Complex Formation between Indole-3-acetic Acid and Phospholipid Membrane Components in Aqueous Media. 3. Interaction of Indole-3-acetic Acid with Amphiphiles Containing the Trimethylammonium Group†

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ABSTRACT: Specific structural aspects of the interaction between the plant hormone indole-3-acetic acid (IAA) and phosphatidylcholine (PC) have been investigated by nuclear magnetic resonance (NMR) techniques. IAA-induced ^1H changes in chemical shift of compounds containing a trimethylammonium group have been monitored. Small IAA-induced changes are observed (<0.05 ppm) for the PC head group moieties, choline chloride, phosphocholine chloride, and glycerylphosphocholine. Similar chemical shift changes are observed for short chain length (less than eight carbon atoms) acylcholine, acylcarnitine, acyllysophosphatidylcholine, and alkyltrimethylammonium bromide compounds. However, large IAA-induced chemical shift changes (up to 1 ppm) are observed with longer chain homologues of these amphiphiles. The sharp transition from small to large IAA-induced ^1H chemical shift changes is attributed to the formation of organized multimolecular structures (micelles). This is supported by light scattering data on a number of acylcarnitine derivatives. The nature of the amphiphile head group is important in determining the magnitude of the IAA-induced changes in chemical shift; an inverse relationship between head group size and shift change is observed. A model to account for the changes in ^1H chemical shifts is proposed that is based on the anisotropic nature of amphiphiles organized into micelles and bilayers and that invokes an IAA-induced change in orientation of the head group.

The plant hormone indole-3-acetic acid (IAA)[1] induces upfield changes in chemical shifts of head group protons of purified soybean phosphatidylcholine (PC) as monitored by nuclear magnetic resonance (NMR) (Jones et al., 1984). The magnitudes of these changes have been shown to depend upon the solvent system employed, with the largest shift changes being observed in solvents that induce the formation of organized multimolecular PC structures (Marker et al., 1978; Jones et al., 1984). Such solvents include chloroform, in which PC is in the form of inverted micelles (Levine et al., 1972), and sonicated aqueous dispersions, in which unilamellar bilayered vesicles are formed (Huang, 1969).

The interaction between IAA and PC is also dependent on the nature of the ligand in that compounds closely related to IAA (auxins) induce different changes in ^1H chemical shifts of PC head group protons (Jones & Paleg, 1984), although no changes were observed for the acyl or glyceryl protons (Jones et al., 1984). In addition, IAA induces ^1H chemical shift changes in other compounds containing the trimethylammonium group. The choline group protons in acetylcholine are shifted upfield by IAA (Minch et al., 1979) although the magnitude of these effects is substantially smaller than those observed with PC at similar concentrations. The phosphate group in PC has been implicated in the stronger binding both from ^{31}P NMR measurements (Marker et al., 1978) and from model building (Weigl, 1969).

Once it was established that there was a degree of specificity exhibited by PC vesicles for different auxin structures (Jones & Paleg, 1984), it became important to determine whether IAA was able to "distinguish" between structures related to (i.e., having one or more functional groups in common with) PC. Compounds examined include the nonamphiphilic compounds choline chloride (CC), phosphocholine chloride (PCC), and glycerylphosphocholine (GPC) together with several homologous series of amphiphiles containing the trimethylammonium group (acylcholines, acylcarnitines, acyllysophosphatidylcholines, and alkyltrimethylammonium bromides). These derivatives provide not only a wide variation in head group type, but each provides members with varying lipid chain lengths and, therefore, different structure-forming capabilities.

The changes in ^1H chemical shifts induced in PC and other trimethylammonium-containing compounds by IAA and related aromatic compounds (Marker et al., 1978; Burton & Minch, 1974; Grätzel et al., 1974; Fendler et al., 1975; Minch et al., 1979) have been ascribed to aromatic ring current effects. However, the results of the present investigations emphasize the inadequacies of an explanation based solely on aromatic ring current induced changes in chemical shift, and an additional or alternative mechanism is suggested that is based on the anisotropic nature of structured amphiphiles.

Experimental Procedures

Materials

Choline chloride (CC), phosphocholine chloride (PCC) (as the calcium salt), and glycerylphosphocholine (GPC) (as the cadmium chloride complex) were purchased from Sigma Chemical Co. and used as received except that the latter was freed of cadmium chloride by passage through a mixed ion exchange resin bed. Hexanoyl-, octanoyl-, and decanoylcholine

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[1] Abbreviations: NMR, nuclear magnetic resonance; PC, soybean phosphatidylcholine; IAA, indole-3-acetic acid; CC, choline chloride; PCC, phosphocholine chloride; GPC, glycerylphosphocholine; EDTA, ethylenediaminetetraacetic acid; K_d, dissociation constant; Δ, complex shift; ACh, acylcholine compounds; ACarn, acylcarnitine compounds; ALPC, acyllysophosphatidylcholine compounds; ATAB, alkyltrimethylammonium compounds; LCh, lauroylcholine; LCarn, lauroylcarnitine; LTAB, lauryltrimethylammonium bromide; LLPC, lauroyllysophosphatidylcholine; cmc, critical micelle concentration.

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iodides were prepared from their corresponding methyl esters by trans esterification with *N,N*-dimethylethanolamine under catalytic amounts of sodium metal at 100 °C in dioxane. The resulting (*N,N*-dimethylamino)ethyl esters were alkylated with iodomethane in the absence of base to give the appropriate acylcholine iodides, which were purified by 2 times recrystallization from anhydrous methanol/diethyl ether at −20 °C. The off-white crystalline materials were filtered and dried under vacuum at room temperature in the dark. Purities were confirmed by NMR analysis. Acetylcholine chloride, butyrylcholine chloride, lauroylcholine chloride, and iodide (Sigma) were used as received. Myristoylcholine chloride (90–95%) (also Sigma) was 4 times recrystallized from anhydrous methanol/diethyl ether at −20 °C. A trimethylammonium-containing compound was a minor contaminant of the above material, as monitored by NMR, and could not be removed by repeated recrystallization.

Octyl-, decyl-, and dodecyltrimethylammonium bromides were prepared by the alkylation of excess anhydrous trimethylamine with the corresponding alkyl bromide in a pressure vessel at room temperature. The products were again purified by 2 times recrystallization from anhydrous methanol/diethyl ether at −20 °C. The white platelike crystalline materials were filtered and dried under vacuum at room temperature (except octyltrimethylammonium bromide, which forms a gel at room temperature and was dried at −20 °C under vacuum). Myristyl- and palmityltrimethylammonium bromides were purchased from Aldrich and purified as above.

dl-Acetyl-, *dl*-hexanoyl-, *dl*-octanoyl-, and *dl*-lauroylcarnitine hydrochlorides together with lauroyl- and palmitoyllysophosphatidylcholines were purchased from Sigma. Octanoyl- and decanoyllysophosphatidylcholines were obtained from Calbiochem (Australia). All acylcarnitines and acyllysophosphatidylcholines were used as received. IAA (Sigma) was recrystallized from 1,2-dichloroethane and stored under nitrogen at 4 °C. D_2O was purchased from AAEC (Australia).

