^{31}P NMR and X-ray Diffraction Study of the Effect of Photopolymerization on Lipid Polymorphism†

Judith A. Barry,*,‡ Henry Lamparski,‡ Erramilli Shyamsunder,‖ Fredrik Osterberg,‖ John Cerne,‖ Michael F. Brown,‡ and David F. O'Brien‡

Department of Chemistry, University of Arizona, Tucson, Arizona 85721, and Department of Physics, Princeton University, Princeton, New Jersey 08544

Received April 24, 1992; Revised Manuscript Received August 6, 1992

ABSTRACT: It was recently shown that oligolamellar vesicles of 3:1 mixtures of dioleoylphosphatidylethanolamine (DOPE) and the photopolymerizable lipid 1,2-bis[10-(2′,4′-hexadienoyloxy)decanoyl]-*sn*-glycero-3-phosphocholine (SorbPC) are destabilized by polymerization of the SorbPC [Lamparski, H., Liman, U., Frankel, D. A., Barry, J. A., Ramaswami, V., Brown, M. F., & O'Brien, D. F. (1992) *Biochemistry 31*, 685–694]. The current work describes the polymorphic phase behavior of these mixtures in extended bilayers, as studied by ^{31}P NMR spectroscopy and X-ray diffraction. In the NMR experiments, samples with varying degrees of polymerization were slowly raised in temperature, with spectra acquired every 2.5–10 °C. In the unpolymerized mixiture, and in those photopolymerized samples where the monomeric SorbPC was decreased by 33% and 51%, an isotropic signal grew progressively until no signal from the lamellar liquid-crystalline (L_α) phase remained. In the highly polymerized sample with a 90% loss of monomeric SorbPC, less than 20% of the lipids underwent this transition. In none of the samples was an inverted hexagonal phase (H_{II}) observed, under conditions of slow heating to almost 100 °C. The X-ray diffraction studies indicated that samples which exhibit the isotropic NMR signal corresponded to a structure exhibiting no well-defined crystalline order, which upon thermal cycling became an inverted cubic phase belonging to either the *Pn3m* or *Pn3* space groups. The temperature of the transition to the cubic precursor decreased as the extent of polymerization increased, demonstrating that photopolymerization of these lipid bilayers can significantly alter the composition and thermotropic phase behavior of the mixture.

Several classes of biological lipids, including phosphatidylethanolamines (PE),[1] form hydrated membrane systems that display a variety of lamellar and nonlamellar phases as a function of temperature, pressure, and concentration. Nonlamellar lipid phases in membranes have been extensively investigated in recent years due to interest in their possible cell membrane role in the processes of fusion, endo- and exocytosis, and the transmembrane movement of large molecules [reviewed by Verkleij (1984), Cullis et al. (1985), Gruner et al. (1987), Hui (1988), Lindblom and Rilfors (1989), and Seddon (1990)]. It has also been suggested that unique membrane properties required for the function of certain integral proteins may exist near the L_α/nonlamellar phase boundaries (Wiedmann et al., 1988; Hui & Sen, 1989).

Model membranes composed of PEs are capable of forming an inverted hexagonal (H_{II}) phase, which was first observed in the X-ray diffraction studies of Luzzati and co-workers in the 1960s (Luzzati & Reiss-Husson, 1962; Luzzati et al., 1968). It can also be detected by ^{31}P NMR (Cullis & de Kruijff, 1979) and by differential scanning calorimetry (Van Dijck et al., 1976; Cullis & de Kruijff, 1978; Epand, 1985; Ellens et al., 1986a,b) and visualized by freeze-fracture electron microscopy (Deamer et al., 1970; Gulik-Krzywicki, 1975; Hui et al., 1981) as well as cryomicroscopy (Siegel et al., 1989). Frequently a phase with isotropic symmetry is found between the L_α and H_{II} phases (Lindblom & Rilfors, 1989). Freeze-fracture electron microscopy, ^{31}P NMR, and X-ray diffraction studies of mixtures of PE and phosphatidylcholines (PC) (Cullis & de Kruijff, 1978; Cullis et al., 1978a; Hui et al., 1981, 1983; Boni & Hui, 1983; Eriksson et al., 1985) revealed the existence of structures with isotropic symmetry. In those cases where X-ray diffraction methods were used (Gruner et al., 1988; Ellens et al., 1989; Siegel & Banschbach, 1990), these isotropic structures were shown to be well-defined inverted cubic phases (Q_{II}) or precursors to cubic phases. These structures have also been observed directly with cryomicroscopy in DOPE/octyl glucoside liposomes (Siegel et al., 1989) and in DOPE/DOPC/cholesterol (3:2:1) (Frederik et al., 1989).

PE liposomes are generally unstable at physiological pH unless they also consist of other lipids such as PC (Stollery & Vail, 1977). Processes which lead to the phase separation of PE and other lipids appear to modify the local phase behavior of PE (Ellens et al., 1984; Conner et al., 1984; Duzgunes et al., 1985; Ellens et al., 1986b; Leventis et al., 1986; Brown & Silvius, 1989). Research on PE polymorphism has lead to the

† This research was supported by National Institutes of Health (NIH) Postdoctoral Fellowship EY06111 (J.A.B.), the Office of Naval Research Contract N14-90-J-1702 and NIH Grant GM32614 (E.S.), NIH Grants GM41413 and RR03529 (M.F.B.), and National Science Foundation Grant DMR-8722341 (D.F.O.). Preliminary reports of this research were presented at the 35th Annual Biophysical Society Meeting, Feb 1991 (Barry et al., 1991; Osterberg et al., 1991).

* Author to whom correspondence should be addressed.

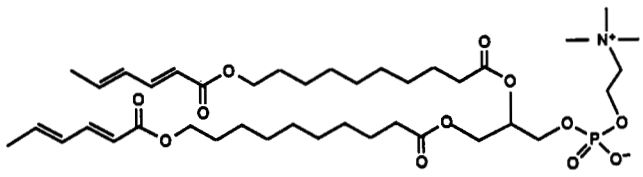
‡ University of Arizona.

‖ Princeton University.

