Lowry, O. H., Rosebrough, N. J., Farr, A. L., & Randall, R. J. (1951) *J. Biol. Chem. 193*, 265–275.
Martinez-Carrion, M., & Raftery, M. A. (1973) *Biochem. Biophys. Res. Commun. 55*, 1156–1164.
Mebs, D., Narita, K., Iwanaga, S., Samejima, Y., & Lee, C. Y. (1972) *Hoppe-Seyler's Z. Physiol. Chem. 353*, 243–262.
Merrifield, R. B. (1969) *Adv. Enzymol. 32*, 221–296.
Mihovilovic, M., & Martinez-Carrion, M. (1979) *Biochemistry 18*, 4522–4528.
Mihovilovic, M., & Richman, D. P. (1984) *J. Biol. Chem. 259*, 15051–15059.
Mihovilovic, M., & Richman, D. P. (1987) *J. Biol. Chem. 262*, 4978–4986.
Moore, H. H., & Raftery, M. A. (1980) *Proc. Natl. Acad. Sci. U.S.A. 77*, 4509–4513.
Mulac-Jericevic, B., & Atassi, M. Z. (1986) *FEBS Lett. 199*, 68–74.
Neumann, D., Barchan, D., Safran, A., Gershoni, J. M., & Fuchs, S. (1986a) *Proc. Natl. Acad. Sci. U.S.A. 83*, 3008–3011.
Neumann, D., Barchan, D., Fridkin, M., & Fuchs, S. (1986b) *Proc. Natl. Acad. Sci. U.S.A. 83*, 9250–9253.
Noda, M., Takahashi, H., Tanabe, T., Toyosota, M., Furutani, Y., Hirose, T. Asai, M., Inayama, S., Miyata, T., & Numa, S. (1982) *Nature 299*, 793–797.
Noda, M., Takahashi, H., Tanabe, T., Toyosota, M., Furutani, Y., Hirose, T., Asai, M., Inayama, S., Miyata, T., & Numa, S. (1983) *Nature 302*, 528–532.
Oswald, R. E., Heidmann, T., & Changeux, J. P. (1983) *Biochemistry 22*, 3128–3136.
Quast, U., Schimerlik, M. I., & Raftery, M. A. (1979) *Biochemistry 18*, 1891–1901.
Raftery, M. A., Hunkapiller, M. W., Strader, C. D., & Hood, L. E. (1980) *Science 208*, 1454–1457.
Raftery, M. A., Dunn, S. M. J., Conti-Tronconi, B. M., Middleman, D. S., & Crawford, R. D. (1983) *Cold Spring Harbor Symp. Quant. Biol. 48*, 21–33.
Ralston, S., Sarin, V., Lam-Thanh, H., Rivier, J., Fox, J. L., & Lindstrom, J. (1987) *Biochemistry 26*, 3261–3266.
Ratnam, M., Sargent, P. B., Sarin, V., Fox, J. L., LeNguyen, D., Rivier, J., Criado, M., & Lindstrom, J. (1986a) *Biochemistry 25*, 2621–2632.
Ratnam, M., LeNguyen, D., Rivier, J., Sargent, P. B., & Lindstrom, J. (1986b) *Biochemistry 25*, 2633–2643.
Reynolds, J. A., & Karlin, A. (1978) *Biochemistry 17*, 2035–2038.
Sator, V., Raftery, M. A., & Martinez-Carrion, M. (1977) *Arch. Biochem. Biophys. 184*, 95–102.
Schimerlik, M. I., Quast, U., & Raftery, M. A. (1979a) *Biochemistry 18*, 1884–1890.
Schimerlik, M. I., Quast, U., & Raftery, M. A. (1979b) *Biochemistry 18*, 1902–1906.
Schmidt, J., & Raftery, M. A. (1973) *Anal. Biochem. 52*, 349–354.
Shaker, N., Eldefrawi, A. T., Miller, E. R., & Eldefrawi, M. E. (1981) *Mol. Pharmacol. 20*, 511–518.
Tzartos, S. J., & Lindstrom, J. M. (1980) *Proc. Natl. Acad. Sci. U.S.A. 77*, 755–759.
Tzartos, S. J., Kokla, A., Walgrave, S. L., & Conti-Tronconi, B. M. (1988) *Proc. Natl. Acad. Sci. U.S.A. 85*, 2899–2903.
Watters, D., & Maelicke, A. (1983) *Biochemistry 22*, 1811–1819.
Wilson, P. T., Lentz, T. L., & Hawrot, E. (1985) *Proc. Natl. Acad. Sci. U.S.A. 82*, 8790–8794.

