# On the use of deuterated phospholipids for infrared spectroscopic studies of monomolecular films: A thermodynamic analysis of single and binary component phospholipid monolayers

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# On the use of deuterated phospholipids for infrared spectroscopic studies of monomolecular films: a thermodynamic analysis of single and binary component phospholipid monolayers

David D. Baldyga, Richard A. Dluhy *

*Department of Chemistry, University of Georgia, Athens, GA 30602-2556, USA*

## Abstract

A thermodynamic study of monolayer mixing behavior was performed using binary lipid mixtures of DPPC + DOPG (7:1 mol:mol) as well as a mixture containing the phosphocholine acyl chain perdeuterated analog of DPPC, DPPC-$d_{62}$ (i.e. 7:1 mol:mol DPPC-$d_{62}$ + DOPG). An analysis was performed on these isotherms that calculated the Helmholtz excess free energy of mixing to determine potential thermodynamic differences in the fully protiated DPPC:DOPG monolayer versus the identical monolayer substituted with acyl chain perdeuterated DPPC (i.e. DPPC-$d_{62}$). The main conclusions of this study are that: (1) the temperature-dependent thermodynamic phase behavior of DPPC-$d_{62}$ differs substantially from that of DPPC, and these differences appear magnified when these molecules are studied as monomolecular films at the A/W interface as opposed to bulk phase systems; and (2) the calculated excess free energy of mixing values, $\Delta F_{xs}(A)$, for the deuterium-containing 7:1 DPPC-$d_{62}$:DOPG binary monolayer film show a type of near-ideal mixing behavior above 20°C that is consistent with the 'squeezing-out' of the DOPG component in the binary mixture containing the DPPC-$d_{62}$ component. This type of behavior does not occur in the graph of the excess free energy of mixing for the fully protiated 7:1 DPPC:DOPG binary monolayer film. The use of acyl chain perdeuterated DPPC (i.e. DPPC-$d_{62}$) in binary monomolecular mixtures with other phospholipids produces a real and measurable difference on the thermodynamic properties of the monolayer when compared to the case of the fully protiated monomolecular film. In particular, the presence of DPPC-$d_{62}$ in a 7:1 mol:mol DPPC-$d_{62}$:DOPG binary monomolecular film may over-state the fluid nature of the monolayer at any given temperature, thereby leading to an over-estimate of the amount of material potentially 'squeezed out' of the monomolecular film. These results have implications for a commonly used IR spectroscopic method that relies on the

incorporation of deuterium-labeled phospholipids into monolayers for vibrational spectroscopic analysis of individual components in a multicomponent monomolecular film. © 1998 Published by Elsevier Science Ireland Ltd. All rights reserved.

## 1. Introduction

For many years vibrational spectroscopy (both infrared and Raman) has been used to study biological membrane structure (for reviews of the application of vibrational spectroscopy in this area (Casal et al., 1984; Levin et al., 1984; Mendelsohn et al., 1986; Dluhy et al., 1995). Vibrational spectroscopy has several unique spectroscopic properties that make this method especially valuable in the study of membrane biophysics. Both IR and Raman vibrations depend upon changes in conformation and configuration of the bonds making up the normal mode of vibration. Also, unlike other spectroscopic methods, vibrational spectroscopy has the advantage of noninvasively monitoring absorptions due to all regions of the lipid molecule. Finally, the time scale of the vibrational experiments ($\sim 10^{-12}$ s) assures that the interpretation of experimental results is not complicated by time scale averaging of anisotropic motions.

Much of the previous work in the area of biomembrane vibrational spectroscopy has shown that the C–H stretching region of phospholipid vibrational spectra has great utility in determining lateral packing order as well as conformation and mobility of the hydrocarbon chains. In studies of lipid mixtures, however, the C–H region produces an overall band profile that reflects the weighted contributions of all components in the mixture. The resulting spectra therefore reflect the average behavior of the whole system and information concerning individual components is lost.

A method that circumvents this problem was first used for bulk phase hydrocarbon mixtures in the 1970s (Mendelsohn et al., 1976, 1980). This method relies on the isotopic substitution of $^2$H for $^1$H in the acyl chains of phospholipids, which results in a shift of the C–D stretching modes to lower frequency, the magnitude of the shift depending upon the ratio of the square root of the reduced masses, as predicted by the harmonic oscillator model. The spectral window of the C–D stretching region (2000–2200 cm$^{-1}$) is free from interference from other lipid bands or most other non-lipid components, therefore the C–D modes directly sense only the structural alterations in the deuterated environment.

For bulk phase phospholipids, the incorporation of perdeuterated fatty acids into the acyl chains of phospholipids results in minimum thermodynamic perturbation to the molecule. Previous Raman and IR studies have shown that DPPC-d$_{62}$ behaves essentially identically to the normal, protiated DPPC molecule (Bunow et al., 1977; Gaber et al., 1978; Sunder et al., 1978) with the only reported difference being a 5°C reduction in its phase transition temperature relative to protiated DPPC. In addition, bulk phase mixtures containing deuterated phospholipids have been demonstrated to exhibit identical phase diagrams to normal, protiated mixtures, indicating that the use of a deuterated component does not significantly disrupt the thermodynamics of the binary mixture (Mendelsohn et al., 1980; Dluhy et al., 1985).

While bulk phase studies are important, in recent years it has become progressively more obvious that many important biological processes occur at interfaces. Biophysical monomolecular films have received increased attention as sensors and in other 'soft matter' materials science applications. As a consequence, there has been an interest in the use of monomolecular manipulation techniques and surface-sensitive spectroscopic methods for the study of biophysical monolayer systems. Recent advances in instrumentation and methodology have enabled IR and Raman spectroscopies to

become true surface-sensitive analytical methods for thin films and monolayers (Dluhy et al., 1995). These new surface-sensitive vibrational techniques are being increasingly applied to the study of biophysical monolayers.

As the use of IR spectroscopy has been extended to the study of biophysical monomolecular films, researchers have also begun to incorporate synthetic, isotopically labeled deuterated lipids into the monolayer film systems under study. The advantage of the use of deuterated compounds in the study of monomolecular films is exactly the same as for bulk phases, namely, the ability to spectroscopically analyze individual constituents in multicomponent mixtures. This method has been particularly applied to binary monomolecular films of acyl chain perdeuterated DPPC (i.e. DPPC-$d_{62}$) in complexes with other defined protiated lipids as specific biomembrane models (Rana et al., 1993; Pastrana-Rios et al., 1994).

Although the use of deuterated phospholipids in IR studies of biophysical monomolecular films has increased in recent years, there has not been a systematic study of the underlying thermodynamic assumptions inherent in this experiment, namely that the incorporation of a deuterated phospholipid as a component in a multicomponent monolayer film results in a minimum thermodynamic perturbation to the system. The goal of the research described in this paper is to test that hypothesis using a thermodynamic analysis of the temperature-dependence of the isotherms obtained from two separate binary monomolecular phospholipid films. The specific binary phospholipid monolayer systems studied here are DPPC:DOPG (7:1 mol:mol) as well as a mixture containing the phosphocholine acyl chain perdeuterated analog of DPPC, DPPC-$d_{62}$ (i.e. 7:1 mol:mol DPPC-$d_{62}$:DOPG).

## 2. Materials and methods

### 2.1. Acquisition of the $\pi$-A isotherms

The phospholipids used for this study were synthetic and obtained from Avanti Polar Lipids (based in Alabaster, AL) at 99% stated purity. No further purification of these phospholipids was performed. Approximately 1–2 mg/ml solutions of phospholipid were prepared with high performance liquid chromatography (HPLC) grade chloroform ($CHCl_3$) used as the solvent. The concentration of these phospholipid solutions was then quantified by an inorganic phosphorus assay (Chen et al., 1956) that involved acid-digestion of portions of the phospholipid solutions. The quantified solutions were then stored at $-20°C$.

A 50 $\mu$l Hamilton syringe was used to deliver 20–40 $\mu$l of a phospholipid solution to a 150 mM NaCl subphase, pH 5.6, using a Joyce-Loebl (Gateshead, UK) film balance. The $\pi$-A isotherms were digitally recorded as the film was compressed. A temperature bath that circulated water within the walls of the trough held the temperature of the subphase constant to $\pm 0.5°C$. The temperatures at which the $\pi$-A isotherms were measured were 15, 20, 25, and 30°C. After the raw $\pi$-A data were acquired, the thermodynamic values were calculated from these $\pi$-A isotherms by Lahey FORTRAN 77 (with FORTRAN 90 features) programs. The specific computer source code used in this analysis has been presented separately (Baldyga et al., 1997).

## 3. Results and discussion

### 3.1. The $\pi$-A isotherms of DPPC and DPPC-$d_{62}$

All $\pi$-A isotherms, graphed in Fig. 1, are averages of three $\pi$-A isotherms that were measured at the indicated temperature. The relative standard deviations of the surface pressures are estimated to be $\pm 5\%$. For the sake of legibility, error bars were not included in the figures. All the surface pressure, isotherm and thermodynamic data in the figures contained in this manuscript have been tabulated with their associated errors and are presented elsewhere (Baldyga et al., 1997).