[1] Abbreviations: DOPC, dioleoylphosphatidylcholine; DOPE, dioleoylphosphatidylethanolamine; DOPE-Me, monomethylated dioleoylphosphatidylethanolamine; DOPE-Me$_2$, dimethylated dioleoylphosphatidylethanolamine; EM, electron microscopy; T_l, lamellar liquid-crystalline (L_α) phase to isotropic transition temperature; NMR, nuclear magnetic resonance; PC, phosphatidylcholine; PE, phosphatidylethanolamine; SIT, silicon-intensified target; SorbPC 1,2-bis[10-(2′,4′-hexadienoyloxy)-decanoyl]-*sn*-glycero-3-phosphocholine; TES, *N*-[tris(hydroxymethyl)-methyl]-2-aminoethanesulfonic acid; TLC, thin-layer chromatography.

0006-2960/92/0431-10114$03.00/0

development of several methods to modulate the local phase behavior of lipid membranes and cause their destabilization by the addition of ions, protons, antibiotics, and peptides. Recently Lamparski et al. (1992) [preliminary account by Frankel et al. (1989)] described the photoinduced destabilization of oligolamellar liposomes composed of DOPE (or TPE) and a light-sensitive lipid, SorbPC (**1**). Photoactivation



bis-SorbPC (1)

of SorbPC causes polymerization of lipids within the same monolayer and causes the phase separation of the polymerized lipid from the colipid(s). If the colipid prefers a lamellar structure, the newly formed phase-separated liposome will retain both its size and encapsulation characteristics. If the colipid is polymorphic, the liposome can be destabilized with the release of water-soluble markers. This new method for the destabilization of liposomes opens the possibility of photocontrol of the release of reagents in a spatially and temporally selective manner. The polymerization of liposomes is reviewed by Ringsdorf et al. (1988) and by O'Brien and Ramaswami (1989).

The potential importance of light-induced bilayer membrane destabilization prompted the current study into the nature of the lipid structures which are formed in DOPE/SorbPC membranes. This paper describes both ^{31}P NMR and X-ray diffraction studies of the polymorphic phase behavior of extended bilayers of DOPE/SorbPC (3:1) membranes as a function of temperature and extent of photopolymerization of SorbPC. In all mixtures, a slow increase in temperature lead to a progressive conversion of the lamellar ^{31}P NMR signal to an isotropic signal. The temperature of the transition from the lamellar to the isotropic phase decreased as the fraction of SorbPC photopolymerization increased. X-ray diffraction data showed that although the isotropic NMR signal did not correspond to a phase with well-defined crystalline order, thermal cycling of the sample converted it to an inverted cubic phase.

EXPERIMENTAL PROCEDURES

Materials and Preparation of Extended Bilayers. The photopolymerizable SorbPC was prepared according to the procedure of Lamparski et al. (1992). The DOPE was purchased from Avanti Polar Lipids, Inc. (Pelham, AL) and used without further purification (one spot TLC, 65:24:4 $CHCl_3$/MeOH/H_2O). All procedures were performed under yellow safelights to protect against UV radiation. Four mixtures of 3:1 mol/mol DOPE/SorbPC were prepared; three of these samples were polymerized to varying degrees by exposure to ultraviolet radiation as described below, while one sample was left unpolymerized. The samples were prepared for polymerization (or for NMR in the case of the unpolymerized sample) by transferring weighed quantities of the DOPE and SorbPC to an 8-mm glass tube with $CHCl_3$. The $CHCl_3$ was evaporated with argon, and the lipids were lyophilized from benzene to remove any residual $CHCl_3$ and water. The lipids were dried in the dark under vacuum for 8–12 h, hydrated with buffer (2 mM TES, 2 mM imidazole, and 150 mM NaCl at pH 7.4) to a concentration of about 100 mg/mL (90 wt % H_2O), and vortexed. In order to ensure that small, unilamellar vesicles were not present, the samples were freeze-thawed at least 10 times in either liquid nitrogen or 2-propanol/dry ice and allowed to warm from the last freeze at 4 °C for 20–30 min. A sample of pure, unpolymerized SorbPC was also prepared in this way.

Photopolymerization of Hydrated Bilayers. Polymerization of the DOPE/SorbPC bilayers was accomplished by irradiation of about 400 μL of the hydrated lipid mixture in a quartz cuvette with a path length of 1 mm. The large surface of the cuvette was placed 15 mm from a low-pressure mercury arc lamp (254-nm emission) for 1.0, 1.25, 3.5, and 12.8 h to achieve the following loss of monomeric SorbPC: 28%, 33%, 51%, and 90%, respectively. The incident light energy from the lamp was $\sim 5 \times 10^{14}$ photons/s (Lamparski et al., 1992). During exposure the temperature was maintained at 30.0 ± 0.2 °C via a thermostated cell. The loss of monomer was determined spectrophotometrically from the loss of sorbate absorption at the λ_{max} (258 nm) as a function of UV exposure on a Hewlett-Packard 8452 dioide array spectrophotometer. The photoinduced loss of monomer is accompanied by the formation of an immobile spot on TLC in addition to the spots for PE and PC.

^{31}P NMR Spectroscopy. For the ^{31}P NMR experiments, 20–30 mg of lipid was sealed in an 8-mm tube which was placed in a 10-mm NMR tube containing ethylene glycol. This provided thermal contact between the sample and the probe and a uniform distribution of temperature in the sample. The temperature was controlled to ±0.2 °C with the variable temperature of the NMR instrument. In order to maintain constant hydration during the course of the experiments, a reservoir of water was placed in the NMR tube above the sample. Proton-decoupled ^{31}P NMR spectra were acquired on a General Electric GN-500 spectrometer operating at 202.5 MHz (11.7 T) using the phase-cycled pulse sequence $(90°-\tau_1-180°-\tau_2-\text{acquisition})_n$. The number of acquisitions n ranged from 4500 to 6000, the 90° pulse length was 25 μs, the delay τ_1 was 13 μs, the time before acquisition τ_2 was 10 μs, and the recycle time between sequences was 2 s. A line broadening of 80 Hz was used by applying an exponential multiplication function to the free induction decay before Fourier transformation.

For each sample, a series of spectra was acquired over a temperature range of 25–62 °C. Before acquiring data, each sample was allowed to equilibrate thermally for at least 30 min. Depending on the sample, spectra were acquired at either 11 or 13 temperatures, with intervals between temperatures of 2.5, 5, and 10 °C. The 2.5 °C increments were used over a 15–25 °C range which bracketed the formation of the isotropic NMR signal. Upon completion of the 62 °C spectrum, the sample was cooled to 25 °C and another spectrum acquired. Following this series of experiments, which lasted from 39 to 48 h, the sample appearance changed from a white, viscous material to a translucent, manila-colored material which sat as a discrete, irregularly shaped ball surrounded by clear buffer. The sample with 90% polymerized PC was an exception to this, keeping the same appearance regardless of temperature. Each sample was then subjected to at least 10 freeze-thaw cycles, and a final spectrum was acquired; freeze-thawing invariably returned the lipids to the same white color they had prior to heating. Thin-layer chromatographic analysis (65:25:4:$CHCl_3$/MeOH/H_2O) of the NMR samples revealed no evidence of change in the lipid sample composition during the NMR experiment.