Thermodynamics of Phospholipid Bilayer Assembly

N. L. Gershfeld

Laboratory of Physical Biology, National Institute of Arthritis and Musculoskeletal and Skin Diseases, National Institutes of Health, Bethesda, Maryland 20892

Received November 4, 1988; Revised Manuscript Received January 25, 1989

ABSTRACT: Thermodynamic properties of bilayer assembly have been obtained from measurements of the solubility of the sodium salt of dimyristoylphosphatidylglycerol (DMPG) in water. The standard free energy of bilayer assembly $\Delta G°_a$ is shown to be $RT \ln Xs + zF\Psi_0$ where Xs is the mole fraction of dissolved lipid, F is the Faraday constant, z is the valence of the counterion (Na^+), and Ψ_0 is the electrical double-layer potential of the ionized bilayer. The function $d \ln Xs/dT$ was found to be discontinuous at 24 °C, the gel–liquid-crystal transition temperature (T_m) for DMPG. This function was unaffected when solubilities were measured in 0.001 M NaCl solutions; thus, Ψ_0 is constant in the experimental temperature interval (4–40 °C). Using a value of $\Psi_0 = -180$ mV [Eisenberg et al. (1979) *Biochemistry 18*, 5213–5223], and the temperature dependence of $\Delta G°_a$, values for $\Delta H°_a$ and $\Delta S°_a$ at 24 °C were calculated for the gel and liquid-crystal states of DMPG. For the gel, $\Delta H°_a$ and $T\Delta S°_a$ are −26.2 and −12.7 kcal/mol, respectively; for the liquid-crystal, $\Delta H°_a$ and $T\Delta S°_a$ are −19.2 and −5.7 kcal/mol, respectively. The calculated value for the latent heat of the gel–liquid-crystal transition is 7 kcal/mol, in agreement with calorimetric measurements.

The process of self-assembly of phospholipid bilayers from their components in aqueous solution may be represented by the equilibrium relation

$$[\text{phospholipid}]_{\text{solution}} \rightleftharpoons [\text{phospholipid}]_{\text{bilayer}} \quad (1)$$

In principle, all the thermodynamic properties of bilayer assembly may be obtained by conventional measurements of the chemical activity of the components that participate in this equilibrium. These measurements, however, have been hampered primarily because activities of phospholipids in solution are difficult to obtain due to the very low solubility of these compounds in water. It is, therefore, understandable that

much of what is known about the assembly process is limited to the physical properties of phospholipid bilayers including structure (Bangham, 1968; Small, 1986), bilayer mechanics (Helfrich, 1973), and interbilayer forces (LeNeveu et al., 1977). The emphasis on the structural properties of bilayers is also reflected in a theory of bilayer assembly which relies on the molecular shape of the phospholipid constituents to predict whether a particular lipid will form micelles or bilayers (Israelachvili et al., 1977). However, as eq 1 indicates, evaluation of the thermodynamic properties of bilayer assembly requires phospholipid solubility data. Indeed, as the following study will show, a complete thermodynamic description of bilayer assembly can be developed solely on the basis of phospholipid solution properties.

In a preliminary study (Gershfeld et al., 1986), the solubility of the sodium salt of dimyristoylphosphatidylglycerol (DMPG) in water was found high enough to suggest that its chemical activity may be accessible for a rigorous evaluation of the thermodynamic properties of bilayer assembly. Because DMPG bilayers are ionized, eq 1 and the concomitant free energy expression must be modified to include a term that expresses the contribution of the ionic charge to the work of forming the bilayer. In the present study, this contribution is obtained by measuring the solubility of DMPG in solutions of neutral salt. Since DMPG forms typical bilayers that exhibit a well-defined latent heat for the gel–liquid-crystal transition (Findlay & Barton, 1978), the thermodynamics of bilayer assembly are developed for both the gel and liquid-crystal states.