Methods

Solutions of the amphiphilic compounds were made up in 0.2 M sodium acetate/acetic acid–D_2O buffer containing 1 mM EDTA (pH 3.85) at approximately 65 mM except where stated. Solutions of IAA, as the sodium salt, at concentrations that were approximately isotonic with the amphiphile solutions were made up by titrating the free acid in D_2O with NaOD. Increments of 5, 10, or 20 μL of the NaIAA solution were added to the NMR tube with a microliter syringe.

All of the amphiphiles used in this study, except myristoylcholine chloride, are soluble at 65 mM in acetate buffer (pH 3.85) at room temperature. The latter compound formed clear solutions at 40 °C and was measured at this temperature.

Light Scattering. Light scattering measurements on aqueous solutions of acetyl-, ocanoyl-, and lauroylcarnitine hydrochloride were made on a Sopheca 42000 light scattering photometer with 5461-Å wavelength light from a high-pressure mercury vapor lamp at 21 ± 0.5 °C.

Amphiphile solutions were dedusted by ultrafiltration through 0.22-μm Millipore filters. Solutions of known concentration were diluted with solvent to give solutions of progressively lower concentration.

NMR. NMR spectra at 90 MHz were recorded on a JEOL FX-90Q Fourier-transform spectrometer with a spectral width of 800 Hz accumulated into 8K data addresses at a probe temperature at 24 °C except where stated. Dioxane was used as an internal reference. Chemical shift reproducibility was better than ±0.005 ppm. Dissociation constants (K_d) and complex shifts (Δ) for the interaction between IAA and amphiphile or head group entities were determined as reported previously (Jones et al., 1983), primarily using changes in $-N^+(CH_3)_3$ chemical shifts caused by added IAA.

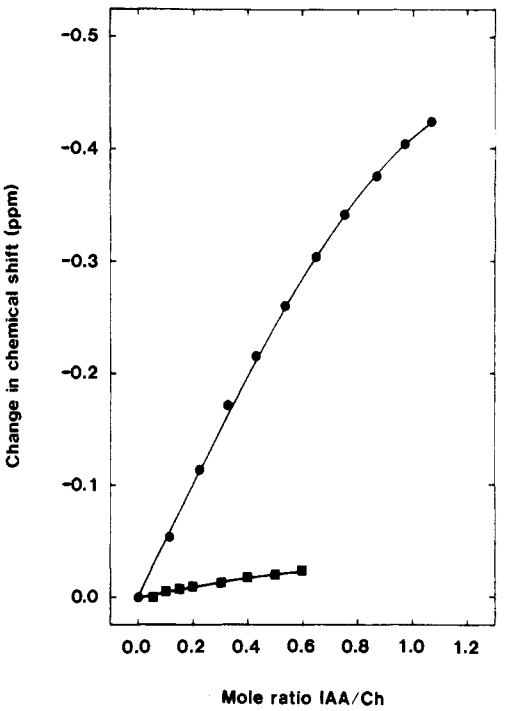

FIGURE 1: Upfield changes in chemical shift of $-N^+(CH_3)_3$ protons, relative to an internal 1,4-dioxane standard, of ca. 65 mM CC, PCC, and GPC (■) in acetate buffer, pH 3.85, with increasing concentration of IAA. The data for the three compounds are identical within experimental error. The data obtained by titrating PC with IAA (same conditions as above) are also shown (●).

Results

Head Group Components. The addition of IAA to solutions of CC, PCC, and GPC in acetate buffer (pH 3.85), up to a mole ratio of approximately 0.6:1, produced small upfield changes in the chemical shift of the trimethylammonium protons in these compound (Figure 1). Small changes in other resonance positions were also detected without observable changes in line shapes. These IAA-induced $-N^+(CH_3)_3$ chemical shift changes are very similar for the three compounds, and they enabled a composite K_d of $(4.2 \pm 0.3) \times 10^{-1}$ M and a Δ of −0.349 ± 0.009 ppm to be calculated. These values may be compared with those obtained from the IAA-induced shift changes of PC $-N^+(CH_3)_3$ protons (also shown in Figure 1) of 4.2×10^{-3} M and −0.550 ppm, respectively.

Amphiphile Systems. The effects of IAA on amphiphile systems have been investigated in terms of the changes in all observed protons resonances with increasing concentrations of IAA for the four classes of trimethylammonium-containing compounds and the effects of chain length of the IAA-induced head group shifts within an homologous series of amphiphiles.

The former is discussed in detail for amphiphiles with a C_{12} (lauroyl or dodecyl) chain by using lauroylcholine as a model system by which other amphiphiles are compared. Similarly, the acylcholines have been used as the model system in comparing the effects of chain length on IAA-induced head group shifts within a class of amphiphiles.

Figure 2 illustrates a typical ^1H NMR spectrum of a long-chain ($>C_8$) acylcholine compound in the absence (solid line) and presence (broken line) of a 1:1 mole ratio of IAA; the one depicted is that of lauroylcholine chloride (LCh). The peak assignments were made by comparison with other

