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Small concentrations of alamethicin induce a cubic phase in bulk phosphatidylethanolamine mixtures

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Abstract

Under normal conditions, excess water dispersions of liquid crystalline 1,2-dielaidoyl-*sn*-glycero-3-phosphoethanolamine (DEPE) are known to convert from a liquid crystalline lamellar (L_α) to inverse hexagonal (H_{II}) phase at about 60°C. The nonlamellar phase behavior of lipid systems is also known to depend on the monolayer spontaneous curvature. The single-channel activity of alamethicin in black lipid bilayer membranes has been shown to be dependent upon the lipid composition of the membrane. Since the monolayer spontaneous curvature properties (e.g., the monolayer spontaneous curvature, curvature coefficients and bilayer thickness) vary with lipid composition, the single-channel activity of alamethicin presumably also correlates with the monolayer spontaneous curvature properties. Accordingly, we reasoned that if alamethicin couples to the curvature properties of a lipid film, then the curvature properties must, in turn, be perturbed by the presence of alamethicin and that this perturbation should be observable in the lipid phase behavior. Here X-ray diffraction and NMR are used to show that the presence of as little as 1% alamethicin introduces a large region of cubic phase into the thermal phase diagram. This suggests that perturbation of the nonlamellar phase behavior of a lipid system may be a method to survey different channel-forming molecules for possible behavior that indicates that the ion channel is sensitive to the monolayer spontaneous curvature properties.

Keywords: Alamethicin; Cubic phase; Lipid–protein interaction

1. Introduction

Alamethicin is a small polypeptide (mol. wt. approx. 1600) which has been extensively studied as a model for ion channels in lipid bilayers (for reviews, see Hall [1], Latorre and Alvarez [2], Woolley and Wallace [3], and Sansom [4,5]). The effect of alamethicin on typical lipids such as phosphatidylcholines (PC's) and phosphatidylserines (PS's) has been extensively investigated (Chapman et al. [6], Hauser et al. [7], Lau and Chan [8,9], McIntosh et al. [10], and Das and Balaram [11]). These lipids tend to form lamellar bilayers when purified and hydrated under physiological conditions. However, there has been little exploration of the phase behavior of mixtures of alamethicin and lipids which tend to form nonlamellar phases, most notably H_{II}-phases, such as unsaturated phosphatidylethanolamines (PE's).

H_{II}-prone lipids, which frequently comprise large fractions of biomembrane bilayer compositions (Cullis et al. [12]), possess a large spontaneous curvature which is responsible for the formation of relatively low temperature H_{II} phases (Gruner [13]). It has been postulated that the presence of H_{II}-prone lipids in bilayer membranes induces a frustrated curvature stress which may couple to the conformational states of embedded membrane proteins and, consequently, may alter the activity of these proteins (Gruner [13,14], Hui and Sen [15], and Tate et al. [16]). Recent experiments on mammalian proteins have provided support for this model (Streicher-Scott [17], Gibson and Brown [18]). Single-channel conductance experiments on alamethicin have reported a large effect which appears to be non-site-specific, i.e., influenced not by the particular binding of the protein to the lipid (Keller et al. [19]). It would be of considerable interest to know if embedded membrane molecules interact energetically with the curva-

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ture stress field. In the case of alamethicin, this interaction can be probed by the effect on the relative probability of the conductance levels of the peptide. Unfortunately, the activity of most molecules is not associated with a readily probed signal, such as multiple conductance levels. In consequence, it might be difficult to assay if there is an energetic interaction of the molecule with the curvature field.

A substantial energetic interaction of the bilayer lipids with embedded molecules through a curvature stress field must perturb both the molecules and the field. An alternative to searching for effects of lipid composition (and, hence, curvature stress) upon the molecule, which might be difficult to assay in a membrane system, is to search for the effect of the molecule on the lipids. Since the activity of alamethicin is known to be sensitive to lipid composition (and presumed to be sensitive to a bilayer's curvature stress field; Keller et al. [19]), we sought to test this idea by examining the effect of small concentrations of alamethicin upon the lipid phase behavior. The rationale behind this experiment is that the nonlamellar phase behavior is known to be sensitive to the curvature stress (Gruner [13], Tate et al. [16]), and therefore, effects of alamethicin upon this stress field should be apparent as perturbations of the phase behavior of the lipid. It was found that the presence of very small weight fractions of alamethicin in excess water dispersions of 1,2-dielaidoyl-*sn*-glycero-3-phosphoethanolamine produces striking nonlamellar phase behavior.

