. These results suggest that when the film thickness is negligible in comparison to the wavelength

of light, it may be more accurate to treat this system as two semi-infinite bulk phases when calculating the electric field amplitudes within the lipid monolayer. These results require us to reexamine and, in some cases, to reinterpret earlier conclusions about molecular order based on calculated values for the electric field amplitudes within a membrane.

THEORY

Molecular species to be examined by internal reflection infrared spectroscopy are placed in close proximity to an internal reflection element and subjected to an evanescent electric field (Harrick, 1967). Electromagnetic energy is absorbed by these molecules in proportion to the mean square amplitude of the evanescent field at a some distance d from the surface of the internal reflection element. Hansen (1968) provides expressions for calculating the electric field amplitude components in a stratified system composed of three distinct phases (as depicted in Fig. 1), from which we obtain the following time-averaged expressions:

$$\langle E_y^2 \rangle = (1 + R_\perp) + 2\sqrt{R_\perp}\cos\left(\delta_\perp - \frac{4\pi d\xi_1}{\lambda}\right) \quad \text{Phase 1} \quad (3)$$

$$= \left|(1 + r_\perp)\cos\left(\frac{2\pi d\xi_2}{\lambda}\right) + i\frac{\xi_1}{\xi_2}(1 - r_\perp)\sin\left(\frac{2\pi d\xi_2}{\lambda}\right)\right|^2 \quad \text{Phase 2} \quad (4)$$

$$= |t_\perp|^2 e^{(-4\pi \mathrm{Im}\xi_3/\lambda)\cdot(d-h)} \quad \text{Phase 3} \quad (5)$$

$$\langle E_x^2 \rangle = \cos^2\phi\left\{(1 + R_\parallel) - 2\sqrt{R_\parallel}\cos\left(\delta_\parallel - \frac{4\pi d\xi_1}{\lambda}\right)\right\} \quad \text{Phase 1} \quad (6)$$

$$= \left|\cos\phi(1 - r_\parallel)\cos\left(\frac{2\pi d\xi_2}{\lambda}\right) + i\left(\frac{\xi_2 n_1}{\hat{n}_2^2}\right)(1 + r_\parallel)\sin\left(\frac{2\pi d\xi_2}{\lambda}\right)\right|^2 \quad \text{Phase 2} \quad (7)$$

$$= \left|\frac{\xi_3}{\hat{n}_3}\cdot t_\parallel\right|^2 e^{(-4\pi \mathrm{Im}\xi_3/\lambda)\cdot(d-h)} \quad \text{Phase 3} \quad (8)$$

$$\langle E_z^2 \rangle = \sin^2\phi\left\{(1 + R_\parallel) + 2\sqrt{R_\parallel}\cos\left(\delta_\parallel - \frac{4\pi d\xi_1}{\lambda}\right)\right\} \quad \text{Phase 1} \quad (9)$$

$$= \left|\frac{n_1^2\sin\phi}{\hat{n}_2^2}(1 + r_\parallel)\cos\left(\frac{2\pi d\xi_2}{\lambda}\right) + i\left(\frac{n_1\sin\phi}{\xi_2}\right)\cos\phi(1 - r_\parallel)\sin\left(\frac{2\pi d\xi_2}{\lambda}\right)\right|^2 \quad \text{Phase 2} \quad (10)$$

$$= \left|\frac{n_1\sin\phi}{\hat{n}_3}\cdot t_\parallel\right|^2 e^{(-4\pi \mathrm{Im}\xi_3/\lambda)\cdot(d-h)}, \quad \text{Phase 3} \quad (11)$$

where phases 1 and 3 are semi-infinite bulk layers; h is the thickness of a weakly absorbing thin film (phase 2); d is the distance from the interface along the z axis; $t_\perp$, $t_\parallel$, $r_\perp$, and $r_\parallel$ are the Fresnel transmittance and reflectance coefficients for light polarized perpendicular and parallel to the plane of incidence; $R_\parallel = |r_\parallel|^2$, $R_\perp = |r_\perp|^2$; n_1 is the real part of the refractive index in phase 1; $\hat{n}_j = n_j + i\kappa_j$ is the complex refractive index for phase j; κ is the absorption coefficient; ϕ is the angle of incidence; λ is the wavelength of electromagnetic radiation; δ represents a phase change upon reflection or transmission; and $\xi_j = \hat{n}_j\cos\phi_j = (\hat{n}_j^2 - n_1^2\sin^2\phi_1)^{1/2}$. Note that Eq. 10 corrects a minor typographical error in equation 48 of Hansen (1968). The Fresnel coefficients for reflection and transmission of electromagnetic radiation for a three-phase system are are given by

$$t_\perp = \frac{t_{\perp 12}t_{\perp 23}e^{i\beta}}{1 + r_{\perp 12}r_{\perp 23}e^{2i\beta}} \qquad t_\parallel = \left(\frac{t_{\parallel 12}t_{\parallel 23}e^{i\beta}}{1 + r_{\parallel 12}r_{\parallel 23}e^{2i\beta}}\right)\cdot\frac{n_1}{\hat{n}_3} \quad (12)$$