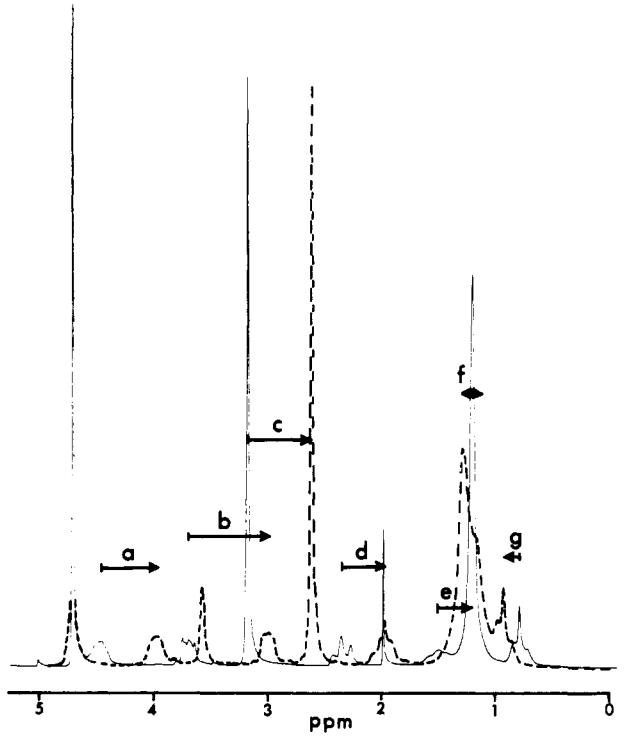

FIGURE 2: Proton NMR spectra of lauroylcholine chloride (ca. 65 mM) in acetate buffer, pH 3.85, at 24 °C in the absence of IAA (solid line) and in the presence of 65 mM IAA (broken line). Resonances that have an IAA-induced shift (indicated by arrows) are (a) $-O-CH_2-$, (b) N^+-CH_2-, (c) $-N^+(CH_3)_3$, (d) $CO-CH_2-$, (e) $CO-CH_2CH_2-$, (f) $-(CH_2)_n-$, and (g) $-CH_2-CH_3$. The sharp peaks at 1.99 (relative to tetramethylsilane) and 4.71 ppm are due to acetate and HDO resonances, respectively. The peak at 3.58 ppm (in the presence of IAA) is due to the IAA methylene resonance.

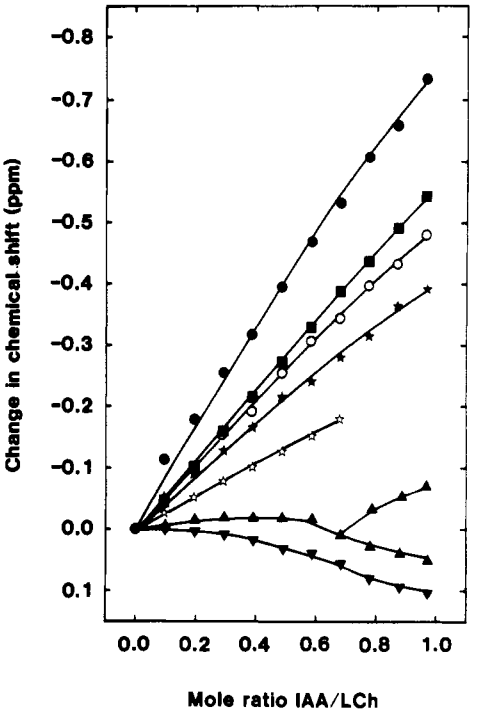

FIGURE 3: Changes in proton chemical shifts of lauroylcholine chloride (ca. 65 mM) in acetate buffer (pH 3.85) with increasing concentrations of IAA. Peak positions were measured relative to an internal 1,4-dioxane standard. (●) N^+-CH_2-; (■) $-N^+(CH_3)_3$; (○) $-CH_2-O-$; (solid star) $CO-CH_2-$; (☆) $CO-CH_2-CH_2-$; (▲) $-(CH_2)_n-$; (▼) $-CH_2-CH_3$.

long-chain ester- and choline-containing compounds. On incremental addition of IAA, a number of spectral changes were observed as indicated by arrows in Figure 2. Choline-group protons were shifted upfield by IAA in a concentration-dependent manner, together with the α- and β-methylene protons of the lauroyl chain (Figure 3). Upfield chemical shift changes of ca. −0.75, −0.55, and −0.5 ppm were observed for the $-N^+-CH_2-$, $-N^+(CH_3)_3$, and $-O-CH_2-$ protons, respectively, in the presence of a 1:1 mole ratio of IAA. The resonance associated with the remaining-chain methylene protons moved upfield only slightly at low concentrations of IAA (<0.6 mole ratio) and began to broaden, but at higher concentrations, this peak split into two components, one of which moved upfield whilst the other moved downfield (see Figure 3). The terminal methyl group protons moved progressively downfield with increasing concentrations of IAA although this movement was not regular.

Similar spectral changes were observed when lauroylcarnitine (LCarn; Figure 4a), dodecyltrimethylammonium bromide (LTAB; Figure 4b), and lauroyllysophosphatidylcholine (LLPC; Figure 4c) were titrated with IAA. The magnitudes of these shift changes vary considerably, however, between the various classes of amphiphile. The N^+-CH_2- protons in LTAB were shifted upfield approximately −1.0 ppm in the presence of a 0.8:1 mole ratio of IAA to LTAB compared to ~−0.5 ppm for LCarn and ~−0.34 ppm for LLPC. Similar differences were observed for the $-N^+(CH_3)_3$ protons. In LCarn and LLPC, the $-O-CH_2-$ protons experienced smaller shifts than the $-N^+(CH_3)_3$ protons although the equivalent group in LTAB ($N^+-CH_2CH_2-$) experienced larger IAA-induced shifts. In accordance with the effects observed with lauroylcholine, the terminal methyl group on the acyl (alkyl) chain was progressively shifted downfield in LCarn and LTAB with increasing concentrationsof IAA. In LLPC, however, this resonance was shifted upfield (Figure 4). In this system the chain methylene protons also showed different behavior. With increasing concentrations of IAA the methylene proton envelope gradually broadened (from 6.1 Hz at 0 IAA to 13.8 Hz at a mole ratio of 1:1 IAA to LLPC) and moved upfield but did not split into two components (glycerol protons remained unshifted). In contrast, the methylene protons in LTAB showed splitting at low concentrations of IAA.

In all the amphiphile systems, the head group signals were reduced in intensity only slightly with minimal line broadening due to the addition of IAA. Furthermore, changes in shape of the N^+-CH_2- multiplet with added IAA were seen. This was most clearly observed with LLPC. At low IAA concentrations, this signal appeared as a well-formed triplet, but at higher IAA concentrations the signal appeared as an unsymmetrical doublet. Whilst somewhat similar changes were observed with other amphiphile systems, they were less well-defined.

Effects of Amphiphile Chain Length. The effects of chain length on the ability of IAA to induce changes in chemical shift of $-N^+(CH_3)_3$ protons in acylcholine homologues are shown in Figure 5. Decanoylcholine solutions showed similar ^1H chemical shift changes to those of lauroylcholine systems on titration with IAA. However, hexanoyl and shorter chain homologues showed only very small IAA-induced shift changes, which were of similar magnitude to those of the head group component systems (i.e., CC, PCC, and GPC) described above. Octanoylcholine represents a system intermediate between those that have small and those that have large IAA-induced shifts. This may also be suggested by the upward curvature of the titration curve for this compound (Figure 5).