2. Materials and methods

Avanti Polar Lipids (Alabaster, AL) provided DEPE, 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine (DOPE), and 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC) which have molecular weights of 743, 743 and 786 g/mol, respectively. These lipid powders were used without further purification. The lipids were shown to be at least 98% free of fatty acid and lysolipids by thin-layer chromatography.

Alamethicin A 4665 was obtained from Sigma (St. Louis, MO). This material contained at least three homologues, one of which may have been charged. Alamethicin powder was dissolved into methanol (1 mg/50 μl). The alamethicin solution was then added to DEPE in benzene in the desired concentration to a cone-bottom 1 ml vial containing DEPE and benzene. The vial and its contents were sealed in a larger plastic vial and frozen at -80°C for 15 min. The contents of the plastic vial were placed under vacuum overnight while the plastic vial sat in a bath of evaporating liquid nitrogen. The lyophilized lipid was fully hydrated by adding deionized water, with extensive stirring, until bulk excess water was observed to coexist with the lipid dispersion (usually $\approx$ 1:1 lipid/water by weight).

The phase behavior was determined via X-ray diffraction and NMR. X-ray diffraction samples were prepared by transferring some of the lipid mixture, including excess water, to acid-cleaned 1.5 mm diameter glass X-ray capillaries by means of a positive displacement pipette. The capillaries were sealed with 5-min epoxy, leaving a small air gap between the top of the lipid-water mass and the epoxy plug. Capillaries were stored at room temperature and at least a week usually elapsed between when the capillaries were made and when they were analyzed. After X-ray diffraction was performed on the capillaries, they were stored at room temperature. These samples could be re-analyzed at later times.

The X-ray diffraction apparatus and procedures are described in Gruner et al. [20] and [21]. Briefly, the samples were step-wise temperature-ramped from 10°C to 90°C in 5 C° steps. After at least a 10-min equilibration time at each temperature, a diffraction pattern, typically 100 to 400 s in duration, was acquired on a 2-dimensional X-ray detector. The sequences of powder-pattern rings in the diffraction patterns were analyzed to determine both the phase of the lipid dispersion and the lattice constant of the phase at each temperature. NMR experiments were performed from 30°C to 90°C on a Bruker MSL 300 spectrometer as previously described in Gawrisch et al. [22].