$$r_\perp = \frac{r_{\perp 12} + r_{\perp 23}e^{2i\beta}}{1 + r_{\perp 12}r_{\perp 23}e^{2i\beta}} \qquad r_\parallel = \frac{r_{\parallel 12} + r_{\parallel 23}e^{2i\beta}}{1 + r_{\parallel 12}r_{\parallel 23}e^{2i\beta}} \quad (13)$$

$$r_{\perp jk} = \frac{\xi_j - \xi_k}{\xi_j + \xi_k} \qquad t_{\perp jk} = \frac{2\xi_j}{\xi_j + \xi_k} \quad (14)$$

$$r_{\parallel jk} = \frac{\hat{n}_k^2\xi_j - \hat{n}_j^2\xi_k}{\hat{n}_k^2\xi_j + \hat{n}_j^2\xi_k} \qquad t_{\parallel jk} = \frac{2\hat{n}_k^2\xi_j}{\hat{n}_k^2\xi_j + \hat{n}_j^2\xi_k} \quad (15)$$

and $\beta = 2\pi h\xi_2/\lambda$. We have employed notational conventions consistent with those of Hansen (1968), in which the complex refractive index is defined with a positive imaginary component. In later publications, Hansen defines this using a negative imaginary component, which changes the sign of several terms in Eqs. 3–13 (Hansen, 1973). It should also be noted that Eqs. 3–11 have been multiplied by a normalization factor of 2, and there is a minor difference in

the way the Fresnel transmittance coefficients are defined-between Hansen (1968) and Hansen (1973). However, the two definitions are mathematically equivalent, provided they are consistently applied.

The forgoing expressions explicitly account for the thickness of the intermediate phase. For mid-IR radiation, however, the electric field amplitudes in an evanescent field do not decay significantly for values of h that correspond to the thickness of a lipid membrane, and one may assume that the amplitudes throughout the membrane are approximately equal to those at $h = 0$. Using this assumption, and for a system composed of two nonabsorbing phases (i.e., zero intermediate phase thickness), Eqs. 5, 8, and 11 reduce algebraically to

$$\langle E_{y_0}^2 \rangle = \frac{4 \cos^2 \phi}{(1 - n_{31}^2)} \tag{16}$$

$$\langle E_{x_0}^2 \rangle = \frac{4 \cos^2 \phi (\sin^2 \phi - n_{31}^2)}{(1 - n_{31}^2)[(1 + n_{31}^2)\sin^2 \phi - n_{31}^2]} \tag{17}$$

$$\langle E_{z_0}^2 \rangle = \frac{4 \cos^2 \phi \sin^2 \phi}{(1 - n_{31}^2)[(1 + n_{31}^2)\sin^2 \phi - n_{31}^2]}, \tag{18}$$

where $n_{jk} = (n_j/n_k)$, the plane of incidence is parallel to the xz plane, o subscripts indicate that the expressions pertain to the interface ($d = 0$), and n_1 and n_3 are the refractive indices of the semi-infinite bulk phases. These expressions were originally derived by Harrick (1965) to calculate the electric field amplitudes for two adjacent bulk phases. For amplitudes in a three-phase system, Harrick employs Eqs. 16 and 17 (x and y components for a two-phase system) without modification, but incorporates the factor n_{32}^4 in the numerator of the z component expression, yielding:

$$\langle E_z^2 \rangle = \frac{4 n_{32}^4 \cos^2 \phi \sin^2 \phi}{(1 - n_{31}^2)[(1 + n_{31}^2)\sin^2 \phi - n_{31}^2]}. \tag{19}$$

This approximation is based on the relationship

$$(n_3^2 + k_3^2)^2 \langle E_z^2 \rangle_3 = (n_2^2 + k_2^2)^2 \langle E_z^2 \rangle_2, \tag{20}$$

which, in turn, follows from the requirement that charge flow at an interface must be continuous (Hansen, 1973). In the following text, we refer to Eqs. 16, 17, and 18 as the two-phase approximation, and Eqs. 16, 17, and 19 as the thin-film approximation (Harrick and du Pre, 1966; Harrick, 1967; Mirabella and Harrick, 1985).

EXPERIMENTAL METHODS

Spectra were collected with a Bio-Rad Digilab FTS 60A spectrometer equipped with a rotatable KRS-5 aluminum wire grid polarizer (Molecular Detector, Portland, OR) and a narrow-band MCT detector. The infrared beam was directed into a 9 × 9 × 36 mm germanium crystal (Harrick Scientific, Ossining, NY), which served as an internal reflection element (IRE) and membrane support. Before each experiment, the crystal surface was meticulously cleaned, hand polished, and treated with octadecyltrichlorosilane to render it hydrophobic. After the application of samples, the crystal IRE is mounted in a custom-built spectrometer accessory and may then be immersed into a Langmuir-Blodgett lipid film deposition system. Detailed descriptions of the instrumentation, crystal preparation, and spectral processing have been published elsewhere (Axelsen et al., 1995a,b).