Myristoylcholine displayed somewhat anomolous behavior when compared with other long-chain homologues. At 40 °C,

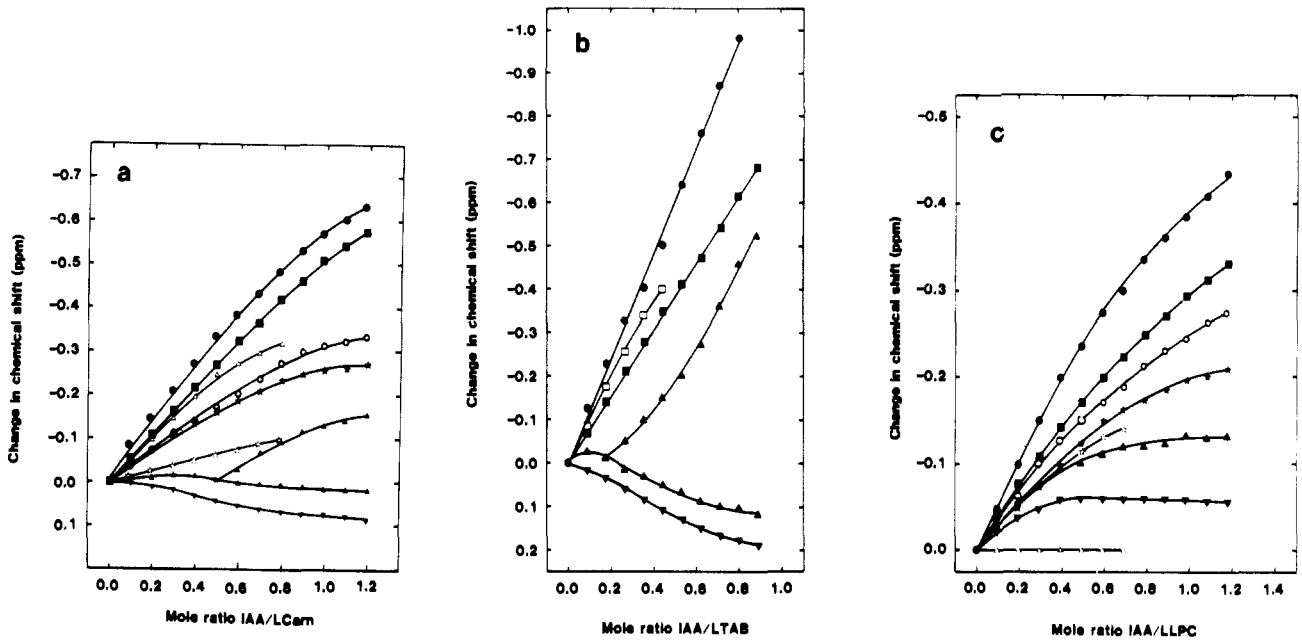

FIGURE 4: IAA-induced changes in proton chemical shifts of (a) lauroylcarnitine hydrochloride (LCarn), (b) dodecyltrimethylammonium bromide (LTAB), and (c) lauroyllysophosphatidylcholine (LLPC) in acetate buffer (pH 3.85) at amphiphile concentrations of ~65 mM. Peak positions were measured relative to a 1,4-dioxane reference. (a–c) (●) N^+–CH_2; (■) $-N^+(CH_3)_3$; (▲) $-(CH_2)_n-$; (▼) $-CH_2CH_3$. (a and c) (solid star) $CO-CH_2-$; (☆) $CO-CH_2CH_2-$; (○) $O-CH_n-$. (a) (△) $-CH_2COOH$. (b) (□) $N^+-CH_2CH_2$.

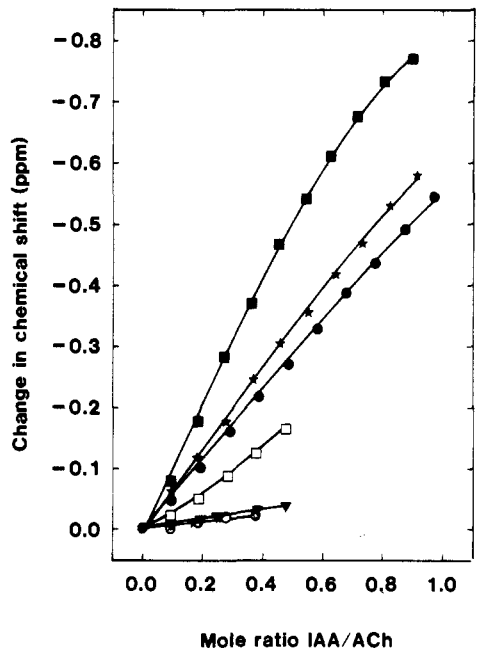

FIGURE 5: Effects of chain length on the IAA-induced changes in chemical shift of $-N^+(CH_3)_3$ protons for a homologous series of acylcholine compounds. All compounds were measured at a concentration of ca. 65 mM in acetate buffer. (○) acetylcholine; (☆) butyrylcholine; (▼) hexanoylcholine; (□) octanoylcholine; (solid star) decanoylcholine; (●) lauroylcholine; (■) myristoylcholine. Shift changes were measured with respect to an internal 1,4-dioxane reference.

Table I: Dissociation Constants (K_d) and Complex Shifts (Δ) Determined for the Interaction between IAA and Various Amphiphiles[a]

compd	K_d	Δ (ppm)	stoichiometry (IAA to compd)
C_{14}-TAB	(8.5 ± 1.3) × 10^{-5} M^2	−1.695 ± 0.009	2:1
C_{16}-TAB	(10.3 ± 2.6) × 10^{-5} M^2	−1.664 ± 0.026	2:1
C_{12}-Ch	(29 ± 2.6) × 10^{-6} M^2	−1.232 ± 0.008	2:1
C_{14}-Ch	(13 ± 1) × 10^{-3} M	−1.373 ± 0.014	1:1
C_{16}-Carn	(49 ± 8) × 10^{-3} M	−1.084 ± 0.043	1:1
C_{10}-LPC	(39 ± 4) × 10^{-3} M	−0.652 ± 0.017	1:1
C_{12}-LPC	(30 ± 4) × 10^{-3} M	−0.614 ± 0.014	1:1
C_{14}-LPC	(28 ± 2) × 10^{-3} M	−0.611 ± 0.007	1:1
C_{16}-LPC	(27 ± 2) × 10^{-3} M	−0.635 ± 0.008	1:1

[a] Data for other amphiphiles could not be successfully fitted to theoretical curves to obtain K_ds. Observed IAA-induced changes in chemical shift of $-N^+(CH_3)_3$ protons were used to obtain the derived parameters.

the peaks in the ^1H NMR spectrum were significantly broader than those of other members of this series. When it was titrated with IAA, the intensities of these peaks progressively decreased. The observed IAA-induced change in chemical shift of the trimethylammonium peak was also significantly greater than that of other members of the homologous series. These anomolous results are not due to elevated temperature since the results with other members of the series were not significantly altered by raising the temperature to 40 °C. A stoichiometry of 1:1 IAA to ACh best fits the data for myristoylcholine compared with a stoichiometry of 2:1 for lauroylcholine (see Table I). Calculated Δ's are −1.37 and −1.23 ppm, respectively. It was not possible to satisfactorily calculate K_ds and Δ's for the remaining members of the series, nor was it possible to calculate K_ds and Δ's for IAA-induced effects on other protons of these compounds.