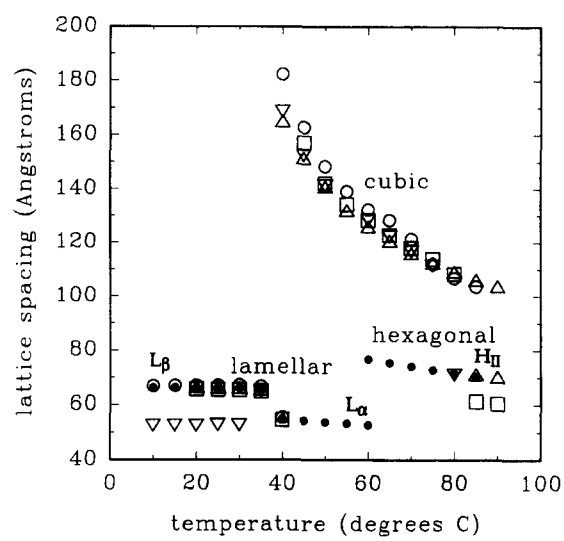

Fig. 1. Phase diagram and lattice repeat spacings of DEPE/alamethicin mixtures in excess water. These lattice spacings, or lattice basis lengths, were obtained from the X-ray diffraction patterns acquired during heating runs of the sample (see Methods); similar plots were observed upon cooling. Filled symbols denote the behavior of DEPE in the absence of alamethicin. Without alamethicin, as the temperature ascends, the system passes through the L_β and L_α lamellar phases and into the H_{II}. The H_{II} spacing shown here is the hexagonal basis. Open circles, squares, downward triangles and upward triangles denote the presence of alamethicin in DEPE as 1%, 3%, 5%, and 10% respectively. Now the system is observed to pass through lamellar, cubic, and hexagonal phases. The L_β spacings of samples of 5% alamethicin are the same as those with 1%, 3% or 10% alamethicin. Exposures are usually separated in time by approx. 10 min. As the equilibration time increases, coexistence of two phases occurs over smaller temperature spans.

3. Results

The phases and lattice dimensions of excess-water DEPE/alamethicin mixtures are shown in Fig. 1. In the absence of alamethicin, DEPE exhibits a L_β–L_α phase transition at about 38°C and a L_α–H_{II} transition at about 60°C, consistent with the results of So [23]. The trends of lattice repeat spacings are similar in form to those observed in the DOPE excess water system (e.g., see Tate and Gruner [24]); the L_β phase repeat is practically temperature invariant. The L_β–L_α phase transition is seen as a sharp drop in spacing to a mildly temperature-sensitive value followed by a sharp rise to the repeat spacing of the H_{II} phase. The H_{II} phase lattice repeat is characterized by a slowly arching decrease with temperature. This decrease has been shown in the DOPE system to correspond to a decrease in the spontaneous radius of curvature with temperature, which is driven by a very small, progressive thinning of the lipid layer thickness (Tate and Gruner [24]). As the spontaneous radius of curvature falls, the radius of the water cores of the H_{II} tubes also falls and water is expelled into the bulk water phase.

^{31}P-NMR spectra agree with the transition temperatures and phase assignments of the X-ray data. Fig. 3a, b, and c show DEPE in the L_β (30°C), L_α (50°C), and H_{II} (90°C) phases, respectively. The L_β–L_α transition of DEPE is characterized by a change in the chemical shift anisotropy ($\Delta\sigma$) from −50 ppm to −40 ppm. Hexagonal phases possess $\Delta\sigma$ of about 17 ppm.

The phase behavior of DEPE in the presence of even very small amounts of alamethicin is dramatically different (Fig. 1). Even for concentrations as low as 1% alamethicin to DEPE by weight, as temperature rises, a cubic phase spontaneously self-assembles between 40°C and 85°C (the upper temperature limit of the X-ray work). The behavior seen upon cooling is similar, except for a small amount of hysteresis in the transition temperature, an effect which is not uncommon in H_{II}-forming systems (Shyamsunder et al. [25]). The X-ray lattice spacing and temperature range of this cubic phase does not change over an alamethicin concentration range of 1% to 10%, suggesting that the effect of the addition of alamethicin is saturated at a concentration of about 1%. Although this is not direct evidence of phase separation of lipid and alamethicin above 1% alamethicin concentration, it is highly suggestive. At 0.5% alamethicin, a cubic phase still appears, but its lattice spacing is longer and its temperature span shorter. Alamethicin concentrations below 0.5% and above 10% were not explored.

Fig. 2 exhibits the assignment of diffraction peaks. The pattern is symmetric about the main beam (obscured here by the beamstop). For a system of 5% alamethicin in DEPE at 60°C, diffraction peaks are spaced in the ratios $\sqrt{2}:\sqrt{3}:\sqrt{4}:\sqrt{6}:\sqrt{8}:\sqrt{9}:\sqrt{10}:\sqrt{11}:\sqrt{12}:\sqrt{14}$, consistent with the $Pm\bar{3}n$ 'double diamond' cubic space group (Intl. Tables for X-ray Crystallography [26]) and a unit cell spacing of 127 Å.