PBG (molecular weight 150,00) and PBA (molecular weight 60,000) were purchased commercially (Sigma, St. Louis, MO) and prepared as dilute solutions (~3×10^{-8} M) in dioxane or methylene chloride. Fifty-microliter aliquots of one or the other of these solutions were dropped onto one of the two 9 × 36 mm reflecting surfaces of the crystal that had been positioned horizontally. After the solvent evaporated, the crystal was turned over and additional aliquots of the same solution were evaporated onto the opposite side before the crystal IRE was mounted in the spectrometer. Based on the amount of this solution applied to the crystal surface, the area of the surface, and the cross-sectional area expected for a helical polymer, we estimate that the polypeptides cover no more than 10% of the crystal surface in any region (i.e., they form an incomplete and sparse monolayer). We determined when the solvent had thoroughly evaporated by observing the disappearance of its characteristic spectral features. Background spectra were obtained by rinsing the applied polypeptide off the surface with polymer-free solvent and collecting another spectrum. Thus, we obtain sample and background spectra without without manipulating the crystal or any other component in the beam path. This technique provides for exceptionally noise-free spectra and flat baselines.

Spectra were derived from 1024 single-beam scans recorded at 2 cm^{-1} resolution, with triangular apodization and one level of zero filling. Experiments were performed in triplicate, and the reported data represent average values from these experiments. Dichroic ratios were determined from band areas using a single Gaussian/Lorentzian function fitted to the amide I band between 1700 and 1600 cm^{-1}. Spectral baselines were adjusted to zero at 1800 cm^{-1} but were not deconvolved, smoothed, or otherwise manipulated.

RESULTS

The polarized IR spectra obtained by evaporating PBG and PBA from dioxane onto the crystal surface are shown in Fig. 2, *A* and *B*, and those obtained by evaporating PBA from methylene chloride are shown in Fig. 2 *C*. Amide I (~1655 cm^{-1}) and amide II (~1550 cm^{-1}) bands, as well as those attributable to ester carbonyl stretching (~1740 cm^{-1}), are readily identified. We assume that PBA and PBG are α-helical and that their longitudinal axes are oriented isotropically in the plane of the crystal surface (Fig. 1 *A*; support for these assumptions is discussed below).

The different approaches for the calculation of electric field amplitudes described above were subjected to three separate tests. In each test, we attempted to use the calculated field amplitudes, the measured dichroic ratio, and Eq. 1 to verify that surface-adsorbed PBA and PBG are highly ordered and oriented parallel to the crystal surface. In the first test, the solvent was simply allowed to evaporate in dry air (Fig. 1 *A*). Accordingly, electric field amplitudes were calculated using the two-phase approximation (Eqs. 16–18), the thin-film approximation (Eqs. 16, 17, and 19), and the thickness-dependent three-phase expressions (Eqs. 4, 7, and 10 for phase 2; Eqs. 5, 8, and 11 for phase 3), assuming $n_1 = 4.00$, $n_2 = 1.40$, $h = 25$ Å (to account for the intermediate phase, composed of acyl-silane), $n_3 = 1.00$ (air), and $\lambda = 1650$ cm^{-1}. We have assigned $\kappa_2 = 0.001$ and $\kappa_3 = 0.000$. Although these two assignments are entirely conjecture, the field amplitude results are quite insensitive to their values over a range of 0.000–0.100. The calculated amplitudes are listed in Table 1, and order parameters obtained using each set of results and Eq. 1 are listed in Table 2.