The effects of chain length on the IAA-induced changes in chemical shift of $-N^+(CH_3)_3$ protons in the homologous series of acylcarnitine (ACarn), alkyltrimethylammonium bromide (ATAB), and acyllysophosphatidylcholine (ALPC) are shown in panels a–c of Figure 6, respectively. Again, a chain length of eight carbon atoms (or between eight and ten carbon atoms in the ATAB series) marks the transition between small and

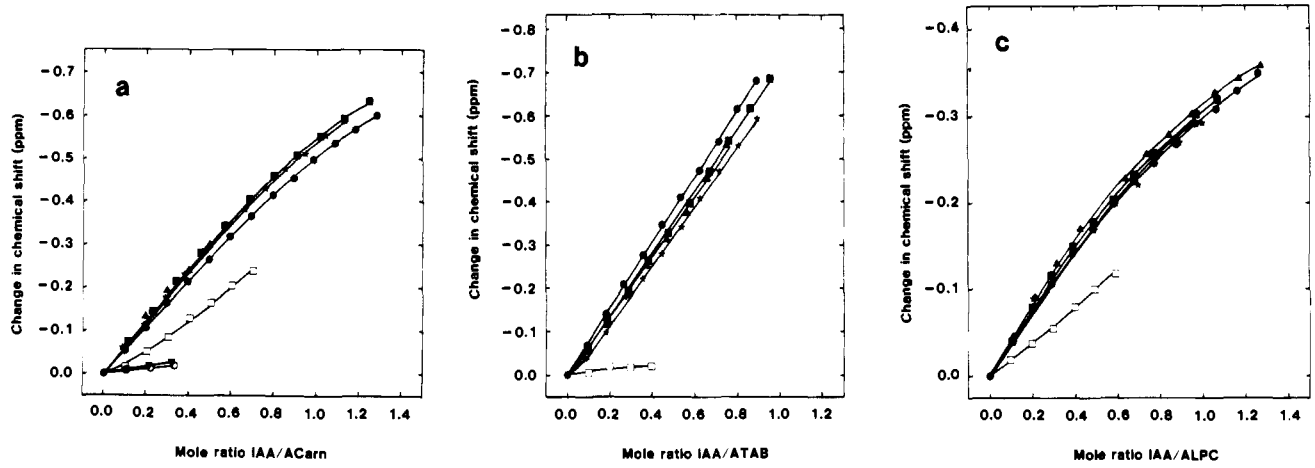

FIGURE 6: Effects of chain length on the IAA-induced changes in chemical shift of $-N^+(CH_3)_3$ protons for a homologous series of (a) acylcarnitine hydrochlorides, (b) alkyltrimethylammonium bromides, and (c) acyllysophosphatidylcholines (ca. 65 mM in acetate buffer, pH 3.85): (○) acetyl, (▼) hexanoyl, (□) octanoyl (octyl), (solid star) decanoyl (decyl), (●) lauroyl (lauryl), (■) myristoyl (myristyl), and (▲) patmitoyl (cetyl) derivatives.

large changes in chemical shift on titration with IAA. It is noteworthy that the magnitude of the changes does not vary significantly amongst members of these series above this transition, unlike the situation observed with the acylcholine compounds.

Attempts to fit theoretical curves to the acylcarnitine data, by assuming a single equilibrium and by using various stoichiometries, failed in all cases with the exception of the C_{16} acylcarnitine hydrochloride. A best fit for this homologue was obtained with a K_d of 49 ± 8 mM and a Δ of −1.084 ± 34 ppm, with an IAA to carnitine stoichiometry of 1:1 (Table I). The limited range of data for the C_{16} compound (gel formation and subsequent loss of NMR signal were observed at IAA concentrations greater than a mole ratio of 0.5:1 IAA to ACarn), compared to the C_{10}, C_{12}, and C_{14} members of this series, suggests that the lack of success in calculations of K_ds for the latter may be due to anomalous chemical shift behavior at higher IAA concentrations. Nevertheless, truncation of the data for these compounds at a mole ratio of 0.5 IAA to ACarn also failed to give satisfactory fits to theoretical K_d curves.

Theoretical curves have been fitted to the observed data for the C_{14} and C_{16} compounds of ATAB (but not for the C_{10} and C_{12} compounds), giving K_ds of approximately 1×10^{-4} M^2 for a stoichiometry of 2:1 IAA to ATAB. Δ's were calculated to be approximately −1.7 ppm (Table I). On the other hand, data for all the lysophosphatidylcholines with a chain length greater than eight carbon attms could be fitted to such curves. K_ds are in the range 27–39 mM with Δ's in the range −0.61 to −0.65 ppm for a stoichiometry of 1:1 IAA to ALPC (Table I). Futhermore, the K_ds calculated for the N^+-CH_2- and $O-CH_2-$ protons of lauroyllysophosphatidylcholine (LLPC) of 38 ± 4 mM (Δ of −0.937 ± 0.010 ppm) and 32 ± 3 mM (Δ of −0.528 ± 0.012 ppm) are in good agreement with these values.