Thermodynamics of Assembly for Ionized Bilayers. The phase relations for DMPG (Gershfeld et al., 1986) indicate that the lipid swells in water to form a jellylike substance comprising an extended matrix of interacting bilayer sheets. The jelly state is maintained up to a critical temperature (T^*) where the bilayer sheets of the jelly transform to macroscopic unilamellar vesicles. The transformation temperature T^* is approximately 8 °C higher than T_m, the gel–liquid-crystal transition temperature for DMPG. The phosphate groups associated with the bilayer are extensively ionized and behave as fixed charges in much the same manner as in an ion-exchange resin. The dissociated Na^+ ions are distributed about the fixed charges of the bilayer, forming an electrical double layer whose potential is Ψ_0. In solution, DMPG is completely ionized, forming the anion ($DMPG^-$) and Na^+. At $T < T^*$, $DMPG^-$ exists as single molecules; micelles do not form in solution until the Krafft point is reached at temperatures above T^* (Gershfeld et al., 1986).

On the basis of these phase relations at $T < T^*$, the equilibrium between the DMPG jelly, as a separate phase, and dissolved $DMPG^-$ is expressed as

$$DMPG^-_s + Na^+_s \rightleftharpoons (Na^+ \cdot DMPG^-)_b \qquad (2)$$

where subscripts s and b refer to solution and bilayer phases, respectively. The equilibrium constant for assembly (K_a) may be expressed in terms of the activities of each of the components in the system. Choosing as standard states the bilayer phase with activity equal to 1, and the pure solute for the dissolved lipid phase, the equilibrium constant for assembly K_a may be written as

$$K_a = (1/fXs)a^+_b/a^+_s \qquad (3)$$

where Xs is the mole fraction of DMPG in solution with f the activity coefficient and a^+_b and a^+_s the activities of Na^+ in the bilayer and solution, respectively. In this study, it is assumed that $f = 1$ because the $DMPG^-$ solutions are very dilute, and micelles are absent (Gershfeld et al., 1986). The ratio of Na^+ activities in the bilayer and solution recognizes the fact that the counterion will distribute itself between the solution and the bilayer phase. This ratio may be written (MacDougall, 1948) in terms of the electrical double-layer potential Ψ_0 as

$$a^+_b/a^+_s = \exp(-zF\Psi_0/RT) \qquad (4)$$

where z is the valence of the counterion, F the Faraday constant, and R the gas constant. Combining eq 3 and 4, the standard free energy of assembly, $\Delta G^\circ_a = -RT \ln K_a$ becomes

$$\Delta G^\circ_a = RT \ln Xs + zF\Psi_0 \qquad (5)$$

Equation 5 explicitly assumes that only electrostatic interactions occur between the counterions and the bilayer. This assumption is reasonable for univalent electrolytes but may not be valid with multivalent counterions, e.g., Ca^{2+}, where extensive cross-linking reactions between bilayer and counterion are likely to occur. From the temperature dependence of ΔG°_a, using the Gibbs–Helmholtz relation, $d(\Delta G^\circ_a/T)/dT = -\Delta H^\circ_a/T^2$, the standard enthalpies and entropies of assembly may be evaluated.

Materials and Methods

The sodium salt of DMPG, washed with EDTA to remove heavy-metal ions (Avanti Polar Lipids, Inc., Birmingham, AL), was shown to have a purity >99% by thin-layer chromatography and was used without further purification. Decomposition of DMPG in water to the lysophosphatidylglycerol compound in control experiments was found to be insignificant. Sodium chloride was AR grade (Fisher). Water was prepared in a double distillation quartz apparatus; all dispersions were made with buffer-free water.

Solubility. To obtain equilibrium solubilities, it is essential that the solubility be a constant value at each temperature and independent of the amount of the excess lipid phase. The procedure for determining the solubility of DMPG in water depends on equilibrating dispersions of the lipid at constant temperature and then separating the excess lipid phase by centrifugation at that temperature (Gershfeld et al., 1986). Generally, 2–5 times the amount of DMPG necessary to saturate the solution was used; the dispersions were shaken in a constant-temperature bath for 3–4 h, sufficient to attain equilibrium. All experiments were conducted in the absence of light. The excess lipid was separated in a Beckmann Model L5-65 centrifuge with an SW-40 rotor; temperature control was maintained from 0 to 45 ± 0.5 °C. Since pressures are generated in the centrifuge tube, the following control experiments were performed. Aliquots of the supernatant were taken at several depths in the tube, thereby varying the pressure by a factor of approximately 2; they were evaporated, and the phosphorus content of each residue was measured (Rouser et al., 1970). Within the error of the analytical method (±10%), no differences in lipid content were detected. This is consistent with the behavior of dilute aqueous solutions of detergents at high pressures (Tanaka et al., 1973); the solutions are "superpressed", and the solubility remains essentially unchanged from values at atmospheric pressure. Centrifugation times of 20 h at 100000*g* were sufficient to yield constant values for the lipid solubility.