The $\pi$-A isotherms for DPPC obtained at temperatures of 15, 20, and 25°C are graphed in Fig. 1A. In comparison, the $\pi$-A isotherms for DPPC-$d_{62}$ obtained at temperatures of 15, 20, 25, and 30°C are graphed in Fig. 1B. Both DPPC and DPPC-$d_{62}$ achieve a surface pressure of 70 mN/m,

which is expected for saturated phospholipid films. However, it is evident from Fig. 1 that the temperature-dependence of the DPPC-$d_{62}$ $\pi$-A isotherms vary significantly compared to those for DPPC, with a uniformly higher surface pressure for DPPC-$d_{62}$ compared to the fully protiated DPPC at any given surface pressure and tempera-



Fig. 1. (A) The $\pi$-A isotherms for DPPC at 20°C ($\bigcirc$), 25°C ($\bullet$), and 30°C ($\triangle$). (B) The $\pi$-A isotherms for DPPC-$d_{62}$ at 15 ($\blacksquare$), 20 ($\bigcirc$), 25 ($\bullet$), and 30°C ($\triangle$).

ture, thus giving rise to the possibility that molecular interactions in DPPC-$d_{62}$ films can vary significantly from those in DPPC monolayers.

### 3.2. Alternative thermodynamic analyses of single-component DPPC $\pi$-A isotherms

There exist two thermodynamic models in the literature that have been used to calculate monolayer thermodynamic quantities as a function of compression from $\pi$-A isotherms. The first model was proposed by Goodrich (Goodrich, 1957) and later expanded upon by Gaines (Gaines, 1966; Gaines et al., 1966). This model allows for the calculation of the Gibbs free energy of compression, $\Delta G_c(\pi)$, by use of the integral presented in Eq. (1).

$$\Delta G_c(\pi) = \int_{\pi_i}^{\pi_f} A(\pi) d\pi \tag{1}$$

Eq. (1) implies that the surface pressure, $\pi$, is the independent variable, controlled by the movement of the Langmuir trough barrier, and that the molecular area $A(\pi)$ is the dependent variable (i.e. the response function of $\pi$). This integral cannot be evaluated in closed form, but can be solved by using numerical methods (e.g. the trapezoidal rule) (Press et al., 1989). After the $\Delta G_c(\pi)$ values have been determined over a range of temperatures, both the entropy, $\Delta S_c(\pi)$, and enthalpy, $\Delta H_c(\pi)$ of compression can be determined by the following expression:

$$\Delta G_c(\pi) = \Delta H_c(\pi) - T\Delta S_c(\pi) \tag{2}$$

In Eq. (2), $T$ is the temperature in degrees Kelvin. A plot of $\Delta G_c(\pi)$ values obtained at several temperature versus $T$ values can be constructed, and a linear least-squares fit performed to find the best-fit straight line through the data points, yielding $-\Delta S_c(\pi)$ as the slope and $\Delta H_c(\pi)$ as the intercept.

The $\Delta G_c(\pi)$ values obtained for DPPC are displayed in Fig. 2A. These values were measured at every 5 mN/m, and are the average of three independent values measured at the same surface pressure. The values of $\pi$ reported in this figure and in the subsequent figures have an estimated standard deviation of $\pm 0.5$ mN/m. Fig. 2A indi-
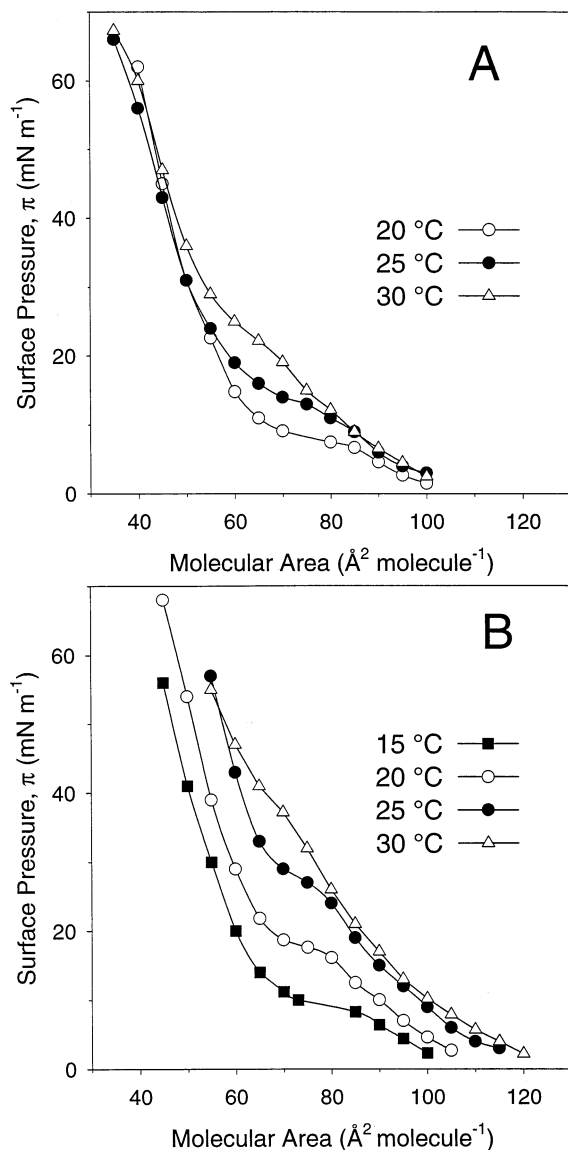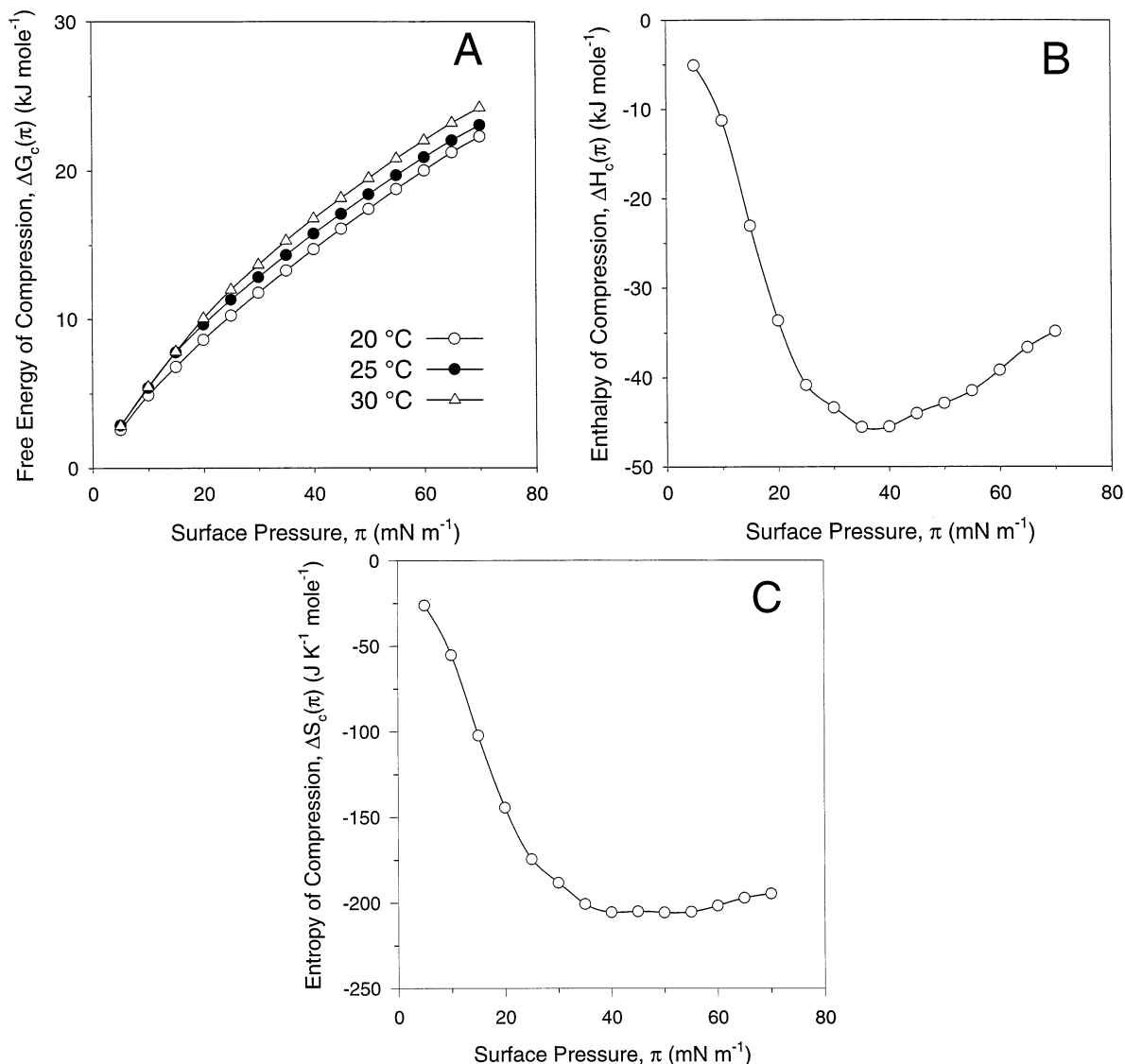
Fig. 2. (A) The $\Delta G_c(\pi)$ values for DPPC, calculated using the Goodrich–Gaines model. Values of $\Delta G_c(\pi)$ obtained at 20 ($\bigcirc$), 25 ($\bullet$), and 30°C ($\triangle$) are plotted here. (B) Values for the enthalpy of compression, $\Delta H_c(\pi)$, for DPPC monolayers, calculated using the Goodrich-Gaines model. Values of $\Delta H_c(\pi)$ were obtained from isotherms measured in the 20–30°C temperature range. (C) Values for the entropy of compression, $\Delta S_c(\pi)$, for DPPC monolayers, calculated using the Goodrich-Gaines model. Values of $\Delta S_c(\pi)$, were obtained from isotherms measured in the 20–30°C temperature range.