The IAA-induced effects observed with amphiphiles of varying chain length are consistent with micelle formation at the concentrations and pHs investigated in these studies. Indeed, it has been recognized that the only structural requirement for the formation of micelles appears to be a chain of more than eight carbon atoms and an effective cross-sectional area of the polar group larger than the cross-sectional area of the hydrocarbon chain (Jain & Wagner, 1980). These conditions are satisfied with the systems that have large IAA-induced shift changes. Since octanoylcarnitine (and the corresponding choline and lysophosphatidylcholine derivatives) produced intermediate IAA-induced shift changes, the effects

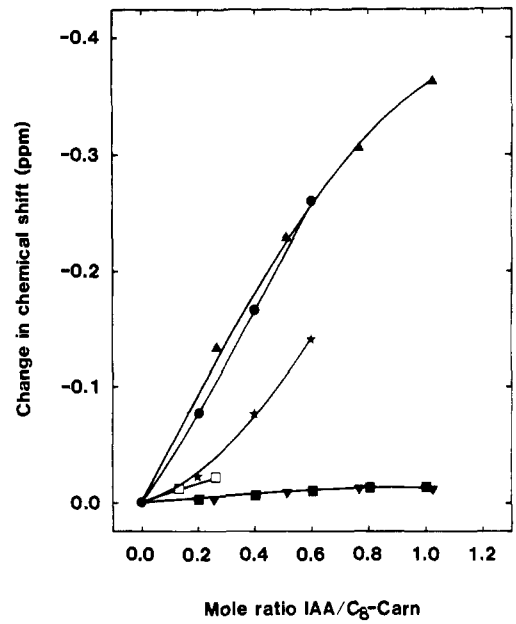

FIGURE 7: Effects of changes in concentration and micelle formation on the IAA-induced changes in the $-N^+(CH_3)_3$ proton chemical shift of octanoylcarnitine: (▼) 10, (solid star) 50, and (●) 100 mM octanoylcarnitine. Data obtained with 10 (■) and 100 mM (□) acetylcarnitine together with 10 mM lauroylcarnitine (▲) are also shown for comparison purposes. All solutions were made up in acetate buffer at pH 3.85.

of changing micelle concentration in this system was investigated. The mole fraction of micelles was altered by changing the amphiphile concentration (Tanford, 1973) over the range 10–100 mM. These systems were then titrated with IAA in the usual manner, and the results are shown in Figure 7 (similar titrations of acetyl- and lauroylcarnitines were also made to examine the effects of overall changes in amphiphile concentration). At low concentration (10 mM), the IAA-induced trimethylammonium shifts are similar to, or smaller than, those observed for the shorter chain homologues whereas at a concentration of 100 mM the shift changes approach the values observed for the longer chain compounds.

These results strengthen the idea that the large IAA-induced changes in amphiphile proton chemical shifts are due to micelle formation and that, at amphiphile concentrations routinely employed in our experiments (ca. 65 mM), octanoylcarnitine is close to its critical micelle concentration (cmc). This is further supported by light scattering experiments. The var-

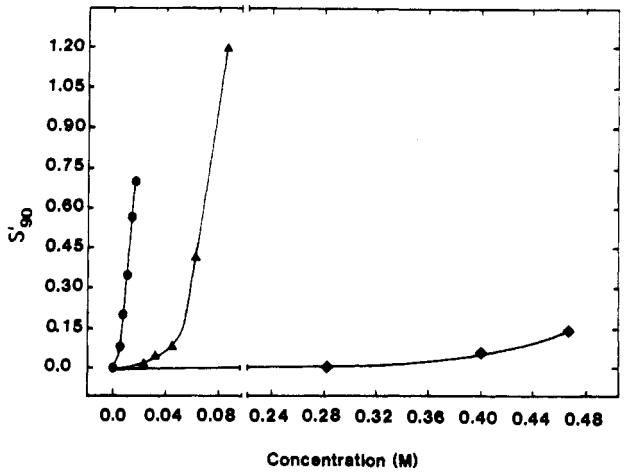

FIGURE 8: Changes in excess light scattered at 90° (S'_{90}) with concentration for (♦) acetylcarnitine, (▲) octanoylcarnitine, and (●) lauroylcarnitine. Solutions were made up in H_2O. Extrapolation of the linear portion of the curves to zero excess scatter gives approximate cmc values for these compounds.

iation in light scattered at 90° by solutions of acetyl-, octanoyl-, and lauroylcarnitine hydrochloride, in excess of that scattered by the pure solvent (S'_{90}), is shown in Figure 8. The data are not corrected for dissymmetry. Extrapolation of the linear portions of the curves to zero excess scatter (Attwood, 1968) gives light scattering cmc values of approximately 4 mM for lauroylcarnitine and approximately 50 mM for octanoylcarnitine. Acetylcarnitine did not give a linear response even at the highest concentration measured (470 mM), indicating a cmc greater than this value.

Discussion

The interactions between IAA and PC is not simply a function of complex formation between IAA and the positively charged trimethylammonium group. This is shown by the weak (in terms of K_ds and Δ's) interaction between IAA and various head group components of PC such as CC, PCC, and GPC, which show only small (<0.05 ppm) IAA-induced ^1H chemical shift changes at pH 3.85. It is also apparent that, under our experimental conditions, a minimum acyl chain length attached to the $-N^+(CH_3)_3$ group is required before any appreciable interaction is observed. The nature of the $-N^+(CH_3)_3$-containing head group seems of lesser importance in that, at the same chain length, the acylcholine, acylcarnitine, acyllysophosphatidylcholine, and alkyltrimethylammonium series all show analogous shift changes induced by IAA. Indeed, since compounds that do not possess the phosphate group appear to have similar abilities in forming complexes with IAA, previous models (Weigl, 1969; Marker et al., 1978) in which the phosphate group was suggested as a requirement for the interaction to take place appear to be invalid.

The nature of the change that occurs at a chain length of eight (or nine), under our experimental conditions, is dependent upon the formation of micelles. This was demonstrated by an examination, with light scattering techniques, of the cmc of three acylcarnitines and by varying the mole fraction of micelles in these systems by changing total amphiphile concentration. C_{12}-Carn forms micellar structures at concentrations well below the 65 mM concentration used in this work, and it also shows pronounced IAA-induced shifts. C_2-Carn does not form micelles even at concentrations 5 time those employed in the NMR experiments, and the IAA-induced chemical shift changes are small. C_8-Carn is intermediate in that at a concentration of 65 mM a proportion of the molecules are in the form of micelles, and this compound responds with intermediate IAA-induced chemical shift changes. The generalization can be made, therefore, that IAA interacts strongly (in terms of a small K_d and a large Δ) with organized lipid systems such as micelles, or membranes, and interacts only weakly with those systems possessing no organization. This generalization is supported by previous results that showed that a strong IAA-PC interaction occurred only in those solvents in which the lipid molecules are found to be ordered (Jones et al., 1984).

Two other interrelated questions are raised by this work: what is the molecular nature of the interaction between IAA and the organized lipid systems, and what is the mechanism that produces the ^1H chemical shift changes observed? An understanding of these phenomena may give insight into the nature of the interaction of IAA with membranes.