These control experiments eliminate the possibility that slowly sedimenting vesicles are present because the concentration of such vesicles would likely be in proportion to the amount of lipid in the dispersion. Most of the reported results were obtained from analysis of aliquots of the supernatant taken from the uppermost portions of each tube.

Differential Scanning Calorimetry. The transition temperature T_m and the latent heat for the gel–liquid-crystal

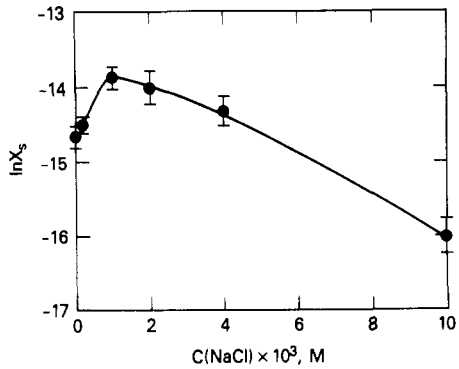

FIGURE 1: Solubility of DMPG (ln *Xs*) as a function of the concentration of NaCl; $T = 18$ °C. Error bars represent ± SE calculated from 6–12 independent determinations.

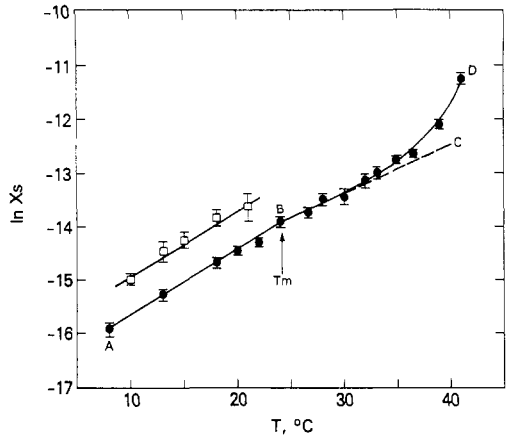

FIGURE 2: Solubility of DMPG (ln *Xs*) in water (●) (Gershfeld et al., 1986) and in 0.001 M NaCl (□) as a function of temperature. Error bars represent ± SE calculated from 6–12 independent determinations. T_m is the gel–liquid-crystal transition temperature, 24 °C. For $T < T_m$, monomer (solution) is in equilibrium with gel state; for temperatures between T_m and 32 °C, monomer is in equilibrium with liquid-crystal. At temperatures above 32 °C, micelles form in solution (Gershfeld et al., 1986).

transition of DMPG were obtained with a Perkin-Elmer DSC Model II scanning calorimeter. The instrument was calibrated with a pure sample of indium. A weighed amount (ca. 1 mg) of anhydrous lipid and water or NaCl solution, in the ratio of 1–2 times the amount of lipid, was added to sample pans, and the pans were sealed. Scanning rates of 1.25–5 °C/min yielded constant values for T_m and for the latent heat.

Results and Discussion

Influence of Ionic Strength on Solubility. The solubility of DMPG at 18 °C in NaCl solutions is shown in Figure 1. The solubility reaches a maximum at approximately 0.001 M; at higher salt concentrations, DMPG solubility falls continuously. For concentrations greater than 0.01 M NaCl, DMPG solubility becomes too small for precise measurement by the method used in this study. Since addition of neutral salt decreases Ψ_0, the solubility of DMPG was expected to decrease. However, because of hydrolysis of phosphate, a small amount of H^+ is bound to the phosphate groups of the bilayers. The small increase in solubility observed at low salt concentrations (0.001 M) is likely due to replacement by Na^+ of this bound hydrogen (Payens, 1955). However, this is a small effect, rapidly overshadowed by the influence of the added electrolyte on Ψ_0. With increasing amounts of added NaCl, the solubility of $DMPG^-$ decreases, as expected.