X-ray Diffraction. Two 3:1 mixtures of DOPE/SorbPC were examined with X-ray diffraction, one unpolymerized

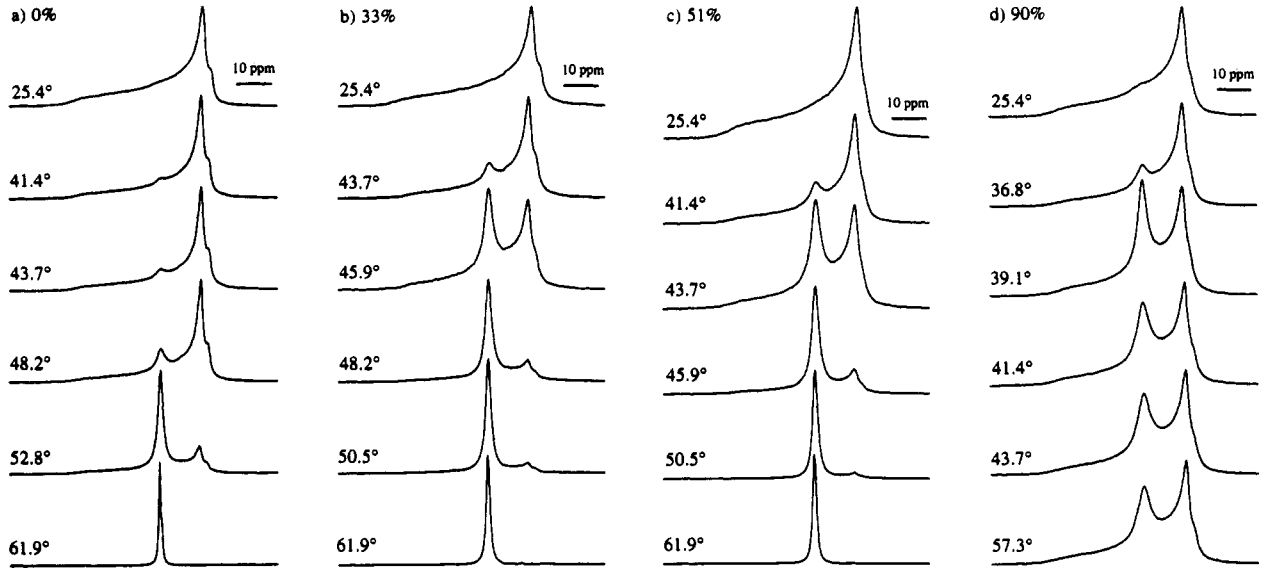


FIGURE 1: Representative proton-decoupled ^{31}P NMR spectra of multilamellar dispersions of DOPE/SorbPC (3:1 molar ratio in excess water) as a function of temperature (in °C, provided next to each spectrum), where the loss of monomeric PC is (a) 0%, (b) 33%, (c) 51%, and (d) 90%. The samples were heated slowly in about 12 increments from 25 to 62 °C, at a rate of 2.5–10 °C per hour. In all cases, the isotropic signal exhibited a pronounced hysteresis, remaining unchanged when cooled to 25 °C; multiple freeze-thaw cycles were required to return the spectra to their original form prior to heating. Partial orientation of the lamellae in the 11.7-T magnetic field can be seen in the enhanced intensity of the edge of the L_α phase spectra corresponding to the $\theta = 90°$ orientation of the bilayer normal with respect to the magnetic field direction.

and one with 28% polymerized PC. The hydrated samples (prepared as described above) were loaded in a dark room into X-ray capillary tubes and sealed. The samples were heated in 10 °C increments every 20 min. X-ray diffraction data were obtained using a Rigaku RU-200 rotating anode X-ray machine equipped with a microfocus cup. The generated Cu Kα X-rays were focused via bent mirror optics (Milch, 1983). Two-dimensional X-ray images were collected with the Princeton SIT area detector as described (Gruner et al., 1982a,b). The digital powder diffraction images were azimuthally integrated along an arc ±10° from the meridional axis to generate plots of X-ray scattering intensity versus scattering angle.

RESULTS

NMR Studies. Proton-decoupled phosphorus NMR spectra of the four DOPE/SorbPC mixtures as a function of increasing temperature are shown in Figure 1. In some of the L_α phase powder patterns, particularly those in Figure 1, panels a and b, there is a shoulder visible on the $\theta = 90°$ edge of the spectrum. This is due to SorbPC, which has a larger chemical shift anisotropy than DOPE, consistent with ^{31}P NMR studies of DOPE/DOPC mixtures (Eriksson et al., 1985). The spectra in Figure 1 outline the phase behavior of the lipid mixtures, showing a progressive increase in the isotropic signal at the expense of the lamellar signal, with increasing temperature. An analogous series of spectra for a hydrated sample of the pure, unpolymerized SorbPC (not shown) remained entirely lamellar with increasing temperature. A decrease in the chemical shift anisotropy was observed between the spectra taken at 25 and 30 °C, which is due to the chain-melting transition of SorbPC at 28 °C.

The ^{31}P NMR line shapes were simulated with a program kindly provided by Per-Olof Eriksson, though the distortion of the spectra due to the orientation of the lipids in the magnetic field (Jansson et al., 1990) reduced the accuracy of the simulations. The L_α phase signals for PE and PC were sufficiently resolved in the unpolymerized and 33% polymerized samples (see Figure 1a,b) to also allow the ratio of PE to PC to be determined for these two samples. The results of the simulation program for the spectra in Figure 1 are given in Table I.