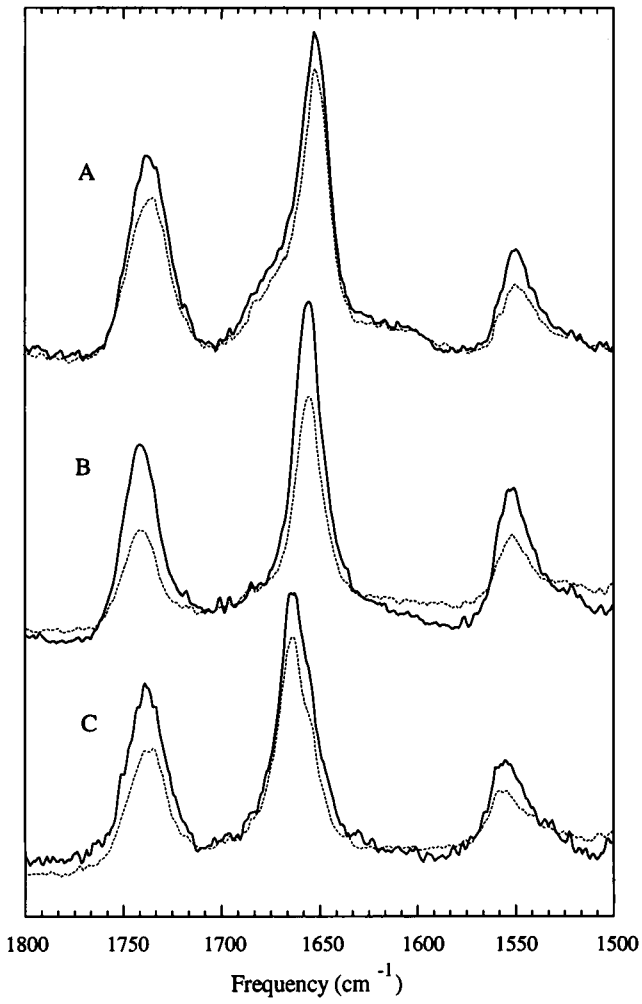

FIGURE 2 PATIR-FTIR spectra of (*A*) PBG evaporated from dioxane, (*B*) PBA evaporated from dioxane, and (*C*) PBA evaporated from methylene chloride. Solid lines, parallel polarization; dotted lines, perpendicular. The sample conditions correspond to Fig. 1 *A*. The spectra were zeroed at 1800, but were not otherwise corrected, smoothed, or deconvolved. Bands assignable to ester carbonyl stretching in the polypeptide side chains are seen at ~1740, and amide II is seen at ~1550. These are shown for context; only the amide I bands at 1655–1664 were analyzed in this work.

Electric field amplitudes calculated using the thin-film approximation suggest that amide I transitions in PBG are almost completely disordered, whereas those in PBA (both right- and left-handed forms) are highly ordered and perpendicular to the crystal surface. The two-phase approximation, on the other hand, yields order parameters that are consistent with our expected result, namely that the amide I transition moments in all three polypeptide forms are preferentially aligned parallel to the plane of the crystal surface. The thickness-dependent equations for phase 3 (air) yield results that are virtually identical to the two-phase approximation, and those for phase 2 (acyl-silane) yield results that are virtually identical to the thin-film approximation. These results suggest that these polypeptides are treated most accurately if they are treated as if they are subject to electric fields in the third (bulk) phase.

In the second test of the various electric field amplitude expressions, we immersed the crystal with adsorbed polypeptide into D_2O (Fig. 1 *B*). Because α-helical PBA and the crystal surface are both hydrophobic, we assumed that the PBA would remain adsorbed and would not alter its conformation or its orientation upon immersion into D_2O. In support of this assumption, we point out that that D_2O immersion did not change the intensities, frequencies, or dichroism of the amide I absorption bands (Table 2). Changing the bulk phase refractive index (n_3) from 1.00 (air) to 1.32 (D_2O), however, changes the electric field amplitudes in both the second and third phase (Table 1). Equations that properly account for these changes should, therefore, indicate that the polypeptides remain parallel to the crystal surface. Contrary to this expectation, the thin-film approximation suggests that D_2O immersion changes the orientation of amide I transitions in the polypeptide from perpendicular to parallel with respect to the crystal surface, whereas the two-phase approximation yields order parameters that are virtually unchanged (Table 2). As in the previous test, the thickness dependent equations for phase 3 yield results that are virtually identical to the two-phase approximation, and those for phase 2 yield results that are virtually identical to the thin-film approximation. Again, these results suggest that these polypeptides are most accu-

TABLE 1 Mean square electric field amplitudes

		Thickness dependent equations		Approximations	
Supported phases	Field component	Second phase	Third phase	Two-phase	Thin-film
Silane/air (Fig. 1 *A*)	$\langle E_x^2 \rangle$	1.98	1.97	1.99	1.99
	$\langle E_y^2 \rangle$	2.11	2.10	2.13	2.13
	$\langle E_z^2 \rangle$	0.57	2.26	2.28	0.59
Silane/D_2O (Fig. 1 *B*)	$\langle E_x^2 \rangle$	1.95	1.94	1.97	1.97
	$\langle E_y^2 \rangle$	2.22	2.21	2.24	2.24
	$\langle E_z^2 \rangle$	1.98	2.48	2.52	1.99
Silane/lipid/D_2O (Fig. 1 *C*)	$\langle E_x^2 \rangle$	1.93	1.93	1.97	1.97
	$\langle E_y^2 \rangle$	2.19	2.19	2.24	2.24
	$\langle E_z^2 \rangle$	1.97	2.48	2.52	1.99

TABLE 2 Calculated values of $S(R_z)$ for amide I

Experimental results			Thickness-dependent expressions		Approximations	
System	Absorption maximum (cm^{-1})	R_z*	Phase 2	Phase 3	Two-phase	Thin-film
PBA#-air	1664	1.40	0.22	−0.23	−0.23	0.24
PBA-air	1655	1.60	0.32	−0.14	−0.14	0.37
PBA-D_2O	1655	1.60	−0.06	−0.14	−0.13	−0.02
PBA/lipid/D_2O	1655	1.65	−0.04	−0.12	−0.12	−0.00
PBG-air	1652	1.20	−0.02	−0.33	−0.33	−0.02
PBG/lipid/D_2O	1652	1.23	−0.26	−0.30	−0.30	−0.25

* Experimental uncertainty is less than ±0.05 for three to five measurements in each system.
# Evaporated from methylene chloride to yield a left-handed α-helical form.

rately treated when they are considered to be subject to electric fields in the third (bulk) phase.