Influence of Temperature on DMPG Solubility. Figure 2 compares the solubility of DMPG in water and in 0.001 M NaCl over a range of temperatures. In the temperature interval where the solubilities in both solvents have been obtained, a linear regression analysis of the slopes of each of the curves indicates they are identical: 0.123/°C, with correlation coefficients of 0.997 and 0.978 for water and 0.001 M NaCl, respectively. This establishes the fact that temperature has little or no effect on the electrical double-layer potential of DMPG bilayers. The relative insensitivity of Ψ_0 to changes in temperature is also reflected in constant values of the gel–liquid-crystal transition temperature and latent heat obtained by calorimetry for DMPG in various NaCl solutions (0–0.01 M); T_m and ΔH_m are 24 ± 0.3 °C and 6.7 ± 0.5 kcal/mol, respectively, in good agreement with previous measurements (Findlay & Barton, 1978). The latent heat ΔH_m is directly related to the difference in slopes at T_m in Figure 2 (see below). Since ΔH_m is constant in NaCl solutions, it is not necessary to measure the solubility of DMPG in 0.001 M NaCl at $T > T_m$ because it will parallel the solubility curve for DMPG in water. DMPG also exhibits a pretransition at 19 °C with an accompanying heat of 0.8 ± 0.2 kcal/mol. This heat is within the experimental limits of the measurement and therefore cannot be extracted from the solubility data.

Table I: Thermodynamic Properties of Bilayer Assembly for Dispersions of DMPG in 0.001 M NaCl at T_m = 24 °C

physical state	$\Delta G°_a$ (kcal/mol)	$\Delta H°_a$ (kcal/mol)	$T\Delta S°_a$ (kcal/mol)	ΔH_m (kcal/mol)
gel	−13.5	−26.2	−12.7	
liquid-crystal	−13.5	−19.2	−5.7	
				+6.7 ± 0.5 (DSC)
				+7 (solubility)

The standard heats of bilayer assembly may be evaluated from the slopes of the curves in Figure 2. From eq 5 and the Gibbs–Helmholtz relation:

$$\Delta H°_a = \bar{H}_b - \bar{H}_s = zF\Psi_0 - RT^2 \mathrm{d}(\ln Xs)/\mathrm{d}T \quad (6)$$

where $\bar{H}_b$ and $\bar{H}_s$ are the partial molar heat contents of the lipid in the bilayer and solution, respectively. Applying eq 6 to the data in Figure 2 at the gel–liquid-crystal transition temperature T_m, we may obtain $\Delta H°_a$ for both the gel and liquid-crystal states of DMPG. For Ψ_0, a value of −180 mV is used; this value was obtained from the ζ potentials of phosphatidylglycerol vesicles (Eisenberg et al., 1979) by extrapolation to the dilute salt concentrations used in the present study. To calculate $\Delta G°_a$, eq 5 is employed; $\Delta S°_a$ is obtained from the identity $\Delta G°_a = \Delta H°_a - T\Delta S°_a$. The thermodynamic properties for assembly of the gel and liquid-crystal states in 0.001 M NaCl are listed in Table I. The solubility measurements in Figure 2 also reflect the gel–liquid-crystal phase transition. First-order phase transformations, as in the case of the gel–liquid-crystal transition, exhibit a discontinuity in the temperature dependence of the chemical potential. Hence, the presence of a discontinuity in d ln $Xs/\mathrm{d}T$ at T_m (24 °C) in Figure 2 confirms the correctness of applying solubility data to measuring the thermodynamic properties of bilayer assembly. Moreover, since gel, liquid-crystal, and solution coexist at T_m, ΔH_m may be obtained from the slopes of the solubility data at T_m as illustrated by the following relationships:

$$\Delta H_m = \bar{H}_b(\text{liquid-crystal}) - \bar{H}_b(\text{gel}) = (\bar{H}_b - \bar{H}_s)(\text{liquid-crystal}) - (\bar{H}_b - \bar{H}_s)(\text{gel}) \quad (7)$$

From eq 6 and 7, using the slopes along *AB* for the gel, and along *BC* for the liquid-crystal (linear regression analysis yields a slope of 0.0845/°C, correlation coefficient of 0.961 for the latter), a value for ΔH_m may be calculated. It is listed in Table

I along with the calorimetrically derived value. The good agreement between the solubility and calorimetrically derived values of ΔH_m establishes the validity of the thermodynamic analysis, as well as the internal consistency of the experimental data.