cates that the $\Delta G_c(\pi)$ values at any given temperature increase monotonically up to a limiting value of approximately 22 kJ/mol at 70 mN/m. In addition, the value of $\Delta G_c(\pi)$ at any given surface pressure also increases as the temperature is raised from 20 to 30°C. The quantities obtained here for DPPC at both 10 and 40 mN/m agree (to within

approximately $\pm$ 5%) with those previously found in the literature (Vilallonga et al., 1968).

The $\Delta H_c(\pi)$ and $\Delta S_c(\pi)$ values for DPPC were obtained over the temperature range 20–30°C by the use of Eq. (2). These values are plotted in Fig. 2B C, respectively. Both the entropy and enthalpy of compression decrease with increasing surface

pressure and reach a minimum after approximately 35 Mn/m, after which these values again rise. This effect likely has its origins in the diminution and disappearance of the DPPC monolayer phase transition after 30°C.

An alternative thermodynamic model for the analysis of monolayer compression has been proposed by Gershfeld (Gershfeld et al., 1970, 1972; Pagano et al., 1972), and used by Mingins et al. (1975) in the study of ionic monolayers. This formulation calculates $\Delta F_c(A)$, the Helmholtz free energy of compression, instead of $\Delta G_c(\pi)$ the Gibbs free energy of the previous model. The Gershfeld model is expressed by the following integral:

$$\Delta F_c(A) = \int_{A_f}^{A_i} \pi(A) dA \qquad (3)$$

In Eq. (3) $A_f$ or $A_i$ are the molecular areas up to which the film is compressed from infinite dilution. In this integral, $A$ is the independent variable, and $\pi(A)$ is the dependent variable (i.e. the response function of $A$). In terms of a typical $\pi$-$A$ isotherm, this is a $y$–$dx$ integration. As with the Goodrich model described above, this integral cannot be evaluated in closed form, but can be solved numerically.

After the $\Delta F_c(A)$ values have been calculated over a range of temperatures, both the entropy, $\Delta S_c(A)$, and enthalpy, $\Delta H_c(A)$, of compression can be calculated according to the following expression:

$$\Delta F_c(A) = \Delta H_c(A) - T\Delta S_c(A) \qquad (4)$$

Both $\Delta S_c(A)$ and $\Delta H_c(A)$ can be calculated from a linear fit of the $\Delta F_c(A)$ versus $T$ values in the same manner as described above for $\Delta S_c(\pi)$ and $\Delta H_c(\pi)$ using the previous model. The $\Delta F_c(A)$, $\Delta H_c(A)$ and $\Delta S_c(A)$ thermodynamic quantities for DPPC calculated as a function of compression according to the current model are graphed in Fig. 3A B, and C, respectively.

Due to the fact that these two thermodynamic models have different underlying assumptions and yield different results, we have investigated the assumptions behind both models, prior to the use of one in the analysis of multicomponent monolayer films.

### 3.3. Analysis of the Helmholtz free energy model

Gershfeld first proposed this model in 1970 (Gershfeld et al., 1970), and it has been used to analyze the entropies of compression in charged monolayers in a later paper (Mingins et al., 1975), which also gave a brief analysis of its thermodynamic derivation. The model begins by defining the free energies involved in creating the A/W interface in a monolayer compression experiment. Both the Helmholtz and Gibbs free energies of compression, under conditions of constant pressure, volume, and temperature are defined using Eq. (5) (Guggenheim, 1933).

$$dF = dG = \gamma dA \qquad (5)$$

In this case, $A$ is the molecular area and the independent variable, and $\gamma$ is the surface tension and also the dependent variable (i.e. the response function of $A$).

For a Langmuir–Adam film balance experiment, the system under study can be thermodynamically defined as being the A/W interface. The A/W interface in the film balance can further be defined as containing two separate sections. The first section is the monolayer film at the A/W interface confined within the compression barriers; the second section is the monolayer-free, pure A/W interface outside of the barrier that is created as the film is compressed. The work done by each section of the system as the film is compressed must be determined, with their sum total being the work done by the entire system. In the case of the film balance, the work done by the entire system is equivalent to the free energies involved (Guggenheim, 1933).

The work done to create the A/W interface with the film confined within the barrier when compressing the film from $A_i$ to $A_f$ ($A_i > A_f$) is represented by the integral in Eq. (6).

$$w = -\int_{A_f}^{A_i} \gamma(A) dA \qquad (6)$$

The sign of this integral is negative because the compression of the film is nonspontaneous, meaning that an external force (i.e. the barrier) must be used to perform positive work on this section of the system by compressing the film. Positive work
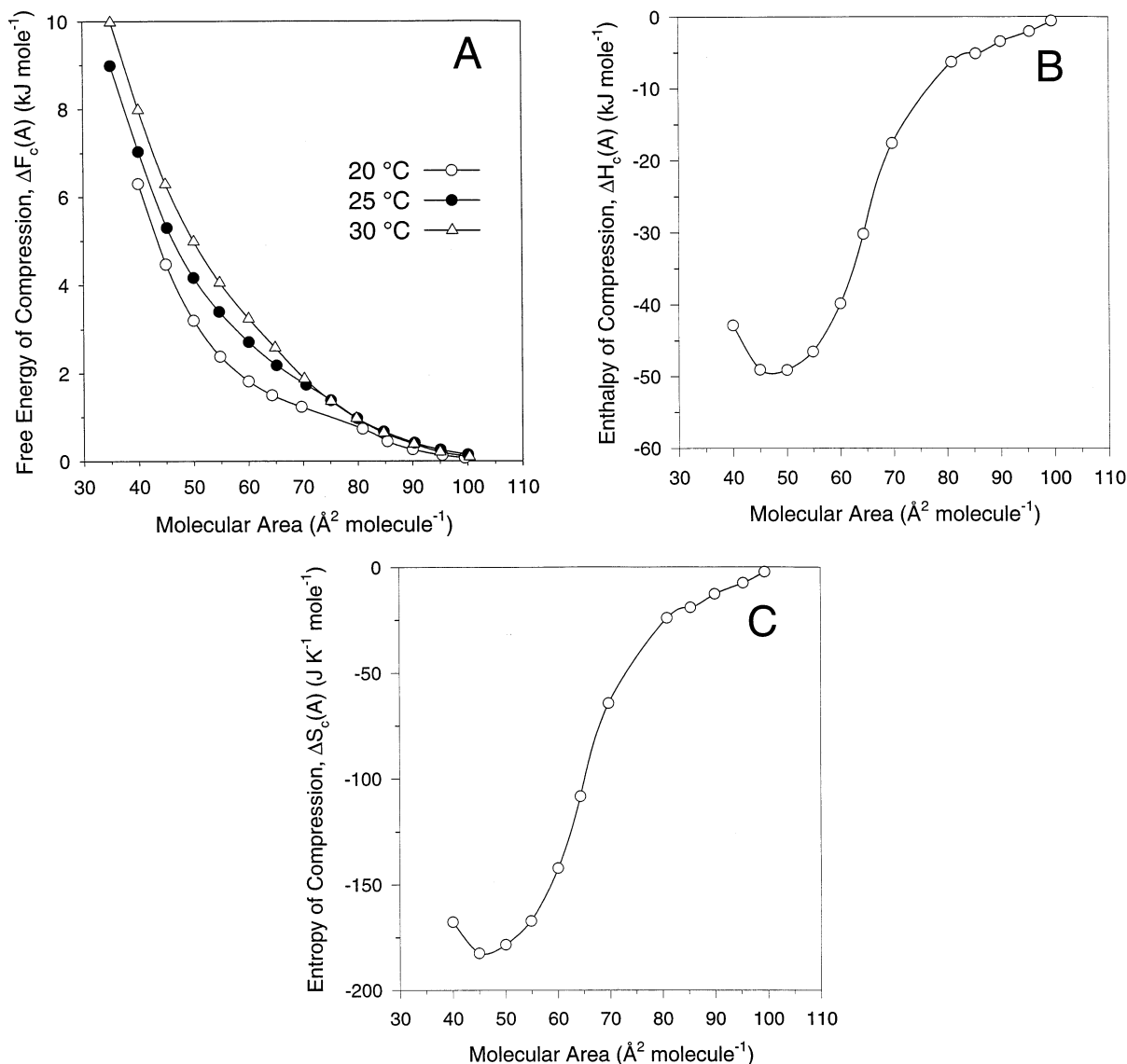
Fig. 3. (A) The Helmholtz free energies of compression, $\Delta F_c(A)$, for DPPC monolayers, calculated using the Gershfeld model. Values of $\Delta F_c(A)$ obtained at 20 (○), 25 (●), and 30°C (△) are plotted here. (B) Values for the enthalpy of compression, $\Delta H_c(A)$, for DPPC monolayers, calculated using the Gershfeld model. Values of $\Delta H_c(A)$ were obtained from isotherms measured in the 20–30°C temperature range. (C) Values for the entropy of compression, $\Delta S_c(A)$, for DPPC monolayers, calculated using the Gershfeld model. Values of $\Delta S_c(A)$ were obtained from isotherms measured in the 20–30°C temperature range.

done on the system corresponds to negative work done by the system. This integral is illustrated graphically in Fig. 4A.