Because the results of both preceding tests may be interpreted as indicating merely that the polypeptides are physically situated within the third phase and thus are not under the influence of fields within the second (acyl-silane) phase, another test of these expressions was performed in which we attempted to position the polypeptide unequivocally within the acyl-chain milieu of the second phase. This was done by applying a 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) monolayer at 36 dyne/cm to the silanized crystal surface on top of adsorbed PBG or PBA using the Langmuir-Blodgett technique (Axelsen et al., 1995a). We assume that this results in a supported planar lipid monolayer with helical polymer situated among the hydrophobic acyl chains of the lipid and the alkyl-silane (Fig. 1 *C*). Electric field amplitudes were again calculated by treating the silane/polypeptide/lipid as a single intermediate phase, and assuming $h = 50$ Å, $n_3 = 1.32$ (Table 1).

According to the thickness-dependent equations, electric field amplitudes change little upon increasing h from 25 to 50 Å. The two-phase and thin-film expressions are thickness-independent, and therefore they do not change for these experimental circumstances. The results, listed in Table 2, indicate that the two-phase expressions yield order parameters for PBA and PBG under these conditions, which are consistent with those determined in air and in D_2O. The thin-film approximation also yields an order parameter for polypeptides under the POPC monolayer that is similar to that found in D_2O, but the result differs substantially from the order parameter derived using the two-phase approximation. The thickness-dependent expressions yield results that are similar to those in the previous two tests, and the results overall are again consistent in suggesting that the amide I dichroism of these polypeptides is most accurately analyzed by using electric field values pertaining to the third (bulk) phase.

The orientation of the amide I transition moment with respect to the α-helical axis, θ, was evaluated using the order parameters listed in Table 2. Under these conditions, $\gamma = 90°$ by definition, and $S_m = -0.5$. Given values of $S(R_z)$ from a measured dichroic ratio and the different approaches used to calculate electric field amplitudes, we calculated $\langle P_2(\cos\theta)\rangle$ using Eq. 2. Although it is unlikely that $\langle P_2(\cos\theta)\rangle = P_2(\cos^2\langle\theta\rangle)$ (Axelsen et al., 1995b), it is of value to calculate $\langle\theta\rangle$ by this means to compare our PATIR-FTIR data with the earlier results for PBG of Miyazawa and Blout (1961), and for PBA by Bradbury et al. (1962) and Tsuboi (1962), using transmission-mode techniques. These results are listed in Table 3. The two-phase approximation and thickness-dependent expressions pertaining to the third phase yield values of $\langle\theta\rangle$ that are consistent with those reported earlier for PBG and PBA. The thin-film approximation, and thickness-dependent expressions pertaining to the second phase, yield values that are much higher than previously reported and results that are plainly unphysical in the case of PBA as a right-handed helix.

DISCUSSION

Our results suggest that the thin-film approximation is not suitable for studies of supported lipid membranes by PATIR-FTIR spectroscopy. This approximation yields results that are extremely unlikely and, in the case of PBA as a

TABLE 3 Calculated values of $\langle\theta\rangle$ for amide I

	Thickness-dependent expressions		Approximations		
System	Phase 2	Phase 3	Two-phase	Thin-film	Reported values
PBA*-air	79	37	39	79	33–41#
PBA-air	§	44	43	§	
PBA-D_2O	50	44	43	50	
PBA/lipid/D_2O	51	45	45	51	
PBG-air	54	28	28	54	28–34¶
PBG/lipid/D_2O	36	31	31	35	

* Evaporated from methylene chloride to yield a left-handed α-helical form.
# Miyazawa and Blout (1961).
§ No possible value.
¶ Bradbury et al. (1962).

right-handed helix, results that are plainly unphysical. Surprisingly, the two-phase approximation yields results that are more plausible, and we cannot improve on either approximation by employing more rigorous expressions that explicitly consider membrane thickness and absorption.

The data in Table 1 show that all four approaches yield similar results for the tangential (x and y) components of the electric fields, and that the discrepancies are due solely to differences in the calculated value of $\langle E_z^2 \rangle$. Because the two-phase approximation and the thin-film approximations differ only in the presence of n_{32}^4, a term in the z component of the latter, differences between the two-phase and thin-film approximations diminish as $n_3 \rightarrow n_2$. Accordingly, one finds that the discrepancies are considerably greater when the bulk phase is air rather than water, because $n_{\text{lipid}} = 1.40$ (assumed) is much closer to $n_{D_2O} = 1.32$ than it is to $n_{\text{air}} = 1.00$. Nevertheless, the discrepancy remains substantial, even in the case of water (Tables 1 and 2).