The enthalpies and entropies of assembly for both the gel and liquid-crystal bilayers are all negative (Table I). These relatively large negative values reflect the crystalline character of the gel and liquid-crystal bilayer states, as well as the fact that the bilayers form as precipitates when the dissolved lipid exceeds its solubility limit. In marked contrast, micelles exhibit positive values for the enthalpy and entropy of assembly (Tanford, 1980). Micelles are essentially liquidlike aggregates, and their assembly from dissolved lipid occurs entirely in solution; under these conditions, hydrophobic effects are likely to dominate, and micelle assembly will be entropically driven.

Although the thermodynamics of bilayer assembly have been obtained for only one lipid, several general properties about bilayer assembly may be deduced. The results indicate that as long as the solubility of the lipid increases with temperature, the heat and entropy of assembly will always be negative. This will be true regardless of the value of Ψ_0, for the value of $zF\Psi_0$ must always be negative or zero. Detergent solubility generally increases with temperature, and phospholipids, as a subset of detergents, are likely to behave similarly. It is anticipated, therefore, that phospholipid bilayer assembly will always involve negative enthalpies and entropies. Neutral phospholipids such as the phosphatidylcholines are expected to give similar solubility–temperature relations as DMPG. However, the solubilities may be orders of magnitude lower than those observed with DMPG.

While this thermodynamic analysis cannot distinguish among the contributions made by each of the equilibrium phases, the forces which drive the bilayer assembly process must involve interactions in both the solution and bilayer phases. Specific chemical contributions of the lipid to the assembly process will obviously require an understanding of its aqueous solution properties as well as the structural properties of the lipid in the bilayer. Theories of assembly based solely on the internal energy of the condensed phase are therefore of limited value. Molecular shape factors may contribute to the internal energy of the condensed phase, but these are probably second-order effects and unlikely to significantly influence whether the bilayer or micelle structure assembles.

Finally, this study suggests how bilayer assembly may occur in cell membranes. The enzymes which synthesize membrane lipids are principally located on existing membranes which are enmeshed in an aqueous phase. If the local concentration of the synthesized lipid exceeds its solubility in this aqueous phase, the material will precipitate as bilayer. For most membrane lipids, the solubility may be extremely low, but the assembly process and its thermodynamic properties will be governed by the solution–bilayer equilibrium described in this study. Particular conditions for membrane bilayer assembly have been presented elsewhere (Gershfeld, 1986, 1989).

References

Bangham, A. D. (1968) *Prog. Biophys. Mol. Biol. 18*, 29–95.

Eisenberg, M., Gresalfi, T., Riccio, T., & McLaughlin, S. (1979) *Biochemistry 18*, 5213–5223.

Findlay, E. J., & Barton, P. G. (1978) *Biochemistry 17*, 2400–2405.

Gershfeld, N. L. (1986) *Biophys. J. 50*, 457–461.

Gershfeld, N. L. (1989) *BBA Rev. Biomembr.* (in press).

Gershfeld, N. L., Stevens, W. B., Jr., & Nossal, R. J. (1986) *Faraday Discuss. Chem. Soc. 81*, 19–29.

Helfrich, W. (1973) *Z. Naturforsch. 28C*, 693–703.

Israelachvili, J. N., Mitchell, J. D., & Ninham, B. W. (1977) *Biochim. Biophys. Acta 470*, 185–201.

LeNeveu, D. M., Rand, R. P., Parsegian, V. A., & Gingell, D. (1977) *Biophys. J. 18*, 209–230.

MacDougall, F. H. (1948) *Thermodynamics and Chemistry*, 3rd ed., p 346, Wiley, New York.

Payens, Th. A. J. (1955) *Philips Res. Rep. No. 10*, 425–481.

Rouser, G., Fleischer, S., & Yamamoto, A. (1970) *Lipids 5*, 494–496.

Small, D. M. (1986) *The Physical Chemistry of Lipids*, Plenum Press, New York.

Tanaka, M., Kaneshina, S., Tomida, T., Noda, K., & Aoki, K. (1973) *J. Colloid Interface Sci. 44*, 525–531.

Tanford, C. (1980) *The Hydrophobic Effect: Formation of Micelles and Biological Membranes*, p 77, Wiley, New York.