As the film within the barrier is compressed, a new A/W interface outside the barrier is created,

and the system must expend energy (i.e. perform positive work) in order to create this interface. This work is represented by the integral in Eq. (7).

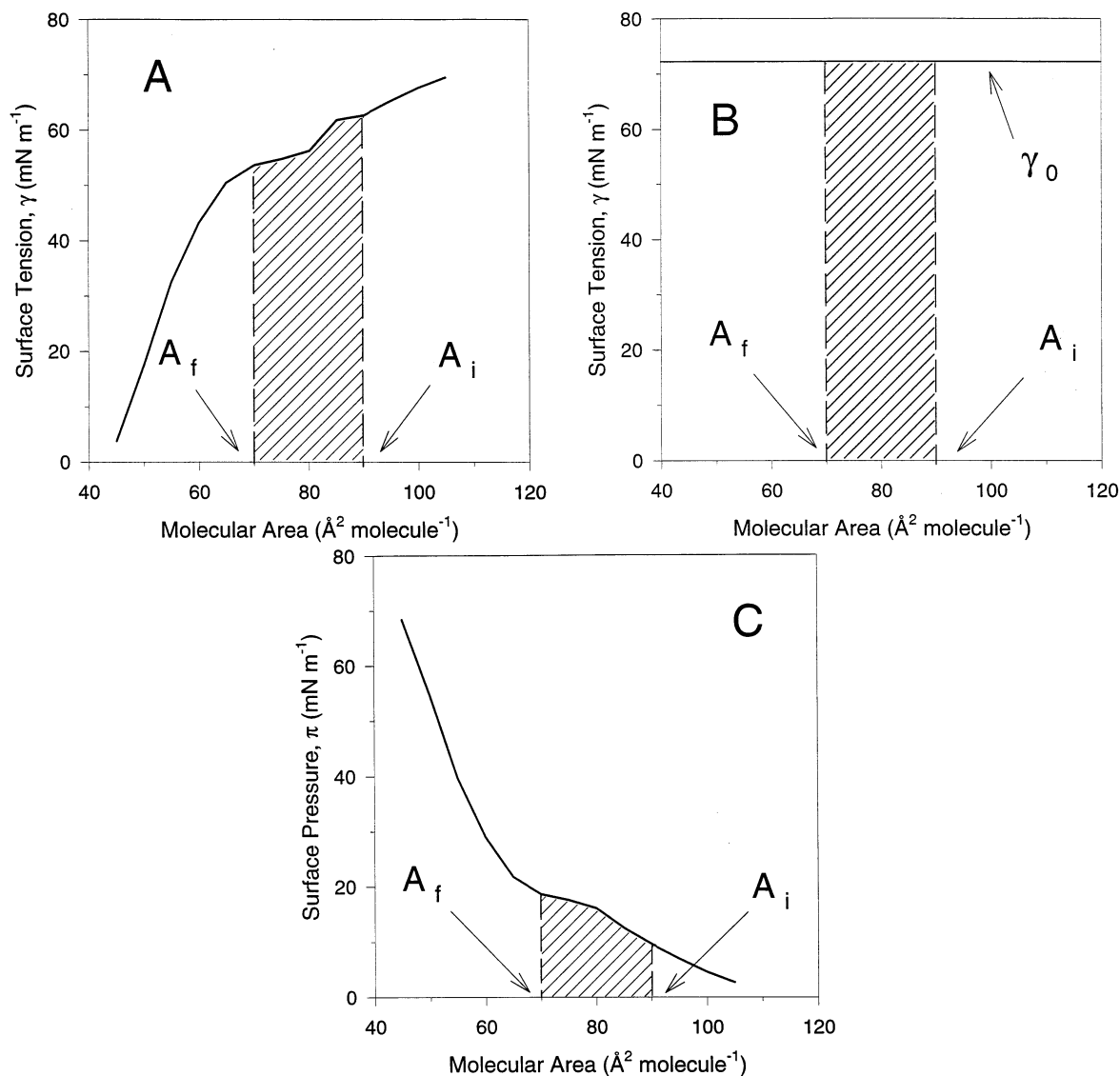$$w = \int_{A_f}^{A_i} \gamma_0(A) dA \tag{7}$$

Fig. 4. A graphical illustration of the Gershfeld method of isotherm integration. (A) A plot of the integral defined by the equation: $-\int_{A_f}^{A_i} \gamma(A)dA$, which illustrates the negative work done by the film as it is compressed. The crosshatched region represents the hypothetical region integrated between the molecular areas $A_i$ and $A_f$ by this integral. (B) A plot of the work done by the system to create the A/W interface outside of the barrier according to the integral: $\int_{A_i}^{A_f} \gamma_0(A)dA$, which illustrates the positive work done by the system to create the A/W interface outside of the barrier. The cross-hatched region represents the hypothetical region integrated between the molecular areas $A_i$ and $A_f$ by this integral. The parameter $\gamma_0$ represents the surface tension of water (72 mN/m). (C) The overall sum of the integrals presented in Fig. 4A, B, which illustrates the work on by thsis system, the Gershfeld model of monolayer thermodynamics, and is represented by the following integral: $\int_{A_f}^{A_i} \pi(A)dA$. The cross-hatched region represents the hypothetical region integrated between the molecular areas $A_i$ and $A_f$ by this integral.

In this Equation, $\gamma_0(A)$ is the surface tension of the A/W interface without the film. This integral is illustrated graphically in Fig. 4B.

The sum total of Eqs. (6) and (7) is equivalent to the work done by the entire system, and hence, to the free energies of compression involved. This is shown in Eq. (8).

$$w = \int_{A_f}^{A_i} (\gamma_0(A) - \gamma(A)) dA \tag{8}$$

The usual definition for surface pressure, $\pi(A)$, i.e. as the difference in surface tensions between a film-free and a film-covered interface, is presented in Eq. (9).

$$\pi(A) = \gamma_0(A) - \gamma(A) \tag{9}$$

Substitution of this definition for surface pressure into Eq. (8) results in Eq. (10), which is the final expression of the work done by this entire system, or the Helmholtz free energy of compression.

$$w = \int_{A_f}^{A_i} \pi(A) dA \tag{10}$$

Since we have already defined $w$, the work done to create the A/W interface with the film confined within the barrier as:

$$w = \gamma dA$$

(see Eq. (6)), hence $w$ may also be defined as

$$w = dF = dG$$

(see Eq. (5)). This, then, is the basis for this particular model of calculating the free energies of monolayer compression, which has the advantage of appearing consistent with the operational methods of a film balance, i.e. the movement of compression barriers (independent variable) resulting in the measurement of surface pressure (dependent variable). The integral in Eq. (10) is illustrated graphically in Fig. 4C.

### 3.4. Analysis of the Gibbs free energy model

The alternative method of defining monolayer thermodynamic properties uses values of the surface tension, $\gamma$, as the independent operational parameter (Goodrich, 1957; Gaines, 1966; Gaines

et al., 1966). If $\gamma$ is chosen as the independent variable, then the free energies of creating the A/W interface are defined as in Eq. (11).

$$dF = dG = -A d\gamma \tag{11}$$

The free energy done by the system as the film is compressed from $\gamma_i$, to $\gamma_f$ ($\gamma_i > \gamma_f$) is represented by the integral in Eq. (12).

$$dF = dG = -\int_{\gamma_i}^{\gamma_f} A(\gamma) d\gamma \tag{12}$$

The value of this integral is negative, and is illustrated graphically in Fig. 5A.