The addition of alamethicin to the DEPE system does

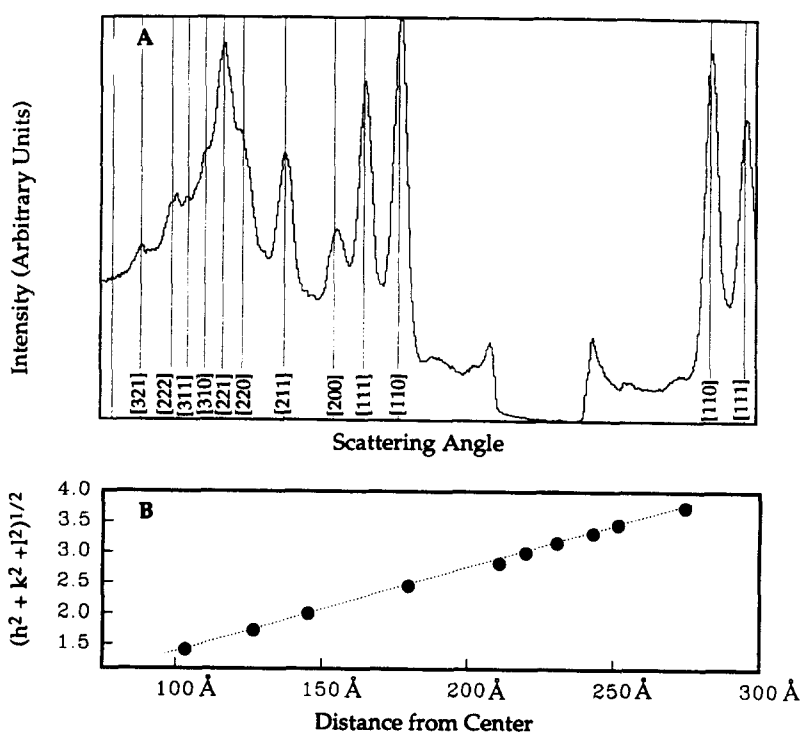

Fig. 2. (A) Slice through powder diffraction rings from mixtures of 5% by weight alamethicin in DEPE and excess water at 60°C. Lines mark predicted peaks in a double diamond cubic lattice of unit cell spacing 127 Å. (B) $(h^2 + k^2 + l^2)^{1/2}$ vs. measured distances from the beam center in Fig. 2A. Characteristics of the $Pm\bar{3}n$ cubic space group; these distances appear in the ratio $\sqrt{2}:\sqrt{3}:\sqrt{4}:\sqrt{6}:\sqrt{8}:\sqrt{9}:\sqrt{10}:\sqrt{11}:\sqrt{12}:\sqrt{14}$.

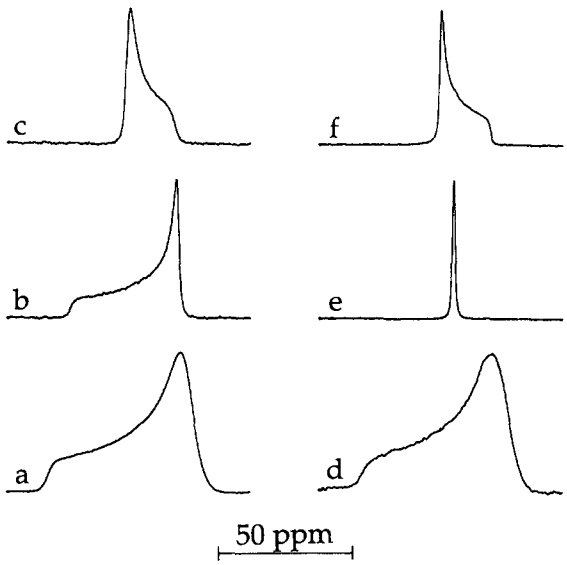

Fig. 3. ^{31}P-NMR spectra of DEPE (a, b, c) and 2% by weight alamethicin/DEPE dispersions (d, e, f) in excess water at 30°C (a,d), 50°C (b,e), and 90°C (c, f). (a) L_β phase, $\Delta\sigma = -50.7$ ppm; (b) L_α phase, $\Delta\sigma = -39.9$ ppm; (c) H_{II} phase, $\Delta\sigma = 16.5$ ppm; (d) L_β phase, $\Delta\sigma = -48.8$ ppm; (e) cubic phase, line width 160 Hz; (f) H_{II} phase, $\Delta\sigma = 18.5$ ppm.

not alter the lattice spacing of the H_{II} phase, only the temperature at which it appears. Note that the cubic and H_{II} phases coexist for some samples and for some temperatures (Fig. 1). In these experiments the sample was allowed to equilibrate for about 10 min after a temperature step. We observed that the coexistence temperature range of the two phases decreases as the equilibration time increases (data not shown) and does not seem to vary with the amount of added alamethicin. The cubic phase is stable for at least 4 months. Often a spurious diffraction ring coexists with the L_β phase, especially during cooling of the sample. Since no higher order diffraction peaks associated with this ring appear, we can not unambiguously assign it a lattice spacing.