A number of NMR studies have shown that small molecules having hydrophobic properties interact with micellar species causing changes in ^1H chemical shifts of both micellar amphiphile and ligand. For example, phenylacetate and benzoate induce chemical shift changes of the $-N^+(CH_3)_3$ protons in cetyltrimethylammonium bromide, which have been ascribed to aromatic ring current effects, where the aromatic nucleus is described as being in close association with the trimethylammonium group (Bunton & Minch, 1974). Aromatic ring current effects have also been invoked to account for the IAA-induced upfield shifts in aqueous solutions of acetylcholine ((Minch et al., 1979) and the shifts induced in butyltrimethylammonium bromide by aromatic anions (Bunton & Minch, 1974). Ionization of the ligand is thought to stabilize the interaction of the aromatic anion with the head group region of the amphiphile (which is in contact with the aqueous phase of the system), whereas suppression of ionization is considered to cause penetration of the ligand into the lipid core of the micelle (Bunton & Minch, 1974). This effect has been demonstrated for the interaction between local anesthetics and phosphatidylcholine model membranes by ^2H NMR techniques (Boulanger et al., 1981).

Since penetration of the aromatic nucleus into the lipid phase distances it from the head group, suppression of ionization should reduce the effects of aromtic ring current shifts on the head group protons. However, the results described previously (Jones et al., 1984), in which it was shown that IAA-induced chemical shift changes of head group protons of PC vesicles were greatly increased by suppressing the ionization of the ligand, suggest that effects other than aromatic ring current shifts cause the observed head group chemical shift changes.

Although there are apparent difficulities associated with ascribing all of the observed head group proton shift changes to aromtic ring effects, it is not yet possible to eliminate it as an explanation. However, an alternative or contributing effect may be developed from a consideration of the nature of the micelle (or membrane) itself. Micelles are dynamic systems composed of a hydrocarbon-like interior and a hydrophilic head group region (which is charged in the systems investigated in this study). In the hydrophobic portion of the micelle, the carbon chains are in the liquid phase (Wishnia, 1963) and, therefore, would be expected to be magnetically isotropic. However, as the micelle surface is approached, the system becomes magnetically anisotropic with respect to the direction normal to the micelle surface. This is due to differences in dielectric constants between the hydrocarbon region and the Stern layer (the region consisting of the hydrophilic head group moieties, counterions, and solvent molecules), which, in turn,

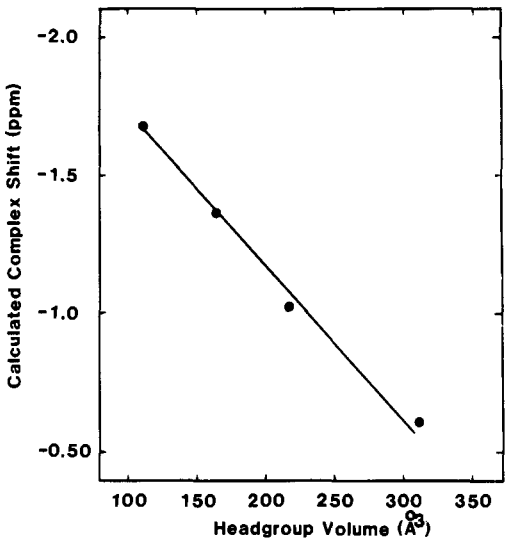

FIGURE 9: Variation in the magnitude of IAA-induced changes in chemical shift of $-N^+(CH_3)_3$ protons with a head group size of amphiphiles in micellar form. Approximate head group volume was obtained by summing the van der Waals volumes of all atoms in the head group (including the carbonyl group for the acyl derivatives, and one methylene group for the alkyltrimethylammonium bromides). Complex shifts for each amphiphile type are averages computed from the data in Table I.

is likely to differ from that of the bulk aqueous phase. Other factors such as variations in hydrogen bonding occurring in the water-deficient lipid phase and the bulk aqueous medium would enhance this difference. Thus, there is a gradient in dielectric constant, hydrogen bonding, and related parameters extending from the hydrophobic core to the bulk aqueous phase and encompassing the head group. Therefore, any change in orientation of the head group that moves it closer to the bulk lipid region would change its magnetic environment, and such a perturbation may give rise to the observed proton upfield chemical shift changes. We suggest that IAA prompts such a change by binding to both hydrocarbon and head group regions, the former by van der Waals interactions and, perhaps, enhanced by the removal of water from the interface region, and the latter by a combination of electrostatic and van der Waals forces. In the presence of IAA, the average orientation of the micellar head groups would be such that a Stern layer of smaller dimensions is formed that in the absence of IAA, forcing water molecules and counterions out of this region. A previous report of the IAA-induced reduction in head group electrical conductivity and capacitance (by a lowering of the dielectric constant) of black lipid membranes (Zimmerman et al., 1977) supports this proposal. A change in head group conformation may also be indicated by the IAA-induced changes in multiplet structure of amphiphile head group protons, although such changes may be due to chemical exchange effects.

The penetration of the indole nucleus of IAA into the hydrocarbon region of the micelles might be expected to cause aromatic ring current induced shifts of other protons. However, the nature of the shifts is likely to be complex and dependent upon the size and shape of the micellar species. Since different micellar forms are anticipated with the various amphiphiles used, the observed complex nature of the chain methylene and methyl proton chemical shift changes induced by IAA is not unexpected.

The magnitude of the IAA-induced shift changes in head group protons is also dependent on the nature of the head group (Table I). Indeed, there appears to be an inverse relationship between the computed complex shift of the $-N^+(CH_3)_3$ protons and head group size (as measured by summing the van der Waals volumes of all atoms in the head group), and such a plot for the different amphiphile series is shown in Figure 9. This finding is in accord with the proposed origin of the IAA-induced chemical shift changes of head group protons. A larger polar head group implies a thicker Stern layer, which, in turn, implies a more gradual change in magnetic properties from the lipid phase to the bulk solvent phase. However, an alternative reason may be that the micelle size and structure is perturbed by the influx of relatively bulky IAA molecules. Alkyltrimethylammonium bromides have been shown to form small micelles due to the high surface charge density [see Tanford (1973)], and these systems are expected to be more perturbed than systems that form larger micelles such as the acyllysophosphatidylcholines.

Although we are not yet able to distinguish between the possible IAA-induced effects (i.e., aromatic ring currents and changes in head group orientation), the incorporation of IAA into micelles (or membranes) is expected to bring about other changes in physicochemical properties of the amphiphile systems. In addition to possible changes in thickness of the Stern layer and the resultant changes in magnetic environment of the head group, IAA-induced changes in the permeability to cations of bilayers composed of phosphatidylcholine are observed (Jones et al., 1984). Changes in head group charge distribution and charge density are also expected together with changes in the ability of the amphiphile ensemble to bind macromolecules. For those amphiphiles forming bilayers, and particularly biological membranes, all of the above parameters are of importance in determining and controlling the functional properties of the membrane.