Our conclusions depend critically on correct assumptions about the conformation and orientation of our polypeptide samples. There are at least five reasons to believe that these assumptions are correct. First, the amide I absorption maxima in Fig. 2, *A* and *B*, at 1652 and 1655 cm^{-1}, are consistent with the earlier reports of Miyazawa and Blout (1961) and Tsuboi (1962) and the x-ray diffraction studies performed on similar preparations by other investigators (e.g., Perutz, 1951), indicating that these adsorbed polymers are α-helical. Likewise, in Fig. 2 *C*, amide I is shifted to 1664 cm^{-1}, consistent with the formation of left-handed α-helices (Bradbury et al., 1968; Giancotti et al., 1972). Second, the position, shape, and width of the amide I band do not change to any detectable degree when we immerse the surface-adsorbed polypeptides in D_2O or place them under a POPC monolayer. This indicates that polymer conformations do not change when the bulk phase is changed from air to water.

Third, in separate experiments, we placed these samples in the spectrometer so that the surface of the crystal was oriented perpendicular to the IR beam path. Transmission spectra collected with the sample in this configuration, and with the beam polarized in the xz and yz planes, revealed no amide I dichroism in the x-y plane. This verified that these polypeptides were oriented isotropically, as is required for analyses using Eq. 1. This examination also verified that the polypeptides are dispersed more or less uniformly over the crystal surface, and that they have not aggregated together at the point from which the solvent last evaporated. Fourth, the polypeptides were applied in quantities that cover less than 10% of the crystal surface. This leaves most of each molecule in direct contact with the crystal surface and, thus, parallel to the surface. Fifth, Takeneka et al. (1980) and Takeda et al. (1981) applied poly-γ-methyl-L-glutamate and PBG, respectively, to the surface of a germanium crystal using a Langmuir-Blodgett technique, and demonstrated that these polypeptides were oriented parallel to the crystal surface using the two-phase approximation as described by Higashiyama and Takenaka (1974). This was true not only for a single continuous monolayer, but also for multilayer preparations (note that this approach was not suitable for our experiments, because the process of applying a monolayer by withdrawing the crystal from a Langmuir-Blodgett trough appears to impart some degree of anisotropy to the polymer chain orientation in the x-y plane).

We believe that electric field amplitude calculations fail within the lipid membrane because of an incorrect assumption and an oversimplification regarding the optical properties of the milieu in which the polypeptides reside. The oversimplification in these approaches is that the second phase is a single homogeneous phase. Banga et al. (1995), in questioning the suitability of the thin-film approximation for PATIR-FTIR studies, focused on the optical consequences of a 50-Å-thick layer of silicon oxide on the surface of their silicon internal reflectance element. In air, an oxide layer quickly forms on crystalline germanium as well and serves as the chemical anchor for the acyl-silane coating. Although we do not know its thickness or its optical properties, the oxide layer is undoubtedly present in all three of the experimental circumstances used in this work. Indeed, the properties of this oxide layer are completely ignored by most investigators, yet it is likely to be thicker than the applied acyl-silane layer and the POPC monolayer combined.

The optical heterogeneity of the interface in these systems is further complicated by stratified layers composed of the acyl-silane reagent and the polar phospholipid headgroups. More rigorous analyses of electric fields in multiple strata are not feasible because values for their thickness and optical density are not available. Furthermore, Anderson and Hansen (1977) suggest that surface roughness may cause the "effective" refractive index near an interface to vary continuously (as opposed to discontinuously). A truly rigorous set of expressions must also account for anisotropic optical properties within some of these phases (e.g., Flournoy and Schaffers, 1966), because the effective optical density almost certainly varies with orientation in systems that are highly ordered. For this reason, it is not clear that simply considering $n > 3$ phases using Hansen's approach would be effective at alleviating the problem. We have performed a four-phase calculation in which the polypeptide is treated as a separate and distinct phase situated between the acyl-silane layer and the bulk phase. Varying the refractive index and thickness of the acyl-silane phase over a wide range made no profound difference in the calculated electric field amplitudes in the polypeptide layer as compared to the calculations performed with the three-phase model (results not shown).

Anderson and Hansen (1977) have considered the consequences of treating an interface composed of multiple anisotropic strata as an idealized single film. Using the thickness-dependent equations, they have shown in such cases that optical constants may be chosen that accurately describe certain aspects of the real physical behavior of the interface. With this approach, of course, the optical "constants" become arbitrary fitting parameters that are no longer related in any direct way to the bulk phase optical properties of the

strata components. This is not a particularly useful approach for our purposes because the best "fit" is provided by setting $n_2 = n_3$, not by setting n_2 to an arbitrary constant value.