^{31}P-NMR spectra of the system of DEPE and excess water with 2 wt.% alamethicin are characteristic of the L_β phase at 30°C and the H_{II} phase at 90°C (Fig. 3), in agreement with the X-ray data. At 50°C, the spectrum exhibits an isotropic line with a width of 160 Hz, consistent with the presence of a cubic phase. A sharp isotropic peak is also observed with mixtures of 3:1 DOPE/DOPC with approx. 2 wt.% alamethicin at 21°C (data not shown).

4. Discussion

Although a double diamond cubic phase occurs in monoelaidin- or monoolein-water systems, it has been observed in atmospheric DOPE- and DEPE-water systems only if the lipids have been repeatedly thermally cycled across the lamellar-hexagonal boundary or if lysolipid has been added. Shyamsunder et al. [25] suggest that a cubic phase may exist at every lamellar/hexagonal interface as a kinetically-hindered state. We speculate that the addition of alamethicin might perturb the lipid system enough to relieve the kinetic hindrance of forming this lower energy cubic phase. However, we do not know how individual alamethicin molecules accomplish this. In general, the understanding of the effects of membrane peptides and proteins on lipid phase behavior is quite limited. For work on gramicidin and melittin, see Killian and De Kruijff [27], and Batenburg et al. [28].

Alamethicin probably lies on the surface of a lipid bilayer or is incorporated as a small aggregates which span the bilayer. Although little is known about the mechanism of interaction between alamethicin and lipids, one expects that at sufficiently high concentrations, adsorbed or incorporated molecules might alter the curvature elastic properties of lipid layers. The ion channel studies of Keller et al. [19] showed a correlation between the monolayer spontaneous curvature and the probability of conductance states of alamethicin, but these studies were done at concentrations of alamethicin which were sufficiently small that the peptide was unlikely to alter the curvature properties of the membrane. In other words, the alamethicin molecules in the ion channel studies would feel stresses from the lipid curvature-elastic field, but were too few in number to significantly alter this field. At the considerably higher concentrations used here, alteration of the monolayer spontaneous curvature, rigidity, and Gaussian curvature coefficient cannot a priori be discounted.

The fact that the dimensions of the inverted hexagonal phase of DEPE changes little upon the addition of alamethicin (Fig. 1) suggests either that the alamethicin is expelled upon the formation of the hexagonal phase or that the presence of alamethicin doesn't alter the monolayer spontaneous curvature. Effects upon the monolayer rigidity and Gaussian curvature coefficient are not readily determined from the measurements presented here. Effects upon the curvature coefficients may arise, for example, if alamethicin changes the bilayer thickness or flexibility. Although we may speculate that such effects might promote the formation of the cubic phase, the understanding of the formation of cubic phases is sufficiently poor that no solid conclusions may be drawn.

The concentration of alamethicin required to induce the transition into the cubic phase is surprisingly small. If one assumes that alamethicin is uniformly dispersed throughout the lipid, then one peptide molecule affects about 200 molecules of lipid. Although the alamethicin concentration is small in our experiments, it is many orders of magnitude above that utilized in single-channel conductance work. We assume that the same peptide–lipid interactions produce a change in alamethicin single channel activity at low alamethicin concentrations that produce a change in phase behavior at high concentrations. However, we do not predict that the formation of a cubic phase at high alame-

thicin concentrations will have any effect upon single-channel studies.