Table I: Percent Isotropic ^{31}P NMR Signal and PE/PC Distribution in Hydrated 3:1 DOPE/SorbPC as a Function of Temperature and SorbPC Photopolymerization[a]

% loss of monomer	T (°C)[b]	% isotropic[c]	PE/PC in L_α phase[c]
none	25.4	0.1 ± 0.1	3.1 ± 0.1
	41.4	1.7 ± 0.1	3.1 ± 0.2
	43.7	4.0 ± 0.2	3.1 ± 0.1
	48.2	8.0 ± 0.4	3.1 ± 0.2
	52.8	46 ± 2	2.0 ± 0.1
	61.9	100	
33.0 ± 0.8	25.4	0.1 ± 0.1	2.9 ± 0.1
	43.7	4.9 ± 0.2	2.8 ± 0.1
	45.9	20 ± 1	2.6 ± 0.1
	48.2	56 ± 3	2 ± 1
	50.5	78 ± 3	3 ± 2
	61.9	100	
51.2 ± 1.6	25.4	0.1 ± 0.1	
	41.4	5.2 ± 0.3	
	43.7	17 ± 1	
	45.9	55 ± 2	
	50.5	82 ± 3	
	61.9	100	
90.4 ± 3.8	25.4	0.8 ± 0.1	
	36.8	6.7 ± 0.3	
	39.1	18 ± 1	
	41.4	16.6 ± 0.8	
	43.7	15.0 ± 0.8	
	57.3	14.5 ± 0.7	

[a] For the ^{31}P NMR spectra in Figure 1. [b] ±0.2 °C estimated error. [c] Determined by simulation of the ^{31}P NMR line shapes.

In all but the most highly polymerized DOPE/SorbPC mixture, the spectra became entirely isotropic between 55 and 60 °C (Figure 1a–c). In the sample where 90% of the SorbPC was polymerized (Figure 1d), only 14% of the lipids contributed to the isotropic signal at 57 °C (Table I). The broad line widths for the isotropic signals in Figure 1d may be due to larger polymer domains (see Discussion). Since the possibility of small unilamellar vesicles in the samples was

eliminated by repeated freeze-thaw cycles prior to heating, the isotropic NMR signals must be due to some arrangement of the lipids forming an extended structure in three dimensions. It is notable that, under the slow heating conditions described under Experimental Procedures, none of the spectra contained an inverted hexagonal phase signal at temperatures up to 62 °C, as normally observed in PE/PC mixtures (Lindblom & Rilfors, 1989). In order to test whether a H_{II} phase occurred at higher temperatures, both polymerized and unpolymerized samples were heated under the same conditions to about 100 °C: the ^{31}P NMR spectra (acquiried on a GN-300 spectrometer at 121.7 MHz) remained purely isotropic.

The isotropic NMR signal was very stable after it was formed. When the samples were cooled to 25 °C following heating, no reduction in the isotropic signal was observed. One sample (46% polymerized) was observed after over 5 months at room temperature, and the 25 °C spectrum still consisted of one isotropic signal. The original lamellar powder pattern could be restored by repeated freezing and thawing between dry ice/acetone or liquid nitrogen temperatures and room temperature. A similar thermal hysteresis has been reported for other hydrated lipid systems which produce isotropic NMR signals between the L_α and H_{II} phases, e.g., in DOPE-Me and mixtures of DOPE and DOPC (Cullis et al., 1978a; Ellens et al., 1986, 1989; Gruner et al., 1988). Deep freezing to temperatures well below the L_α–gel phase transition appears to reset the lamellar structure, so that heating to room temperature gives the L_α phase.

Each isotropic sample (such as those producing the 61.9 °C spectra in Figure 1a–c) appeared as a discrete, manila-colored, irregularly shaped ball of lipids that was surrounded by clear buffer. The ball of lipids maintained its form even when it was jostled about in the buffer with a thin metal rod. This physical state differs drastically from that of hydrated extended bilayers or large liposomes, which form a homogeneous, white mixture. The appearance of lipids as a discrete ball in clear water has been observed in many cubic phase lipid–water systems (G. Lindblom, personal communication). Multiple freeze-thaw cycles invariably caused the discrete ball of lipids to revert to a white, viscous mixture.

It should be noted that the phase behavior of these samples strongly depends on the *rate* at which the samples are heated. Following the slow heating series of NMR experiments, described above, the sample with 90% polymerized PC was cooled to 25 °C and then heated rapidly (in less than a minute) to 66.5 °C. The NMR spectrum acquired from 8 to 49 min after heating was predominantly isotropic, but with a H_{II} signal of 18 ± 1%. A second spectrum of the same sample was taken from 75 to 116 min after heating and showed a drop in the intensity of the H_{II} signal to 8 ± 3%. Therefore, in a sample where slow heating formed none of the H_{II} phase and no more than 18% of the cubic phase, rapid heating produced a small H_{II} signal and a much greater conversion to the cubic phase. In a sample with ~70% polymerization of SorbPC, rapid heating to 70 °C resulted in a spectrum that was completely isotropic except for a trace of the H_{II} phase. However, when the same sample was freeze-thawed and then reheated *slowly*, a small H_{II} signal appeared at 40 °C in addition to the L_α and isotropic signals and continued to increase to about 10% of the total spectral area as the temperature was raised to 57 °C. This dependence of the phase behavior of lipid systems on rates of heating has also been reported by other groups (Gruner et al., 1988; Sjölund et al., 1989; Siegel & Banschbach, 1990).

Three temperatures in the course of the transition were used to characterize the formation of the structure which gives rise to the isotropic NMR signals: (a) the temperature at which 10% of the lipids were isotropic was used to represent the onset of the transition; (b) the midpoint of the transition, where 50% of the lipids were isotropic; and (c) the lowest temperature at which all lipids were isotropic. These values, which we refer to as T_I (10%), T_I (50%), and T_I (end), were obtained by interpolating from graphs of temperature versus the percent of isotropic lipids (calculated from the simulation program). They are compiled in Table II as a function of the loss of monomeric SorbPC. Decreasing the amount of monomeric SorbPC by photopolymerization lowered the transition temperature to the isotropic phase (T_I), consistent with earlier studies on mixtures of PE and PC in which higher proportions of PE decreased the L_α-to-nonlamellar transition temperature (Cullis & de Kruijff, 1978; Tilcock et al., 1982; Boni & Hui, 1983; Eriksson et al., 1985; Ellens et al., 1986).