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Registry No. IAA, 87-51-4; C_8-TAB, 2083-68-3; C_{10}-TAB, 2082-84-0; C_{12}-TAB, 1119-94-4; C_{14}-TAB, 1119-97-7; C_{16}-TAB, 57-09-0; C_2-Ch·Cl, 60-31-1; C_4-Ch·Cl, 2963-78-2; C_6-Ch·I, 18885-38-6; C_8-Ch·I, 26640-66-4; C_{10}-Ch·I, 26640-67-5; C_{12}-Ch·Cl, 25234-60-0; C_{14}-Ch·Cl, 4277-89-8; *dl*-C_2-Carn, 870-77-9; *dl*-C_6-Carn, 68960-69-0; *dl*-C_8-Carn, 4469-11-8; C_{10}-Carn, 22623-14-9; *dl*-C_{12}-Carn, 13962-03-3; *dl*-C_{12}-Carn·HCl, 88729-97-9; C_{14}-Carn, 29874-09-7; C_{16}-Carn, 1935-18-8; C_8-LPC, 45287-18-1; C_{10}-LPC, 22248-63-1; C_{12}-LPC, 20559-18-6; C_{14}-LPC, 20559-16-4; C_{16}-LPC, 17364-16-8; CC, 67-48-1; PCC, 107-73-3; GPC, 563-24-6; methyl hexanoate, 123-66-0; methyl octanoate, 111-11-5; methyl decanoate, 110-42-9; *N,N*-dimethylethanolamine, 108-01-0; iodomethane, 74-88-4; octyl bromide, 111-83-1; decyl bromide, 112-29-8; dodecyl bromide, 143-15-7; trimethylamine, 75-50-3.

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pH-Induced Destabilization of Phosphatidylethanolamine-Containing Liposomes: Role of Bilayer Contact†

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ABSTRACT: The mechanism of pH-induced destabilization of liposomes composed of phosphatidylethanolamine and a charged cholesteryl ester was studied by following the release of encapsulated aqueous contents. The kinetics of release were measured continuously by using the water-soluble fluorophore 8-aminonaphthalene-1,3,6-trisulfonic acid in combination with the water-soluble quencher *p*-xylylenebis(pyridinium) bromide. With this fluorescence assay, release of contents from liposomes composed of phosphatidylethanolamine and cholesteryl hemisuccinate was shown to be a function of pH, ratio of phosphatidylethanolamine to cholesteryl hemisuccinate, and acyl chain composition of the phosphatidylethanolamine. Leakage was very slow at pH 5.5 and increased dramatically with decreasing pH down to 4.0. Replacing phosphatidylethanolamine by phosphatidylcholine eliminated the effect of pH on leakage. Analysis of the kinetics of release by a mass action model demonstrated that bilayer destabilization and leakage occur subsequent to aggregation. The requirement of bilayer contact for destabilization has been found previously for acidic phospholipid bilayers in the presence of divalent cation and for saturated phosphatidylcholine bilayers below the isothermal phase transition temperature. The phosphatidylethanolamine-containing bilayers examined here satisfy the same requirement.

Bilayer destabilization has been shown to require interbilayer contact in a large number of model membrane systems. This prerequisite for membrane fusion has been shown for liposomes composed of acidic phospholipids in the presence of divalent cations (Wilschut et al., 1980; Liao & Prestegard, 1980a,b; Nir et al., 1983; Bentz et al., 1983b) and liposomes composed of saturated phosphatidylcholines below the isothermal phase transition temperature (Suurkuusk et al., 1976; Schullery et al., 1980; Wong et al., 1982). It has been proposed that this is also the case with phosphatidylethanolamine (PE)[1]-containing bilayers (Cullis & Hope, 1978; Cullis et al., 1980; Verkleij et al., 1980; Mantsch et al., 1981) but this has not yet been proven.

Pure PE liposomes can only be made above pH 9.0 (Stollery & Vail, 1977), where the PE is negatively charged. Injection of these liposomes into pH 6.0 buffers, where the PE is protonated, leads to aggregation, leakage, and lipid mixing (Kolber & Haynes, 1979; Pryor et al., 1983). These studies, however, did not show that aggregation, or bilayer contact, was necessary for bilayer destabilization and leakage, since the possibility that leakage occurred independently of aggregation could not be ruled out. Moreover, the protonation of the PE may promote other molecular rearrangements and bilayer perturbations that do not pertain to the interactions between PE-containing bilayers at physiological pH.

Stable PE-containing liposomes can be formed at physiological pH when PE is mixed with acidic phospholipids, due to the stabilization by the negatively charged head groups. Mixing these liposomes with Ca^{2+} (Düzgüneş et al., 1981; Sundler et al., 1981; Hope et al., 1983) or a pH 3.0 buffer (Hope et al., 1983) induces aggregation, destabilization, and fusion of the liposomes. However, neither of these systems is optimal for elucidating the role of PE in bilayer destabilization. With Ca^{2+} the destabilization is dominated by interaction of the acidic phospholipids with the divalent cation. At the pH values necessary to protonate the acidic phospholipids (2.0–3.0), thus allowing aggregation of the liposomes, the further protonation of the PE cannot be excluded. In addition, it is difficult to devise assay systems that are competent over such wide pH ranges.

We have designed a lipid system that can assess the importance of membrane contact in bilayer destabilization of PE-containing liposomes at physiological pH. Stable liposomes can be made with PE and cholesteryl hemisuccinate (CHEMS) at pH 7.5, where the CHEMS is negatively charged. When the CHEMS is protonated, below pH 5.5, the liposome is effectively composed of PE and cholesterol. By inducing the destabilization of the liposomes by H^+ at physiological pH values (4.5–7.5), one can study the effect of contact per se, since the PE remains zwitterionic in this range. To investigate the mechanism of pH-induced release of liposome contents, we developed a quantitative assay that allows for leakage to be monitored at very early times and continu-

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[1] Abbreviations: PC, egg phosphatidylcholine; TPE, phosphatidylethanolamine prepared from egg PC by transesterification; PE, phosphatidylethanolamine; DOPE, dioleoylphosphatidylethanolamine; PS, phosphatidylserine; PA, phosphatidic acid; CHEMS, cholesteryl hemisuccinate; ANTS, 8-aminonaphthalene-1,3,6-trisulfonic acid; DPX, *p*-xylylenebis(pyridinium) bromide; Tris, tris(hydroxymethyl)aminomethane hydrochloride.

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