Since the alamethicin concentration is so low, the enthalpy associated with the incorporation of all of the alamethicin into the lipid (and therefore with the transition to the cubic phase) is probably small. Hence, any effect of alamethicin on the phase diagram of the lipid will most likely manifest itself in a phase transition that is sensitive to small enthalpy changes. Although the gel to liquid crystalline phase transition is fruitfully probed by DSC, its large change in enthalpy is likely to obscure a small change in energy caused by the addition of peptide. The L_α–H_{II} transition is a better candidate. In DOPE, this transition has an enthalpy of 0.3 kcal/mol. An osmotic work equivalent to a change of free energy per lipid molecule of 0.03 kcal/mol ($= 0.05\ k_BT$) is sufficient to trigger the phase transition (Gawrisch et al. [22]). In DOPE-Me, the L_α–Q_{II} (inverted cubic) phase transition enthalpy is even smaller at 0.174 ± 0.034 kcal/mol (Siegel and Banschbach [29]). Hence, nonlamellar phase transitions are sensitive arenas in which to detect small changes in energy upon the addition of small mole fractions of alamethicin.

We suggest that similar effects may be seen in some systems when small concentrations of other integral membrane molecules are mixed with lipids of a nonzero spontaneous curvature and the temperature-dependent phase diagram is probed. The reason that this behavior with alamethicin had not been previously discovered is not for lack of interest in the effect of alamethicin upon the phases of lipids (for a review, see Woolley and Wallace [3]). Rather, it reflects the predominant use of phosphatidylcholines which do not form an H_{II} phase at physiologically interesting temperatures. The reasons for focusing upon phosphatidylcholines as model systems are historical. It is significant that H_{II}-prone lipids, such as unsaturated phosphatidylethanolamines are, in fact, very common in biological membranes.

Several researchers who studied phosphatidylcholines concluded that the introduction of alamethicin changes the properties of the lamellar phase. Chapman et al. [6] saw the repeat spacing of the lamellar phase increase in the presence of 3% alamethicin. Das and Balaram [11] observed that the addition 30 mol% alamethicin broadens the gel to liquid crystal phase transition in PC systems. Irmscher and Jung [30] and Bessler et al. [31] found that alamethicin has hemolytic effects at micromolar concentrations and lyses leukocytes at $5 \cdot 10^{-5}$ molar concentrations. Lau and Chan [9] found that 1% alamethicin barely altered the L_β–L_α phase transition; it was broadened by 1 or 2 C°. 0.4% alamethicin broadened the size distribution of vesicles. The electron micrographs of Hauser et al. [7] showed that small globules replaced regular lamellae in vesicles of egg PC and alamethicin (at 15:1). Hauser et al. [7] inferred from their NMR studies that phosphatidylserine and alamethicin at a molar ratio of 600:1 were creating a new structure. Their data did not imply any similar structure for mixtures of phosphatidylcholine and alamethicin. McIntosh, Ting-Beall and Zampighi [10] examined the addition of 5% to 17% alamethicin to DPPC. They observed that the gel (L_β) *d*-spacing increases and the freeze-fracture planes acquire striations. In the liquid crystalline phase, the fracture faces of vesicles of DPPC with alamethicin have a grainy pattern as seen by freeze fracture.

Observation of the lipids' evolution into nonlamellar phases requires both instrumentation sensitive to this transition and lipids capable of entering the H_{II} phase at biologically interesting temperatures (e.g., typically $<$ 100°C). For the enthalpy reasons described above, these transitions are easier to observe and measure with NMR and X-ray diffraction than with DSC. Experimentation with H_{II}-prone lipids like unsaturated PE's, or mixtures of unsaturated PE's and PC's, may reveal new phenomena and may be more biologically relevant.