Table II: Effect of SorbPC Photopolymerization on the Temperature of the L_α-to-Isotropic Phase Transition of Hydrated 3:1 DOPE/SorbPC[a]

% loss of monomer	DOPE/monomeric PC	temperature (°C)		
		T_I (10%)	T_I (50%)	T_I (end)
none	3.1 ± 0.1	49.1 ± 0.5	54 ± 1	58 ± 3
33.0 ± 0.8	4.3 ± 0.2	44.8 ± 0.5	47.8 ± 0.4	55 ± 2
51.2 ± 1.6	6.1 ± 0.2	42.7 ± 0.5	45.6 ± 0.4	54 ± 1
90.4 ± 3.8	31 ± 1	38.1 ± 0.6		

[a] Temperatures (°C) are given at the points where 10%, 50%, and 100% of the lipids contribute to the isotropic ^{31}P NMR signal, called T_I (10%), T_I (50%), and T_I (end), respectively.

X-ray Studies. The X-ray diffraction data were obtained after azimuthal integration of diffraction images for two types of samples of 3:1 DOPE/SorbPC, unpolymerized and 28% polymerized. The data for the unpolymerized sample at 20 °C (Figure 2) shows three prominent peaks which are consistent with diffraction patterns obtained from a lamellar lattice. The first-order lamellar peak occurs at $(63.1 \pm 1\ \text{Å})^{-1}$. When the sample was heated to 50 °C, the peaks in Figure 2 corresponding to a lamellar lattice disappeared and additional weaker peaks appeared. Four peaks on either side of the beam stop could be discerned on top of a broad background that indicated a lack of long-range order in the sample. The four peaks by themselves were not sufficient for an unambiguous identification of the crystalline lattice. However, these peaks were sufficient to show that the lattice was inconsistent with the presence of either a lamellar or an inverted hexagonal phase in the sample. The positions of the peaks were consistent with the presence of a cubic phase of *Pn3m* symmetry that has been observed previously in similar lipid–water systems (Tate et al., 1991).

A second unpolymerized 3:1 DOPE/SorbPC sample was examined after the sample temperature was slowly raised from room temperature to 62 °C following the protocol used in the acquisition of NMR data. The broad diffraction pattern (Figure 3) shows the absence of long-range order in this sample as well. The diffraction did not change significantly when the sample was cooled to 25 °C. The diffuse patterns in Figure 3 are associated with a sample that exhibits an isotropic line in the NMR data at 62 °C and after cooling to room temperature. In order to identify the structure of the unpolymerized 3:1 DOPE/SorbPC sample at 62 °C, the sample was subjected to extensive thermal cycling as described in Shyamsunder et al. (1988). The lipid–water system then formed a well-defined lattice corresponding to a cubic phase, as shown in Figure 4. The spacing between the peaks falls in the ratio $\sqrt{2}:\sqrt{3}:\sqrt{4}:\sqrt{6}$ as would be expected of a *Pn3m* or a *Pn3* cubic space group. The sample had a pool of bulk

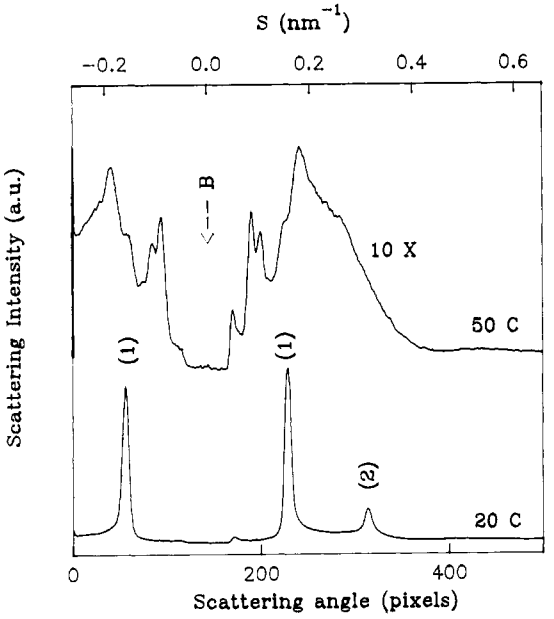


FIGURE 2: Azimuthal integrations of the two-dimensional X-ray diffraction patterns of extended bilayers of 3:1 DOPE/SorbPC that are unpolymerized at 20 and 50 °C. Note that the data for the two temperatures have been displaced for clarity. The arrow indicates the position of the unscattered X-ray beam, blocked by a beam stop. The first-order lamellar peak indicated at 20 °C is at $(63.1 \pm 1\ \text{Å})^{-1}$. S is the magnitude of the scattering vector. At 50 °C, the unpolymerized sample exhibits additional peaks characteristic of a *nonlamellar* lattice (see Figure 4). Note that the data at 50 °C have been scaled by 10×, indicating that the scattering intensity of the Bragg peaks is much smaller than that of the lamellar peaks at 20 °C. B indicates the position of the beamstop.

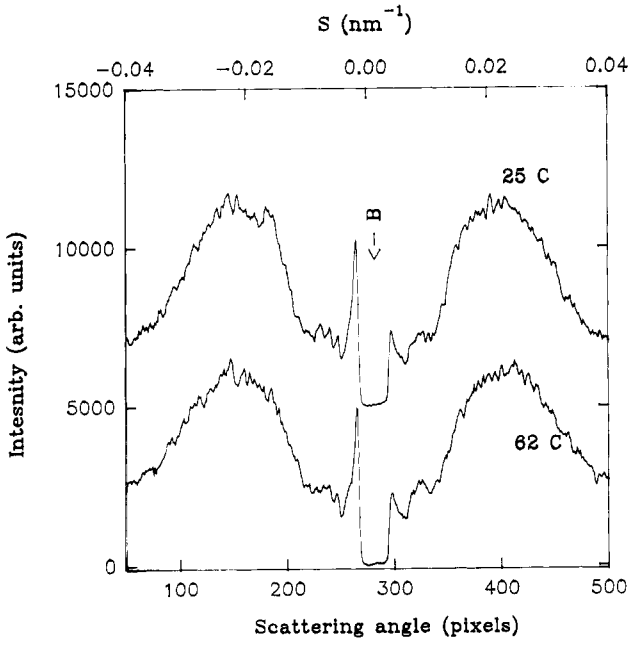


FIGURE 3: Results of the X-ray diffraction studies on the unpolymerized sample after slow heating to 62 °C and then subsequent cooling back to 25 °C. The curves have been displaced vertically for clarity. The most characteristic feature of both patterns is the *absence* of any Bragg peaks that are indicative of either the lamellar or the inverted hexagonal phase and the *presence* of rather broad bumps that signal a lack of long-range order. Again, S is the magnitude of the scattering vector. Thermal cycling of the sample permits the formation of the pattern shown in Figure 4, which is indicative of a cubic phase as discussed in the text. B indicates the position of the beamstop.