The incorrect assumption that probably contributes to the failure of electric field amplitude calculations within the lipid membrane is that macroscopic electromagnetic theory can accurately describe electromagnetic fields within a few angstroms of a surface. The weakness of this assumption has been discussed at length (e.g., Bagchi et al., 1979; Sipe, 1980; Feibelman, 1982; Chabal, 1988), and it is a particular concern in our experiments, where the film thickness is roughly 25–50 Å, whereas the wavelength of IR radiation is on the order of 60,000 Å (i.e., $h \ll \lambda$). Macroscopic treatments of electric fields view the medium as a continuum, and variations in the field are averaged over both time and space. It is less than rigorous to treat a thin dielectric film in this manner because, on a microscopic scale, relationships between electric displacement, current density, and electric fields are nonlocal in nature (Bagchi et al., 1979; Sipe, 1980; Feibelman, 1982; Chabal, 1988). Simple expressions for $\langle E_z^2 \rangle$, based solely upon a local treatment of refraction at a dielectric surface, ignore other components of the chemical environment with the potential to influence $\langle E_z^2 \rangle$ in a membrane. In this sense, our data suggest that a lipid membrane is too thin to be the sole determinant of electric field amplitudes within itself, and that amplitudes within the membrane may be influenced to a large extent by properties of the adjacent bulk phase. This is the essence of the two-phase approximation, and we suspect that this is the reason it succeeds when attempts to determine field amplitudes within the membrane do not. However, we must emphasize that this is merely a conjecture based on the reasonableness of the results we obtain using the two-phase approximation, because electromagnetic theory does not provide a better solution at this point. We acknowledge that the thickness-dependent expressions are mathematically correct on a macroscopic scale; the data merely suggest that they are physically inapplicable to microscopic analyses within a thin supported monolayer or bilayer.

There are a large number of published PATIR-FTIR data based on the thin-film approximation that should be reexamined in light of these results. For example, in Axelsen et al. (1995b), we reported order parameters for two polypeptides and two types of lipid acyl chains. We assumed that the two polypeptides formed transmembrane helices, highlighted our uncertainty over the value of n_2, and examined the impact of this uncertainty on the interpretation of our results. Although we briefly considered whether two-phase expressions were more appropriate, all analyses therein were based on the thin-film approximation.

A reanalysis of our earlier results using the two-phase approximation indicates that the segmental acyl chain order for 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine (8:1 with gramicidin) in a supported monolayer must be revised from 0.49 to 0.73, and the amide I transition order for gramicidin must be decreased from 0.33–0.43 to 0.24. We estimated that the multibilayers used in our earlier work were 240 Å thick (based on our estimate for the number of bilayers in the preparation). We have not yet developed a means of testing the accuracy of electric field amplitude calculations for systems of this thickness; however, 240 Å is still orders of magnitude smaller than λ, and thus the two-phase approximation may still be applicable. For 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine multibilayers with Lys_2-Leu_{24}-Lys_2 (L_{24}), the two-phase approximation suggests that the segmental acyl chain order must be increased from 0.49 to 0.86. The most noteworthy result of this reanalysis, however, is that the transition order for L_{24} becomes 0.00 when the two-phase approximation is used.

L_{24} was designed to model a membrane-spanning helix such as that found in glycophorin. The actual transmembrane segment of glycophorin in a single bilayer preparation was studied using PATIR-FTIR spectroscopy by Smith et al. (1994). These investigators reported a dichroic ratio of 2.14 and interpreted this result using the thin-film approximation to mean that this segment assumes an ordered transmembrane orientation. Using the two-phase expressions, however, we obtain $S = 0.04$ for the amide I transition, and $S = 0.76$ for the segmental acyl chain order. These values suggest that the polypeptide orientation is virtually isotropic with respect to a much better organized lipid membrane, and this differs substantially from the original conclusion. In these studies of the glycophorin segment, and our studies of L_{24}, the polypeptides are added to the lipid in a disordered state. Our revised analysis suggests that the ordering principle that imparts a high degree of order to the lipid acyl chains under these conditions does not also cause these polypeptides to become ordered. Under different (e.g., fully solvated individual liposomal bilayers) circumstances, it remains entirely possible—even quite likely—that both L_{24} and glycophorin are helical and highly ordered in a transmembrane orientation. For example, it has been observed that the amide I dichroism of L_{24} in multibilayers containing anionic lipids undergoes a marked increase upon hydration (R_z from 1.1 → 3.9; J. Blazyk, personal communication). This strongly suggests that L_{24} reorients upon hydration to its expected orientation in the presence of anionic lipids, although we did not observe this change upon hydrating pure POPC multibilayers. These results illustrate a serious potential pitfall in PATIR-FTIR studies of multibilayer preparations.

There is a large body of PATIR-FTIR work on multibilayer preparations, but at least five factors make it difficult to interpret the data. First, the thickness of the multibilayer phase is not known. Second, we do not know how thick the multibilayer preparation must be to render effects of the bulk phase on field amplitudes within the multibilayer phase negligible. Third, even if the multibilayer preparation is quite thick (i.e., $h > \lambda$), so that the preparation is, ostensibly, a two-phase system, it will no longer be valid to assume that the field amplitudes are constant throughout the membrane. Fourth, we have an imprecise knowledge of n_2 for hydrated lipid multibilayers. Finally, the thin-film approximation is particularly inaccurate when the bulk phase is air.