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References

[1] Hall, J.E. (1978) in Membrane Transport in Biology: Concepts and Models, Vol. 1 (Giebisch, G., Tosteson, D.C. and Ussing, H., eds.), pp. 475–531, Springer Verlag, Berlin.
[2] Latorre, R. and Alvarez, O. (1981) Physiol. Rev. 61, 77–150.
[3] Woolley, G.A. and Wallace, B.A. (1992) J. Membr. Biol. 129, 109–136.
[4] Sansom, M.S.P. (1991) Prog. Biophys. Mol. Biol. 55, 139–235.
[5] Sansom, M.S.P. (1993) Eur. Biophys. J. 22, 105–124.
[6] Chapman, D., Cherry, R.J., Finer, E.G., Hauser, H., Phillips, M.C., Shipley, G.G. and McMullen, A.I. (1969) Nature 224, 692–694.
[7] Hauser, H., Finer, E.G. and Chapman, D. (1970) J. Mol. Biol. 53, 419–433.
[8] Lau, A.L. and Chan, S.I. (1974) Biochemistry 13, 4942–4948.
[9] Lau, A.L.Y. and Chan, S.I. (1975) Proc. Natl. Acad. Sci. USA 72, 2170–2174.
[10] McIntosh, T.J., Ting-Beall, H.P. and Zampighi, G. (1982) Biochim. Biophys. Acta 685, 51–60.
[11] Das, M.K. and Balaram, P. (1984) J. Biosci. 6, 337–348.
[12] Cullis, P.R., Hope, M.J., De Kruijff, B., Verkleij, A.J. and Tilcock, C.P.S. (1985) in Phospholipids and Cellular Regulation (Kuo, J.F., ed.), pp. 1–59, CRC Press, Boca Raton, FL.
[13] Gruner, S.M. (1989) J. Phys. Chem. 93, 7562–7570.
[14] Gruner, S.M. (1985) Proc. Natl. Acad. Sci. USA 86, 3665–3669.
[15] Hui, S. and Sen, A. (1989) Proc. Natl. Acad. Sci. USA 86, 5825–5829.
[16] Tate, M.W., Eikenberry, E.F., Turner, D.C., Shyamsunder, E. and Gruner, S.M. (1991) Chem. Phys. Lipids 57, 147–164.

[17] Streicher-Scott, J.L. (1992) Polymorphic Lipids in the Function of the Mitochondrial Adenine Nucleotide Translocator, Ph.D. Dissertation. University of Maryland, Department of Pharmacology and Experimental Therapeutics.
[18] Gibson, N.J. and Brown, M.F. (1993) Biochemistry 32, 2438–2454.
[19] Keller, S.L., Bezrukov, S.M., Gruner, S.M., Tate, M.W., Vodyanoy, I. and Parsegian, V.A. (1993) Biophys. J. 65, 23–27.
[20] Gruner, S.M., Milch, J.R. and Reynolds, G.T. (1982) Rev. Sci. Instrum. 53, 1770–1778.
[21] Gruner, S.M., Tate, M.W., Kirk, G.L., So, P.T.C., Turner, D.C. and Keane, D.T. (1988) Biochemistry 27, 2853–2866.
[22] Gawrisch, K., Parsegian, V.A., Hajduk, D.A., Tate, M.W., Gruner, S.M., Fuller, N.L. and Rand, R.P. (1992) Biochemistry 31, 2856–2864.
[23] So, P.T.C. (1992) High Pressure Effects on the Mesophases of Lipid-Water Systems, Ph.D. Dissertation, p. 249, Princeton University, Department of Physics.
[24] Tate, M.W. and Gruner, S.M. (1989) Biochemistry 28, 4245–4253.
[25] Shyamsunder, E., Gruner, S.M., Tate, M.W., Turner, D.C. and So, P.T.C. (1988) Biochemistry 27, 2332–2336.
[26] International Tables for X-ray Crystallography (1968) Vol. II, p. 147, Kynoch Press, Birmingham, UK.
[27] Killian, J.A. and De Kruijff, B. (1985) Biochemistry 24, 7881–7890.
[28] Batenburg, A.M., Van Esch, J.H. and De Kruijff, B. (1988) Biochemistry 27, 2324–2331.
[29] Siegel, D.P. and Banschbach, J.L. (1990) Biochemistry 29, 5975–5981.
[30] Irmscher, G. and Jung, G. (1977) Eur. J. Biochem. 80, 165–174.
[31] Bessler, W.G., Ottenbreit, B., Irmscher, G. and Jung, G. (1979) Biochem. Biophys. Res. Commun. 87, 99–105.