(excess) water in coexistence with the lipid. Samples prepared with different amounts of water had comparable unit cell

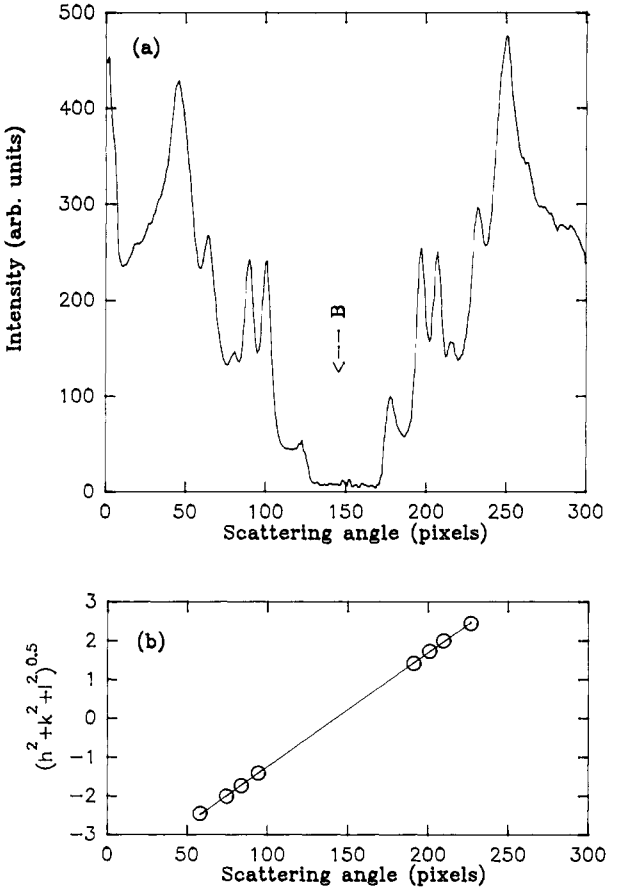


FIGURE 4: (a) Densitometrization of the two-dimensional X-ray diffraction pattern for the unpolymerized sample at 25 °C, after thermal cycling across the lamellar to nonlamellar transition. (b) Plot of $(h^2 + k^2 + l^2)^{1/2}$ versus measured Bragg peak positions in panel a. The spacing between the peaks falls in the ratios $\sqrt{2}$:$\sqrt{3}$:$\sqrt{4}$:$\sqrt{6}$ as would be expected of a *Pn3m* or a *Pn3* cubic space group as observed in aqueous DOPE samples (Shyamsunder et al., 1988). A broad, disordered underlying component is also observed.

spacings, which indicates that the cubic phase is inverted. These results suggest that heating of the unpolymerized sample yields a precursor to the well-defined inverted cubic phase that forms with thermal cycling.

The partially polymerized 3:1 DOPE/SorbPC sample at 20 °C shows a first-order lamellar peak at $(66.2 \pm 1\ \text{Å})^{-1}$. When the sample was heated to 50 °C, the intensity of the lamellar pattern was reduced many fold, indicating that the lamellar phase was being replaced by a poorly defined nonlamellar structure. Thermal cycling of this sample produced the same cubic lattice that was observed in the unpolymerized sample (cf. Figure 4), although higher temperatures were required on the high-temperature end of the cycling to make the transition from the diffuse precursor to the well-defined cubic lattice.

DISCUSSION

The results of this work are consistent with the formation of a precursor to an inverted cubic phase in hydrated 3:1 DOPE/SorbPC mixitures, which upon thermal cycling becomes a well-defined cubic phase. An isotropic ^{31}P NMR signal appeared upon a single slow heating from the L_α phase, which was stable until the lipids were subjected to repeated freeze-thaw cycles. Prior to thermal cycling, the X-ray diffraction pattern obtained with a single slow heating was diffuse, indicating the absence of a well-defined phase with long-range order. The X-ray diffraction patterns obtained

after thermal cycling matched a *Pn3m* or *Pn3* space group associated with a bicontinuous cubic phase; the cubic phase was assigned as inverted because the lattice parameters remained constant with dilution. The diffuse X-ray diffraction patterns and isotropic NMR signals are therefore attributed to nonvesicular structures that are precursors to a well-defined, inverted cubic phase, which will hereafter be referred to in this paper as "the precursor".

It has been suggested that the occurrence of cubic phases as an intermediate between lamellar and inverted hexagonal phases is a universal feature of lipid–water systems (Shyamsunder et al., 1988). Indeed, there are many reports of the formation of stable cubic phases without the need for temperature cycling [e.g., Eriksson et al. (1985) and Ellens et al. (1989)]. In PE systems with lipid molecules having two hydrocarbon tails, the observation of a cubic phase may be hindered by kinetic barriers, leading to phase transitions that can in extreme cases take place over time scales that can be as long as several years (Gruner et al., 1988) before a well-defined Bragg diffraction pattern is observed. A signature of such extraordinarily sluggish phase transitions is a diffuse X-ray scattering, corresponding to the loss of long-range order. When incubated for a long period of time, the diffuse X-ray scattering is replaced by well-defined Bragg peaks characteristic of a cubic lattice. The kinetic barriers can be overcome by the trick of temperature cycling (Shyamsunder et al., 1988; Veiro et al., 1990). The experiments reported here suggest that mixtures of DOPE/SorbPC exhibit the same slow kinetics and exhibit diffuse X-ray scattering which can be induced to form a well-defined cubic phase upon temperature cycling. We may consider the NMR signals as well as the diffuse X-ray scattering to be evidence of the precursor to the cubic phase.

From the PE/PC ratios in the L_α phase determined from ^{31}P NMR (Table I), the PE appears to be preferentially incorporated into the precursor during the early stages of the transition. At 62 °C, however, the NMR data contain no characteristics of either the lamellar or inverted hexagonal phases, indicating that *all* lipids entered into the precursor structure (except in the case of the 90% polymerized mixture, discussed below). Thermal cycling of the unpolymerized and 28% polymerized bilayers produced an inverted cubic phase which was composed of all the lipids. Since SorbPC and DOPE *by themselves* form the lamellar and inverted hexagonal phases, respectively, we would expect to see evidence of these phases in the high temperature NMR and X-ray data if extensive phase separation of these lipids had occurred.