Next, the free energy done to create the A/W interface without the film outside of the barrier is represented by the integral shown in Eq. (13).

$$dF = dG = -\int_{\gamma_0}^{\gamma_0} A(\gamma) d\gamma \tag{13}$$

The solution to the integral presented in Eq. (13) is zero, since the value of the limits in this integral are both $\gamma_0$, meaning that the system does no work to create the pure A/W interface. This result is mathematically a consequence of the underlying assumptions behind the Gibbs free energy model, although it appears physically untenable. Eq. (13) is illustrated graphically in Fig. 5B.

In the Gibbs model, the conversion of $\gamma$ to $\pi$ and the derivation of the final integration expression is accomplished as follows. First, using Eq. (12), the Gibbs free energy of compression is defined between an initial and final surface pressure $\gamma_i$ and $\gamma_f$ ($\gamma_i > \gamma_f$), as shown in Eq. (14).

$$dG = -\int_{\lambda_1}^{\gamma_2} A d\gamma \tag{14}$$

The differential defined in Eq. (15) may be substituted into Eq. (14)

$$d\pi = d\gamma_0 - d\gamma = -d\gamma \tag{15}$$

to obtain the final Gibbs free energy integral expression shown in Eq. (16).

$$\Delta G = \int_{\pi_1}^{\pi_2} A d\pi \tag{16}$$

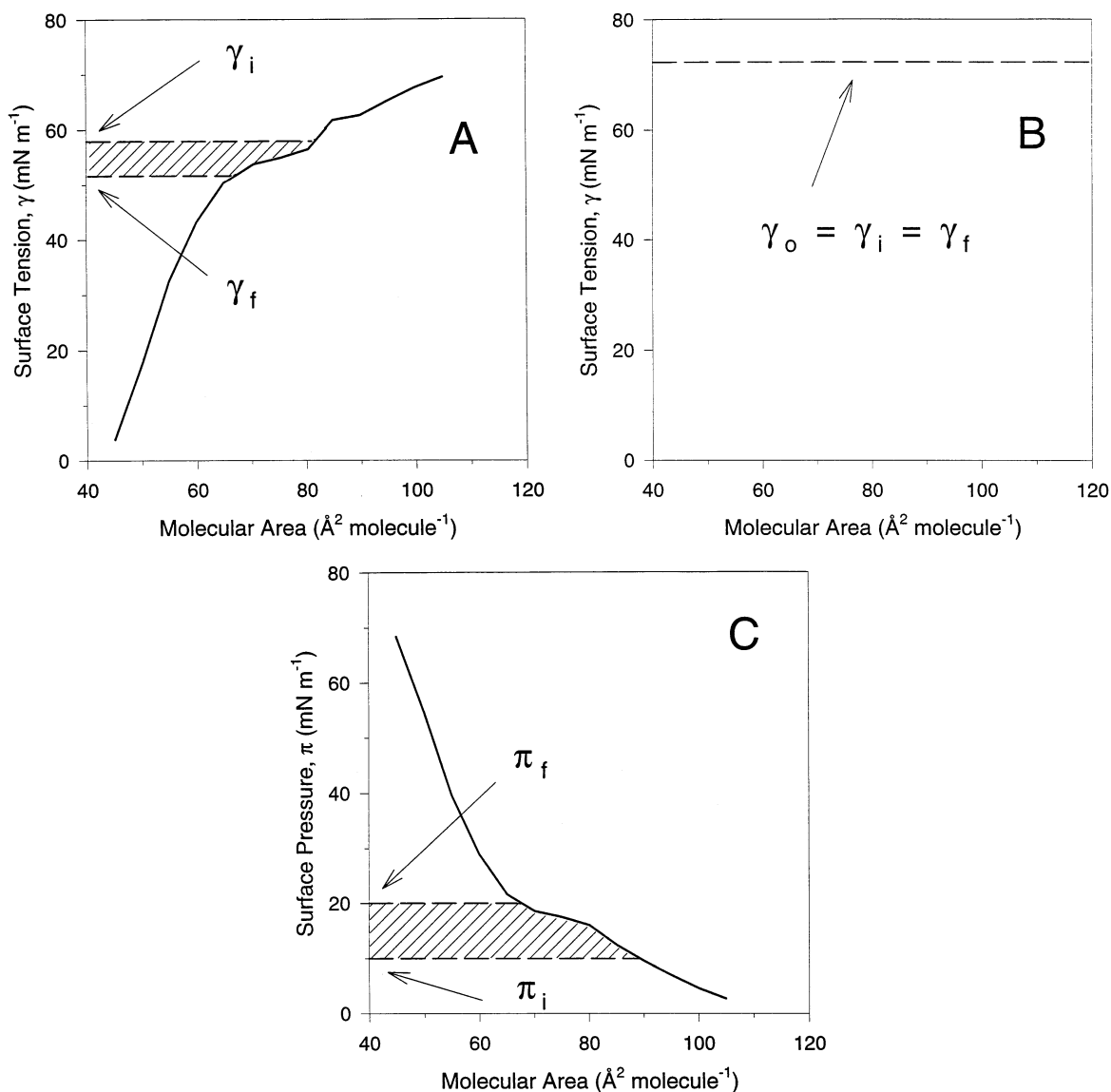This integral is illustrated graphically in Fig. 5C.

Fig. 5. A graphical illustration of the Goodrich-Gaines method of isotherm integration. (A) A plot of the integral defined by the equation: $\int_{\gamma_i}^{\gamma_f} A(\gamma)d\gamma$ which illustrates the negative work done by the system as the barrier is compressed. The cross-hatched region represents the hypothetical region integrated between the surface tensions $\gamma_i$ and $\gamma_f$ by this integral. (B) A plot of the work done by the system to create the A/W interface outside of the barrier according to the integral: $-\int_{\gamma_0}^{\gamma_0} A(\gamma)D\gamma$. The value of this integral equates to zero since by the Goodrich-Gaines definition, $\gamma_0 = \gamma_i = \gamma_f$, where $\gamma_0$ is the surface tension of water (72 mN/m). (C) The overall sum of the integrals presented in Fig. 5A, B, which illustrates the total work done by the system, defines the Goodrich-Gaines model of monolayer thermodynamics, and is represented by the following integral: $\int_{\pi_i}^{\pi_f} A(\pi)d\pi$. The cross-hatched area in the plot represents the hypothetical region integrated between the surface pressures $\pi_i$ and $\pi_f$ by this integral.

Since this second Gibbs free energy model includes an assumption that is physically unsatifying (i.e. that the system does no work to create a film-free interface), as well as defining an independent variable that is somewhat at odds with the operational mode of the film balance (i.e. the use of surface tension as the independent variable and molecular area as the dependent variable), we
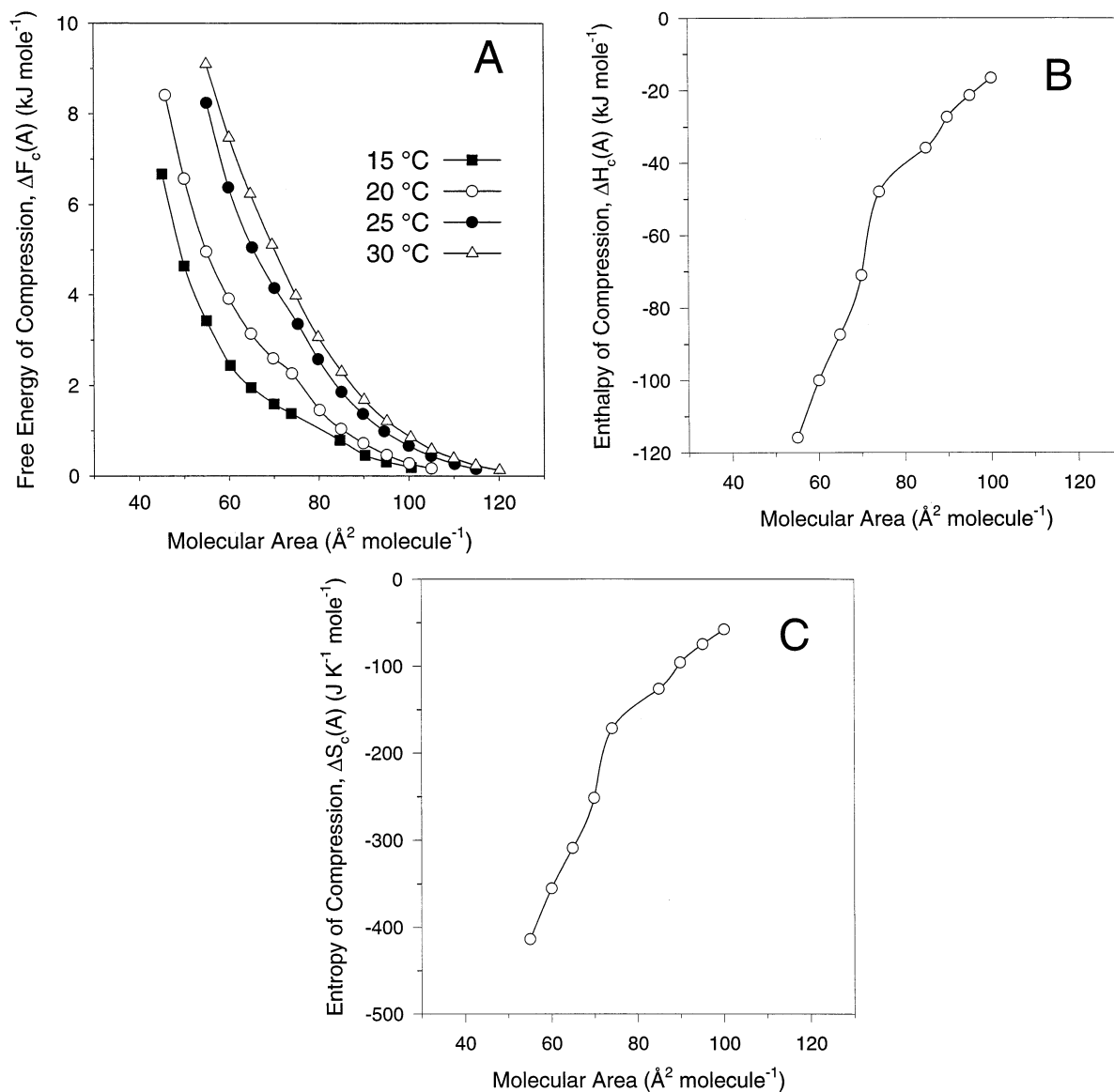
Fig. 6. (A) The Helmholtz free energies of compression, $\Delta F_c(A)$, for DPPC-$d_{62}$ monolayers, calculated using the Gershfeld model. Values of $\Delta F_c(A)$ obtained at 15 (■), 20 (○), 25 (●), and 30°C (△) are plotted here. (B) Values for the enthalpy of compression, $\Delta H_c(A)$, for DPPC monolayers, calculated using the Gershfeld model. Values of $\Delta H_c(A)$ were obtained from isotherms measured in the 20–30°C temperature range. (C) Values for the entropy of compression, $\Delta S_c(A)$, for DPPC monolayers, calculated using the Gershfeld model. Values of $\Delta S_c(A)$ were obtained from isotherms measured in the 20–30 °Ctemperature range.