In light of these ambiguities, we do not know whether it is more appropriate to apply the two-phase or the thin-film approximation to multibilayer studies. Nonetheless, it is valuable to consider the implications of the two-phase approximation. For example, Lüneberg et al. (1995) reported a dichroic ratio of 1.48 for the fusion peptide from an influenza virus, assumed that $\theta = 27°$, and used the thin-film approximation to conclude that this 20-residue peptide situated at a 45° angle in the membrane. Although we do not endorse this practice of interpreting PATIR-FTIR results in terms of an orientation angle (Axelsen et al., 1995b), we infer from this conclusion that these investigators obtained $S_m = 0.25$ and $S(R_z) = 0.36$, which suggests that the fusion peptide is preferentially oriented perpendicular to the membrane surface. If the multibilayer film is negligibly thin, the two-phase approximation yields $S(R_z) = -0.19$, assuming $n_3 = 1.00$. If the multibilayer film is sufficiently thick (i.e., $h > \lambda$) so as to constitute a bulk second phase with $n = 1.45$, the result changes only slightly, to $S(R_z) = -0.17$, and $S_m = -0.25$ to -0.27 for $\theta = 27°$. This analysis indicates that the fusion peptide lies preferentially in the plane of the membranes, irrespective of our assumptions about effective refractive index or bilayer thickness.

Likewise, for the fusion peptide of a wild-type HIV protein (gp41), Martin et al. (1996) report a dichroic ratio of 1.3 and conclude that it assumes an orientation in a membrane that is similar to that of the previous example. Using the two-phase approximation, this result corresponds to $S(R_z) = -0.25$ or -0.28, and $S_m = -0.36$ or -0.41 for $\theta = 27°$ (the two values again corresponding to membrane preparations in which $h \ll \lambda$ or $h > \lambda$ and $n_3 = 1.00$ or 1.45, respectively). This suggests that the fusion peptide in this preparation again lies preferentially in the plane of the membranes—to an even greater degree than in the first example. It remains possible that the thin-film approximation is accurate for some intermediate value of h. At this point, however, we have no data to support or refute this possibility, and no applicable theory.

The 26-residue membrane active polypeptide melittin has been the subject of PATIR-FTIR studies in supported monolayers, bilayers, and multibilayers. Frey and Tamm (1991) reported dichroic ratios of 1.39–1.42 for the amide I transition of melittin in supported POPC/POPG bilayers, corresponding to $S(R_z) = -0.22$ using the two-phase approximation. The dichroic ratio of 1.47 reported in Axelsen et al. (1995a) for supported monolayers yields a similar transition order of -0.19. Thus, the general conclusion that melittin lies oriented in the plane of the membrane seems valid for these preparations, although conclusions about the degree of order must be revised. In contrast, the original studies of melittin in dried lipid suspensions (Vogel et al., 1983) and in multibilayers (Brauner et al., 1987; Frey and Tamm, 1991) concluded that it was aligned more nearly perpendicular to the membrane. Again, applying the two-phase approximation (despite uncertainty as to the bilayer thickness and its effects) to the data of Vogel et al. (1983) yields $S(R_z) = -0.21$ for an Irtran support and $\phi = 60°$, whereas the dichroic ratios of 1.20–1.46 reported by the latter groups yield $S(R_z)$ ranging from -0.27 to -0.36. Thus, applying the two-phase approximation to all of the available PATIR-FTIR data indicates that melittin is predominantly aligned parallel to the plane of the membrane, irrespective of the type of the preparation.

The values of $\langle\theta\rangle$ we obtain for PBA and PBG are of interest for two reasons. First, they confirm the estimates of $\langle\theta\rangle$ made by earlier investigators using dispersive instrumentation in a transmission mode. This agreement constitutes additional support for the validity of the two-phase approximation. Second, they help clarify the differences found in PBG and the two forms of PBA by providing values of $\langle\theta\rangle$ for both polypeptides under the same conditions. Our results clearly demonstrate a substantial difference in the effective value of $\langle\theta\rangle$ for PBA and PBG, despite the small difference between their side chains. This demonstration serves to highlight one of several difficulties involved in the calculation and interpretation of molecular order parameters by PATIR-FTIR: we are currently unable to predict the value of $\langle\theta\rangle$ for a polypeptide helix. Even if a precise value were known, however, additional difficulties remain, because small differences in the calculated order cannot be assigned unique interpretations (Axelsen et al., 1995b).

SUMMARY

The thin-film approximation, while possibly appropriate for relatively thick films, appears to be inappropriate for PATIR-FTIR studies of supported lipid monolayers and bilayers. The two-phase approximation, on the other hand, yields results that are self-consistent, and consistent with those of much earlier investigators using different techniques. Furthermore, the two-phase approximation helps rationalize conflicting data in the literature for peptides such as melittin, and enables us to revise earlier conclusions drawn using the thin-film approximation. In one case, the revised conclusions reveal a serious pitfall to which multibilayer preparations are subject when studying peptides that are expected to assume a transmembrane orientation.

The present work does not rigorously explain the failure of the thin-film approximation or the success of the two-phase approximation. The supported membrane is negligibly thin compared to the wavelength of the light and the penetration depth of the evanescent electric field. For this reason we suggest—but cannot prove—that electric field amplitudes within the membrane are determined more by dielectric properties of the adjacent bulk phase than by properties of the membrane itself. The development of theory to account for nonlocal influences on the amplitude of evanescent electric fields in thin films would be a valuable contribution to the study of membrane biophysics.

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