It is apparent from the restricted formation of the precursor in the highly polymerized sample that there are two corresponding factors introduced by PC polymerization which influence the phase behavior of the DOPE/SorbPC mixtures. Polymerization increases the ratio of DOPE to monomeric PC, which favors nonlamellar phases (as evidenced by the decreasing transition temperature from the L_α phase with increasing loss of monomeric PC). Eventually, however, a point is reached when so many of the SorbPC lipids are polymerized that some lipids are trapped in the lamellar phase. The tradeoff between the two factors may be affected by increased polymer domain size as well as the restricted motion in the individual tails of the PC chains. The fact that complete formation of the precursor was not only allowed but favored with about half of the PC polymerized indicates that sufficiently small polymerized domains are included in the precursor structure giving rise to the isotropic signal. Preliminary data obtained on linear polymers from a mono-SorbPC (Lamparski et al., 1992) indicate that the number of repeat units is only 10–100. Inhibition of formation of the precursor in the 90% polymerized sample suggests that, with increasing exposure to UV radiation, the smaller polymer domains eventually polymerize to one another to form domains too large to form nonlamellar structures. Also, the *monomeric* lipid composition in the highly polymerized sample consists of 97% DOPE. Such a mixture, in the absence of polymerized PC, would be expected to form the H_{II} phase. The absence of the H_{II} phase under these conditions, as well as the significantly curbed formation of the precursor, indicates that, in the limit of nearly complete polymerization of SorbPC, the PE remains mixed with the PC and is to a great extent included in the polymer domains.

The polymerization of SorbPC causes the monomeric domains to be enriched in PE (see Table II), with a consequent decrease in the spontaneous radius of curvature. This increases the probability of bilayer contact due to the decreased hydration of PE relative to PC (Parsegian et al., 1979; Rand, 1981). Multiple points of contact in the extended bilayers increase the probability of formation of isotropic lipid structures, e.g., interlamellar attachments (Siegel, 1986a,b), between the lamellae. Initially, these isotropic lipid structures are expected to be preferentially composed of PE as observed. As more of the assembly changes from lamellar to the precursor, more of the SorbPC and eventually the poly-SorbPC domains participate in the precursor structure as well. In addition, it should be noted that since the poly-SorbPC domains on each side of the bilayer are not covalently linked, they are free to move independently within a leaflet of the bilayer. Even though the polymerizable group is close to the tail of the lipid, the nature of the 1,4-polymerization in these lipids (Lamparski et al., 1992) makes it very unlikely that a growing polymer on one side of the bilayer can react with monomers on the opposite side.

The ^{31}P NMR spectra in this work are similar to those reported by Gruner et al. (1988) for a lipid mixture of 85% soya PE and 15% egg PC (dry wt %) in H_2O. They observed a progressive increase in isotropic signal as the temperature was raised from 20 to 40 °C and then to 60 °C. The phase behavior of this PE/PC mixture closely followed the nonequilibrium behavior of hydrated DOPE-Me bilayers as they were slowly heated and cooled (Gruner et al., 1988). In both the DOPE-Me and PE/PC samples, the isotropic nonlamellar structures were shown by X-ray diffraction to be lipid structures with cubic symmetry. Each of these lipid samples is an example of a system with an intermediate value for the spontaneous radius of curvature, R_0 (Kirk et al., 1984; Gruner, 1985). Lipids with large R_0 values form stable lamellar L_α phases. If the spontaneous radius of curvature of a lipid assembly is small, as in DOPE, the formation of the H_{II} phase is facilitated by a significant drop in free energy. Lipid assemblies with intermediate R_0 values, such as DOPE-Me (Gruner et al., 1988) and selected mixtures of DOPE/DOPC (Ellens et al., 1986), show nonequilibrium phase behavior with the appearance of inverted cubic as well as inverted hexagonal structures. The data presented here indicate that the DOPE/SorbPC system is another example of a lipid mixture with intermediate R_0 values.

In summary, slow heating of both polymerized and unpolymerized extended bilayers of 3:1 DOPE/SorbPC leads to the formation of a structure which produces an isotropic NMR signal and which persists over a wide temperature range. This structure is apparently a precursor to an inverted cubic phase, since thermal cycling yields a well-defined inverted cubic phase appearing to belong to the *Pn3m* or *Pn3* space group.

Photopolymerization of SorbPC changes the composition of the monomeric PE/PC bilayers and therefore the phase equilibrium between the L_α and isotropic precursor. The implication of this new technique has already been demonstrated as a means of destabilizing oligolamellar 3:1 DOPE/SorbPC liposomes in suspension (Frankel et al., 1989; Lamparski et al., 1992). Future applications in the promotion and stabilization of nonlamellar lipid structures can be readily envisioned.

ACKNOWLEDGMENT

We thank Ulrich Liman for participation in the preliminary stages of this work, Göran Lindblom of the University of Umeå and Sol M. Gruner of Princeton University for helpful discussions, and Per-Olof Eriksson of the University of Umeå for the line shape simulation program.