have elected to use Helmholtz free energy model in the further calculation of the thermodynamic values for binary phospholipid monolayers. Thermodynamic values for the Gibbs free energy, enthalpy and entropy of compression as calculated from the second model for all the phospholipids and mixtures used in this study have been calculated and are tabulated elsewhere (Baldyga et al., 1997).

## 3.5. Thermodynamics of compression for DPPC-$d_{62}$

The Helmholtz free energy, enthalpy, and entropy thermodynamic quantities for DPPC-$d_{62}$ are presented in Fig. 6A–Fig. 6C, respectively. At this point, it is instructive to compare these thermodynamic quantities derived for DPPC-$d_{62}$ with those previously derived for the entirely protiated DPPC monolayer (Fig. 3A, B, C). All of the calculated thermodynamic quantities for the acyl chain perdeuterated DPPC-$d_{62}$ are significantly larger than those for DPPC, which indicates that the molecular interactions of DPPC-$d_{62}$ in monolayers vary significantly from those of the fully protiated DPPC monolayer. These results suggest that there could be complications in using DPPC-$d_{62}$ as a substitute for DPPC in vibrational spectroscopic studies of two-component monolayers, of which one component is DPPC. Such studies assume that the molecular interactions of DPPC-$d_{62}$ with the second (protiated) component are identical to those of DPPC with the second (protiated) component. Vibrational spectroscopic studies of these mixed monolayers isolate the C–D bands of the DPPC-$d_{62}$ component from the C–H bands of the second (protiated) component, with the inherent assumption that the spectroscopic information obtained from the C–D vibrations of DPPC-$d_{62}$ faithfully represent the thermodynamic interactions of the normal DPPC molecule (Rana et al., 1993; Pastrana-Rios et al., 1994). This extrapolation is not valid if the DPPC-$d_{62}$ component interacts with the second (protiated) component in the monolayer in a significantly differently manner than does the fully protiated DPPC molecule. To examine the possibility of this, the interaction thermodynamics of two-component films in both DPPC:DOPG and DPPCd$_{62}$:DOPG binary mixed monolayers will be compared.

## 3.6. The $\pi$-A isotherms of DPPC:DOPG and DPPC-$d_{62}$:DOPG

Monomolecular films of DPPC in combination with other lipids have been extensively studied using vibrational spectroscopy as model systems to examine the 'squeeze-out' hypothesis of pul-

monary surfactant function. In one previous IR-ATR study, Rana et al. (1993) combined DPPC-$d_{62}$ with a saturated C-16 phosphoglycerol lipid (DPPG) to form a 7:1 (mol:mol) binary monomolecular film; these binary mixtures were subsequently transferred to germanium crystals for IR analysis. These authors found that no preferential exclusion of the saturated lipid DPPG occurred. In another previously published study, Pastrana-Rios et al. (1994) used external reflection IR at the A/W interface to study mixed binary films of DPPC-$d_{62}$ and DOPG. They showed that the unsaturated DOPG component was selectively excluded from monolayer films at high surface pressures (greater than 50 mN/m) and under conditions of rapid compression rate. The present study uses the techniques of thermodynamic analysis to study the binary monomolecular mixture of DPPC with DOPG as well as the binary mixture of DPPC-$d_{62}$ with DOPG in order to determine how the perdeuteration of the acyl chains in DPPC-$d_{62}$ effects the thermodynamic properties of these monolayers, and hence, how reliable the conclusions can be from monolayer IR studies using DPPC-$d_{62}$ as one of the components in the monomolecular film.

The $\pi$-A isotherms for a monomolecular film of 7:1 (mol:mol) binary mixture of DPPC:DOPG are shown in Fig. 7A, and those for DPPC-$d_{62}$:DOPG (7:1 mol:mol) are shown in Fig. 7B. The data collected at 20 and 25°C are averages of two experiments, with relative standard deviations in surface pressure of approximately ± 5%; the $\pi$-A isotherms for 7:1 DPPC:DOPG collected at 30°C is an average of three independent experiments, with the same relative standard deviation range for the surface pressures. The $\pi$-A isotherm for 7:1 DPPC-$d_{62}$:DOPG collected at 15°C is an average of two independent experiments, with relative standard deviations in surface pressure of approximately ± 5%; while the $\pi$-A isotherm for 7:1 DPPC-$d_{62}$:DOPG collected at 20, 25, and 30°C are averages of three independent experiments, with the same relative standard deviations in the surface pressures.

The $\pi$-A isotherms for 7:1 (mol:mol) DPPC:DOPG monomolecular films show evidence of a plateau region between approximately

90–130 Å$^2$ per molecule that increases in surface pressure from ∼15 to ∼18 mN/m as the subphase temperature is raised from 20 to 25°C (Fig. 7A). This increase in plateau surface pressure reverses itself as the temperature rises from 25 to 30°C and loses its plateau-type character. This observation indicates there may be a rearrange-

ment of the monolayer components taking place with a temperature-dependent threshold, occurring between 25–30°C.

The π-A isotherms for 7:1 (mol:mol) DPPC-d$_{62}$:DOPG monomolecular films show less evidence than does the fully protiated DPPC-containing monolayer of a plateau region between approximately 90 and 130 Å$^2$ per molecule (Fig. 7B). However the isotherm as a whole for the 7:1 (mol:mol) DPPC-d$_{62}$:DOPG monomolecular film increases in surface pressure at any given molecular area as the subphase temperature is raised from 15 to 20°C. This increase in surface pressure reverses itself as the temperature rises from 20 to 25°C as the monolayer loses any plateau-type character at this temperature. There is virtually no change in monolayer isotherm properties for the 7:1 (mol:mol) DPPC-d$_{62}$:DOPG monomolecular film as the temperature is further raised from 25 to 30°C. The temperature-dependence of the isotherms for the DPPC-d$_{62}$ containing monolayer (Fig. 7B) reveals that any structural rearrangement of the monolayer components takes place with a temperature-dependent threshold, occurring between 20–25°C, or approximately 5°C earlier than for the fully protiated DPPC:DOPG monomolecular film.