REFERENCES

Barry, J. A., Lamparski, H., Brown, M. F., & O'Brien, D. F. (1991) *Biophys. J. 59*, 129a.
Boni, L. T., & Hui, S. W. (1983) *Biochim. Biophys. Acta 731*, 177–185.
Brown, P. M., & Silvius, J. R. (1989) *Biochim. Biophys. Acta 980*, 181–190.
Connor, J., Yatvin, M., & Huang, L. (1984) *Proc. Natl. Acad. Sci. U.S.A. 81*, 1715–1718.
Cullis, P. R., & de Kruijff, B. (1978) *Biochim. Biophys. Acta 507*, 207–218.
Cullis, P. R., & de Kruijff, B. (1979) *Biochim. Biophys. Acta 559*, 399–420.
Cullis, P. R., Dijck, P. W. M. V., de Kruijff, B., & de Gier, J. (1978a) *Biochim. Biophys. Acta 513*, 21–30.
Cullis, P. R., Verkleij, A. J., & Ververgaert, P. H. J. T. (1978b) *Biochim. Biophys. Acta 513*, 11–20.
Cullis, P. R., Hope, M. J., de Kruijff, B., Verkeij, A. J., & Tilcock, C. P. S. (1985) in *Phospholipids and Cellular Regulation* (Kuo, J. R., Ed.) pp 1–59, CRC Press, Boca Raton, FL.
Deamer, D. W., Leonard, R., Tardieu, A., & Branton, D. (1970) *Biochim. Biophys. Acta 219*, 47–60.
Duzgunes, N., Straubinger, R. M., Baldwin, P. A., Friend, D. S., & Paphadjopoulos, D. (1985) *Biochemistry 24*, 3091–3098.
Ellens, H., Bentz, J., & Szoka, F. C. (1984) *Biochemistry 23*, 1532–1538.
Ellens, H., Bentz, J., & Szoka, F. C. (1986a) *Biochemistry 25*, 285–294.
Ellens, H., Bentz, J., & Szoka, F. C. (1986b) *Biochemistry 25*, 4141–4147.
Ellens, H., Siegel, D. P., Alford, D., Yeagle, P., Boni, L., Lis, L. J., Quinn, P. J., & Bentz, J. (1989) *Biochemistry 28*, 3692–3703.
Epand, R. M. (1985) *Chem. Phys. Lipids 36*, 387–393.
Eriksson, P.-O., Rilfors, L., Lindblom, G., & Arvidson, G. (1985) *Chem. Phys. Lipids 37*, 357–371.
Frankel, D. A., Lamparski, H., Liman, U., & O'Brien, D. F. (1989) *J. Am. Chem. Soc. 111*, 9262–9263.
Frederik, P. M., Stuart, M. C. A., & Verkleij, A. J. (1989) *Biochim. Biophys. Acta 979*, 275–278.
Gruner, S. M. (1985) *Proc. Natl. Acad. Sci. U.S.A. 82*, 3665–3669.
Gruner, S. M. (1987) in *Liposomes: From Biophysics to Therapeutics* (Ostro, M. J., Ed.) pp 1–38, Marcel Dekker, New York.
Gruner, S. M., Milch, J. R., & Reynolds, G. T. (1982a) *Rev. Sci. Instrum. 53*, 1770–1778.
Gruner, S. M., Milch, J. R., & Reynolds, G. T. (1982b) *Nucl. Instrum. Methods Phys. Res. 195*, 287–297.
Gruner, S. M., Tate, M. W., Kirk, G. L., So, P. T. C., Turner, D. C., Keane, D. T., Tilcock, C. P. S., & Cullis, P. R. (1988) *Biochemistry 27*, 2853–2866.
Gulik-Krzywicki, T. (1975) *Biochim. Biophys. Acta 415*, 1–28.
Hui, S. W. (1988) *Curr. Top. Membr. Transp. 29*, 29–70.
Hui, S. W., & Sen, A. (1989) *Proc. Natl. Acad. Sci. U.S.A. 86*, 5825–5829.
Hui, S. W., Stewart, T. P., Yeagle, P. L., & Albert, A. D. (1981) *Arch. Biochem. Biophys. 207*, 227–240.
Hui, S. W., Stewart, T. P., & Boni, L. T. (1983) *Chem. Phys. Lipids 33*, 113–126.
Jansson, M., Thurmond, R. L., Trouard, T. P., & Brown, M. F. (1990) *Chem. Phys. Lipids 54*, 157–170.
Kirk, G. L., Gruner, S. M., & Stein, D. K. (1984) *Biochemistry 23*, 1093–1102.
Lamparski, H., Liman, U., Frankel, D. A., Barry, J. A., Ramaswami, V., Brown, M. F., & O'Brien, D. F. (1992) *Biochemistry 31*, 685–694.
Leventis, R., Gagne, J., Fuller, N., Rand, R. P., & Silvius, J. R. (1986) *Biochemistry 25*, 6978–6987.
Lindblom, G., & Rilfors, L. (1989) *Biochim. Biophys. Acta 988*, 221–256.
Luzzati, V., & Reiss-Husson, F. (1962) *J. Cell Biol. 12*, 207–219.
Luzzati, V., Gulik-Kuxywicki, T., & Tardieu, A. (1968) *Nature 218*, 1031–1034.
Milch, J. R. (1983) *J. Appl. Crystallogr. 16*, 198–203.
O'Brien, D. F., & Ramaswami, V. (1989) in *Encyclopedia of Polymer Science and Engineering 17*, second ed, pp 108–135, John Wiley & Sons, New York.
Osterberg, F., Cerne, J., Shyamsunder, E., Gruner, S. M., Lamparski, H., & O'Brien, D. F. (1991) *Biophys. J. 59*, 128a.
Parsegian, A., Fuller, N, & Rand, R. P. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 2750–2754.
Rand, R. P. (1981) *Annu. Rev. Biophys. Bioeng. 10*, 277–314.
Ringsdorf, H., Schlarb, B., & Venzmer, J. (1988) *Angew. Chem., Int. Ed. Engl. 27*, 113–158.
Shyamsunder, E., Gruner, S. M., Tate, M. W., Turner, D. C., So, P. T. C., & Tilcock, C. P. S. (1988) *Biochemistry 27*, 2332–2336.
Seddon, J. M. (1990) *Biochim. Biophys. Acta 1031*, 1–69.
Siegel, D. P. (1986a) *Biophys. J. 49*, 1155–1170.
Siegel, D. P. (1986b) *Biophys. J. 49*, 1171–1183.
Siegel, D. P., & Banschbach, J. L. (1990) *Biochemistry 29*, 5975–5981.
Siegel, D. P., Burns, J. L., Chesnut, M. H., & Talmon, Y. (1989) *Biophys. J. 56*, 161–169.
Sjölund, M., Rilfors, L., & Lindblom, G. (1989) *Biochemistry 28*, 1323–1329.
Strolley, J. G., & Vail, W. J. (1977) *Biochim. Biophys. Acta 471*, 372.
Tilcock, C. P. S., Bally, M. B., Farren, S. B., & Cullis, P. R. (1982) *Biochemistry 21*, 4596–4601.
van Dijck, P. W. M., de Kruijff, B., Van Deenen, L. L. M., de Gier, J., & Demel, R. A. (1976) *Biochim. Biophys. Acta 455*, 576–587.
Veiro, J. A., Khalifah, R. G., & Rowe, E. S. (1990) *Biophys. J. 57*, 637–641.
Verkleij, A. J. (1984) *Biochim. Biophys. Acta 779*, 43–63.
Wiedmann, T. S., Pates, R. D., Beach, J. M., Salmon, A., & Brown, M. F. (1988) *Biochemistry 27*, 6469–6474.