### 3.7. The free energy of compression for DPPC:DOPG and DPPC-d$_{62}$:DOPG

The π-A isotherms were analyzed thermodynamically by the model proposed by Gershfeld (Gershfeld et al., 1970, 1972; Pagano et al., 1972; Mingins et al., 1975). For binary monomolecular films, this model yields the Helmholtz free energies of compression, $\Delta F_c(A)_{12}$, given by the integral presented in Eq. (17), where in the case of a binary monomolecular film, the numeric subscript '12' represents the free energy of compression of a two-component mixture consisting of components one and two.

$$\Delta F_c(A)_{12} = \int_{A_f}^{A_i} \pi(A)_{12} dA \qquad (17)$$

The $\Delta F_c(A)_{12}$ values for both the 7:1 (mol:mol) DPPC:DOPG and 7:1 (mol:mol) DPPCd$_{62}$:DOPG films are graphed in Fig. 8A, B, respectively. As
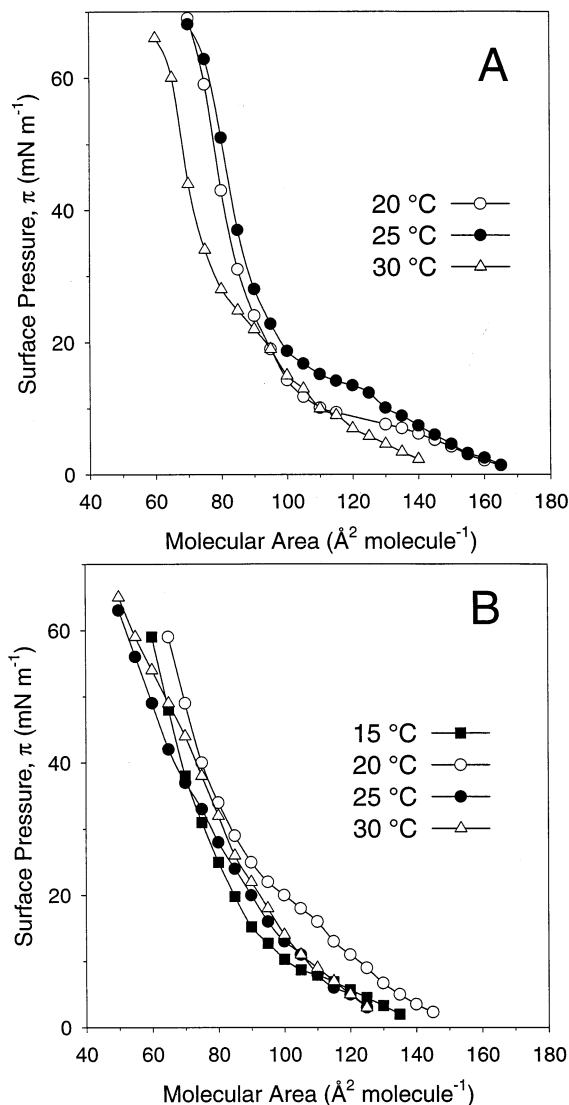


Fig. 7. (A) The π-A isotherms for a 7:1 (mol:mol) DPPC:DOPG binary phospholipid monolayer at 20 (○), 25 (●), and 30°C (△). (B) The π-A isotherms for a 7:1 (mol:mol) DPPC-d$_{62}$: DOPG binary phospholipid monolayer at 15 (■), 20 (○), 25 (●), and 30°C (△).
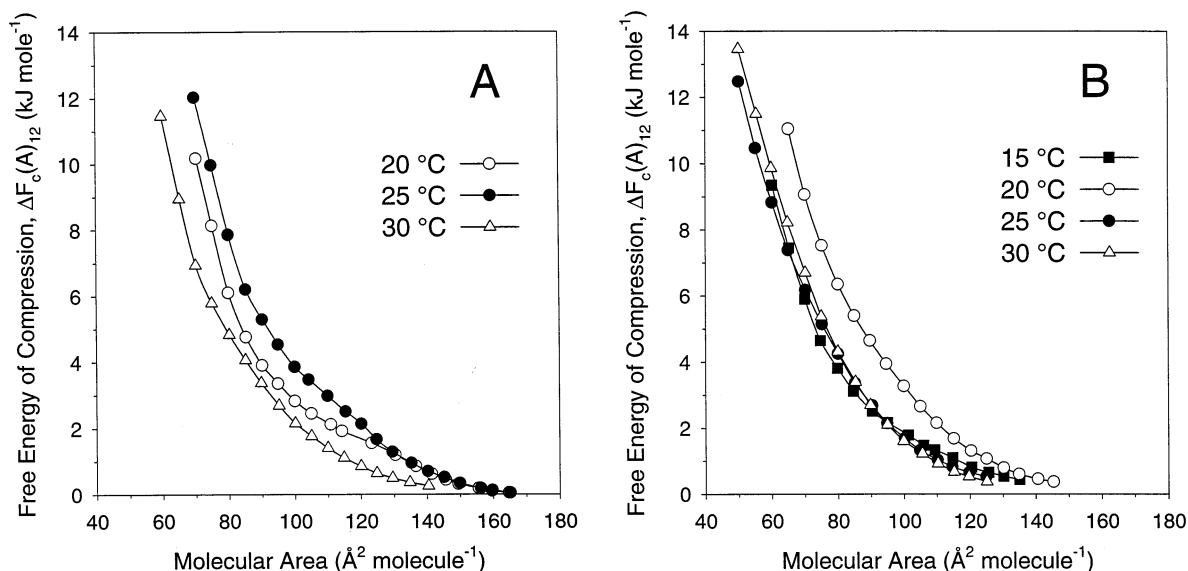
Fig. 8. (A) The Helmholtz free energy of compression values, $\Delta F_c(A)$, as defined in Eq. (17), for a 7:1 (mol:mol) DPPC:DOPG binary phospholipid monolayer at 20 ($\bigcirc$), 25 ($\bullet$), and 30°C ($\triangle$). (B) The Helmholtz free energy of compression values, $\Delta F_c(A)$, as defined in Eq. (17), for a 7:1 (mol:mol) DPPC-$d_{62}$:DOPG binary phospholipid monolayer at 15 ($\blacksquare$), 20 ($\bigcirc$), 25 ($\bullet$), and 30°C ($\triangle$).

might be expected, these thermodynamic values exhibit the same trends as the corresponding $\pi$-A isotherms from which they were derived. That is, the $\Delta F_c(A)_{12}$ values for 7:1 (mol:mol) DPPC:DOPG monomolecular films at any given molecular area rise in magnitude as the subphase temperature is raised from 20 to 25°C (Fig. 8A). This increase in free energy of compression reverses itself as the temperature rises from 25 to 30°C. The $\Delta F_c(A)_{12}$ values for 7:1 (mol:mol) DPPC-$d_{62}$:DOPG monomolecular films also reproduce the pattern seen by their corresponding isotherms (compare Fig. 7B with Fig. 8B). These $\Delta F_c(A)_{12}$ values at any given molecular area as the subphase temperature is raised from 15 to 20°C (Fig. 8B). This increase in free energy of compression reaches a maximum value at 20°C and then reverses itself as the temperature is raised from 20 to 25°C. There is virtually no change in monolayer free energy properties for the 7:1 (mol:mol) DPPC-$d_{62}$:DOPG monomolecular film as the temperature is further raised from 25 to 30°C. Once again, the temperature-dependence of the free energy of compression for the DPPC-$d_{62}$ containing monolayer (Fig. 8B) reveals that any thermody-

namic difference of the perdeuterated monolayer occurs at a temperature approximately 5°C earlier than for the fully protiated DPPC:DOPG monomolecular film.

### 3.8. The excess free energies of mixing for DPPC:DOPG and DPPC-$d_{62}$:DOPG

The excess Helmholtz free energy of mixing, $\Delta F_{xs}(A)$, can be calculated using the model proposed by Gershfeld (Tajima et al., 1975). The $\Delta F_{xs}(A)$ is defined using Eq. (18)–Eq. (20).

$$\Delta F_{xs}(A) = \Delta F_c(A)_{12} - (\chi_1 \Delta F_c(A)_1 + \chi_2 \Delta F_c(A)_2) \tag{18}$$

$$\Delta F_{xs}(A) = \int_{A_f}^{A_i} \pi_{12}(A)dA - \chi_1 \int_{A_f}^{A_i} \pi_1(A)dA$$
$$- \chi_2 \int_{A_f}^{A_i} \pi_2(A)dA \tag{19}$$

$$\Delta F_{xs}(A) = \int_{A_f}^{A_i} (\pi_{12}(A) - (\chi_1 \pi_1(A) + \chi_2 \pi_2(A))) \tag{20}$$

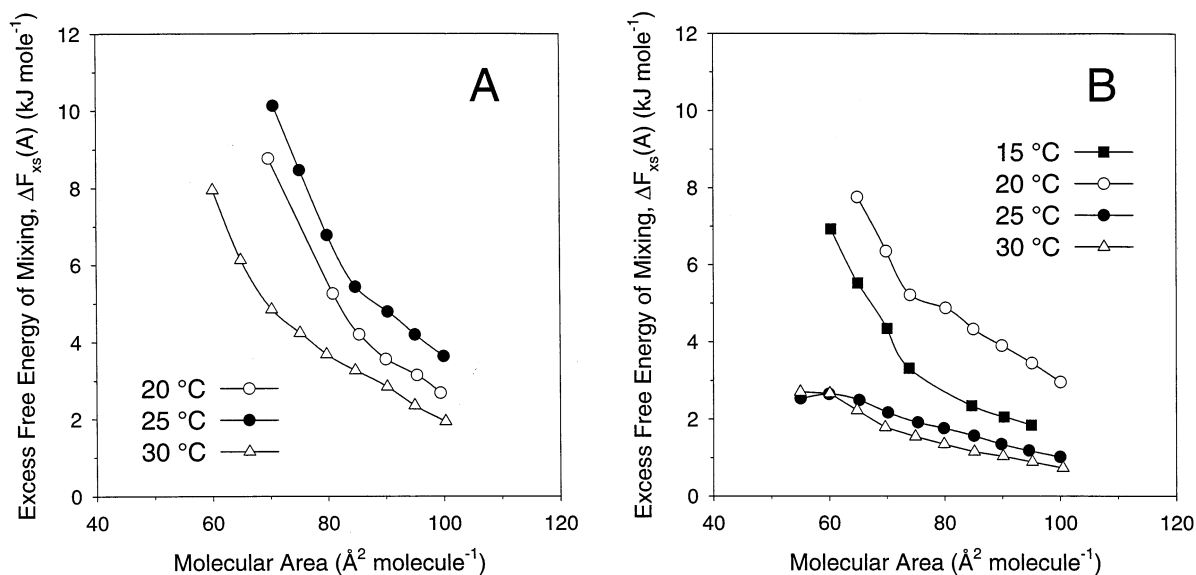In Eqs. (18–20), the numeric subscripts 1 and 2 indicate the individual components of the two-

Fig. 9. (A) The Helmholtz excess free energy of mixing values, $\Delta F_{xs}(A)$, as defined in Eq. (18)–Eq. (20), for a 7:1 (mol:mol) DPPC:DOPG binary phospholipid monolayer at 20 ($\bigcirc$), 25 ($\bullet$), and 30°C ($\triangle$). (B) The Helmholtz free energy of mixing values, $\Delta F_{xs}(A)$, as defined in Eq. (18)–Eq. (20), for a 7:1 (mol:mol) DPPC-d$_{62}$:DOPG binary phospholipid monolayer at 15 ($\blacksquare$), 20 ($\bigcirc$), 25 ($\bullet$), and 30°C ($\triangle$).

component film and $\chi_i$ is the mole fraction of the $i$th component. It is easily seen from these expressions that $\Delta F_{xs}(A)$ is related to a non-ideal excess in the surface pressure isotherm of the mixed binary component monomolecular film when relative to the surface pressure isotherms of the monolayers of the two individual components taken separately.

The calculated $\Delta F_{xs}(A)$ values for both the 7:1 DPPC:DOPG binary monolayer film as well as the 7:1 DPPC-d$_{62}$:DOPG binary monolayer are presented in Fig. 9A, B, respectively. From Fig. 9A it is evident that the calculated $\Delta F_{xs}(A)$ values for the 7:1 DPPC:DOPG binary monolayer film is positive and non-ideal at all molecular areas and temperatures studied. At 20°C the value of the excess free energy of mixing reaches a value of approximately 10 kJ/mol, in very good agreement with previously published calculations of the fully protiated binary monolayer system at 20°C (Williams et al., 1995). After reaching a maximum value at 25°C, however, the values of the excess free energy of mixing measured at 30°C decrease at all molecular areas relative to those $\Delta F_{xs}(A)$

values obtained at 20 or 25°C. The temperature-dependence of this behavior is similar to that observed in the case of the free energy of compression for this system (Fig. 8A).

The calculated $\Delta F_{xs}(A)$ values for the 7:1 DPPC-d$_{62}$:DOPG binary monolayer film are presented in Fig. 9B. These excess free energy of mixing values are positive and non-ideal at all molecular areas studied for subphase temperatures of 15 or 20°C, and (at least for the 20°C data) very closely parallel the $\Delta F_{xs}(A)$ values obtained for the normal, protiated system ($\sim 8$ kJ/mol at 60 $\text{Å}^2$ per molecule and at 20°C). As the temperature is raised from 20 to 25°C, however, the $\Delta F_{xs}(A)$ values calculated for the deuterium-containing binary mixture drop to a value of $\pm 1$–2 kJ/mol, and remain essentially constant over the molecular area range studied.

The type of behavior seen in the $\Delta F_{xs}(A)$ graph in Fig. 9B could indicate a more ideal mixing in the deuterated 7:1 DPPC-d$_{62}$:DOPG binary monolayer film when compared to its protiated analog. However, an equally acceptable explanation that can not be ruled out based on the

thermodynamic evidence alone would indicate that the DOPG has been 'squeezed-out' of the 7:1 DPPC-$d_{62}$:DOPG binary monolayer film. The 'squeezing-out' process leads to a reduction in the excess free energy of mixing values, and serves to make the $\Delta F_{xs}(A)$ curve seem more ideal than it might otherwise appear. There is ample precedence for near-ideal mixing behavior in a squeezed-out monolayer film. The observation of near-ideal excess free energy of mixing has been observed for such classical 'squeezed-out' binary monolayer films as stearic + oleic acid and arachidic + oleic acid (Feher et al., 1977). The interpretation of Fig. 9B to mean that DOPG has been 'squeezed-out' of the perdeuterated binary mixture is also supported by IR evidence (Pastrana-Rios et al., 1994).

Note, however, that the collapse of the $\Delta F_{xs}(A)$ values seen in the case of the 7:1 DPPC$d_{62}$:DOPG binary monolayer film (Fig. 9B) does not occur in the graph of the excess free energy of mixing for the fully protiated 7:1 DPPC:DOPG binary monolayer film (Fig. 9A). Rather, the fully protiated binary mixture shows evidence only for continued non-ideal mixing behavior, even at 30°C, a temperature at which the values of the excess free energy of mixing for the deuterated binary mixture show significant indications of squeeze-out for the DPPC-$d_{62}$-containing monolayer. This is a significantly different result from that of the deuterium-containing 7:1 DPPC-$d_{62}$:DOPG binary monolayer film and points out the potentially important consequences involved in the use of DPPC-$d_{62}$-containing binary monolayers to probe surface film thermodynamics in mixed systems.

## 4. Conclusions

We have performed a study of monolayer mixing behavior using isotherms of binary lipid mixtures of DPPC + DOPG (7:1 mol:mol) as well as a mixture containing the phosphocholine acyl chain perdeuterated analog of DPPC, DPPC-$d_{62}$ (i.e. 7:1 mol:mol DPPC-$d_{62}$ + DOPG). Pressure area isotherms for these binary mixed lipid monolayer systems were acquired between 15 and 30°C. A surface chemistry thermodynamic analysis was performed on these isotherms that calculated the Helmholtz excess free energy of mixing to determine the thermodynamic differences in the fully protiated DPPC:DOPG monolayer versus the DPPC-$d_{62}$:DOPG monolayer that contains the acyl chain perdeuterated DPPC. The main conclusions of this study are as follows:

1. The temperature-dependent thermodynamic phase behavior of DPPC and DPPC-$d_{62}$ differ substantially, and these differences appear magnified when these molecules are studied as monomolecular films at the A/W interface, as opposed to bulk phase systems.

2. Monolayer thermodynamic calculations based on the Helmholtz free energy of compression appear to provide us with the most easily understandable interpretation of the monolayer compression experiment.

3. For any given molecular area, the free energy of compression, enthalphy of compression, and entropy of compression for a single component monolayer containing the perdeuterated molecule DPPC-$d_{62}$ are always much larger than the same quantities calculated for the fully protiated DPPC monolayer.

4. The temperature-dependence of the free energy of compression for the DPPC-$d_{62}$ containing binary monolayer (i.e. DPPC-$d_{62}$:DOPG, 7:1 mol:mol) reveals that $\Delta F_c(A)_{12}$, the free energy of compression for the DPPC-$d_{62}$:DOPG binary monomolecular film, reaches a maximum value at a temperature 5°C lower than for the fully protiated DPPC:DOPG monomolecular film.

5. The calculated excess free energy of mixing values, $\Delta F_{xs}(A)$, for the deuterium-containing 7:1 DPPC-$d_{62}$:DOPG binary monolayer film show a temperature-dependence that results in a small, positive and relatively constant value above 20°C and over the entire molecular area range studied. This type of near-ideal behavior is consistent with the 'squeezing-out' of the DOPG component in the binary mixture containing the DPPC-$d_{62}$ component. The collapse of the $\Delta F_{xs}(A)$ values seen in the case of the 7:1 DPPC-$d_{62}$:DOPG binary monolayer film does not occur in the graph of the excess free energy of mixing for the fully protiated 7:1 DPPC:DOPG binary monolayer film.

6. It appears from the data presented here that the use of acyl chain perdeuterated DPPC (i.e. DPPC-$d_{62}$) in binary monomolecular mixtures with other phospholipids does produce a real and measurable difference on the thermodynamic properties of the monolayer when compared to the case of the fully protiated DPPC:DOPG monomolecular film. In particular, the lower temperatures required to reach the maximum values for the free energy of compression, $\Delta F_c(A)_{12}$, and the collapse of the excess free energy of mixing values, $\Delta F_{xs}(A)$, in the case of the DPPC-$d_{62}$:DOPG monolayer may over-state the fluid nature of the monolayer at any given temperature, thereby leading to an over-estimate of the amount of material potentially 'squeezed-out' of the monomolecular film. The extent of this over-estimation is difficult to determine, however, from solely thermodynamic measurements.

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