Complex Formation between Indole-3-acetic Acid and Phospholipid Membrane Components in Aqueous Media. 1. Parameters of the System†

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ABSTRACT: Changes in proton nuclear magnetic resonance (NMR) chemical shifts have been used to investigate the interaction between the plant hormone indole-3-acetic acid (IAA) and soybean phosphatidylcholine (PC). The addition of IAA to solutions of PC in some organic solvents or to sonicated aqueous dispersions of PC causes upfield changes in chemical shifts of the protons of the choline head group. The magnitudes of these effects are dependent on the concentrations of the interactants, and they enable dissociation constants and complex shifts to be calculated. The interaction is pH dependent and is significantly stronger with un-ionized IAA than with ionized IAA. Na^+ and K^+ ions did not produce differential effects on the IAA-induced upfield proton chemical shift changes in PC, and different concentrations of K^+ ion did not affect the interaction. IAA also effects cation movement through phospholipid bilayers as monitored with NMR by the effects of Pr^{3+} and Mn^{2+} ions on the inside and outside trimethylammonium groups of the PC vesicles.

Since the discovery of the endogenous growth hormone indole-3-acetic acid (IAA)[1] by Kögl and Haagen-Smit in 1931, plant growth hormones have received much attention in terms of their structure–activity relationships, intracellular localization, regulation of biochemical pathways, effects on transport phenomena, etc. There is at present, however, no accepted understanding of the molecular basis of IAA action.

Our research program is designed to investigate the presence or absence of IAA–phospholipid interactions and the nature and characteristics of any complex that forms. Previous studies in this laboratory have demonstrated an interaction between IAA and phospholipids in chloroform with ^1H NMR to monitor chemical shift changes induced by the interaction (Marker et al., 1978). Phosphatidylcholine (PC) forms inverted micellar structures in some organic solvents such as chloroform, whereas in water dispersions are formed. Huang (1969) showed that ultrasonic irradiation of aqueous dispersions of PC produces an ordered lipid structure in the form of spherical vesicles, bounded by a single bilayer wall of lipid. Such vesicles are now widely accepted as useful models in the investigation of membrane-associated biological phenomena. These vesicles lend themselves to high-resolution NMR investigation since they give rise to sharp absorption signals (Penkett et al., 1968) in contrast to the broad signals obtained from hydrated phospholipids (Chapman et al., 1967) or non-sonicated aqueous dispersions (Chan et al., 1971). We report here on the aqueous interaction between IAA and PC with ^1H NMR to monitor chemical shift changes of the PC protons.

Experimental Procedures

Materials

PC was isolated from crude soybean PC (Sigma, type III) by column chromatography on neutral alumina (Merck, activity II–III) with a stepwise gradient of chloroform/methanol (Singleton et al., 1965). A total of 20 g of crude soybean PC usually yielded approximately 4.6 g of pure PC. The purified phospholipid gave a single spot on silica gel thin-layer chromatography plates developed in chloroform/methanol/acetic acid/water (50:25:7:4) and sprayed with molybdate reagent. Purified phospholipids were stored in chloroform/ethanol solution (4:1) under nitrogen at 4 °C in darkness. The purity of the phospholipid preparations remained unchanged throughout the course of the experiments (and, under the conditions of storage, for at least 3 months).

Phospholipid concentrations were determined both gravimetrically and from the phosphorus content with Barlett's (1959) method, and agreement between the two methods was better than 5%. Fatty acid content was determined by trans esterification in methanol/H_2SO_4, and the methyl esters, determined by GLC, were the same as previously reported (Marker et al., 1978).

IAA (Sigma) was recrystallized from 1,2-dichloroethane (Snyder & Pilgrim, 1948) and stored under N_2 at 4 °C. Deuterated solvents were obtained from Merck Sharp & Dohme (Canada), and D_2O was from AAEC (Australia). Praseodymium nitrate hydrate (Pr^{3+}) and manganese chloride tetrahydrate (Mn^{2+}) were purchased from Fluka and BDH BDH (Australia), respectively.

Methods

Solvent systems of soybean PC, at concentrations of ca. 65 mM, were prepared by dissolving known weights of the phospholipid in measured volumes of the deuterated solvents. PC is readily soluble in $CDCl_3$ and MeOH-d_1 but dissolves only slowly in Me_2SO-d_6. In order to prepare solutions of PC in this solvent, the mixture was sonicated for 5 min in an ice bath. IAA in the same solvent was added to the phospholipid solution in microliter increments (with the exception of the $CDCl_3$ systems). Because of the low solubility of IAA in $CDCl_3$, weighed amounts of IAA were added to NMR tubes containing the phospholipid solution.

pH Determinations. pH values of phospholipid dispersions in D_2O were measured at room temperature (ca. 21 °C), with a combination glass electrode that was calibrated for H^+ measurements, and are uncorrected for deuterium isotope effects.

Preparation of Vesicles. Soybean PC (typically 180–200 mg) was hydrated under nitrogen in 4 mL of buffer and so-

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[1] Abbreviations: NMR, nuclear magnetic resonance; IAA, indole-3-acetic acid; PC, soybean phosphatidylcholine; MeOH-d_4, tetradeuterated methanol; Me_2SO-d_6, hexadeuterated dimethyl sulfoxide; EDTA, ethylenediaminetetraacetic acid; TSP, (trimethylsilyl)propionic acid sodium salt; Tris, tris(hydroxymethyl)aminomethane.

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nicated in ice with a Branson B12 ultrasonicator equipped with a microtip, at a power setting of 6. The sonicating vessel was a 10-mL round-bottom glass tube with less than 6-mm clearance between probe tip and the tube wall. The tube was flushed with dry nitrogen, and a nitrogen blanket was maintained above the sample during sonication. Sonication was carried out for 5 min, followed by a 5-min pause to allow dissipation of heat, and continued for a total sonicating period of 30 min. After sonication, the clear vesicle dispersions were centrifuged for 30 min at 30000*g* at 20 °C (fixed-angle rotor) to remove titanium particles and multilamellar liposomes. Usually, however, no upper phase corresponding to multilamellar systems was observed. Samples for NMR analysis were withdrawn immediately after centrifugation. It should be noted that during centrifugation the large unilamellar and multilamellar vesicles prepared in D_2O migrate toward the surface of the preparation, leaving the homogeneous minimal-sized vesicles below. This is different from the situation observed when vesicles are prepared in H_2O; in this solvent the large vesicles move to the bottom of the preparation (Barenholz et al., 1977).

Vesicles were usually prepared in 0.2 M sodium acetate/acetic acid buffer (pH 3.85) containing 1 mM EDTA. In investigation of the pH effects, the following buffer systems at 0.2 M, containing 1 mM EDTA, were also used: sodium citrate/citric acid, pH 3.45, 4.80, and 5.31; sodium maleate/sodium hydrogen maleate, pH 5.30 and 6.00; Tris-HCl, pH 7.10, 7.88, and 9.45.

Solutions of IAA as the sodium or potassium salt, at concentrations that were approximately isotonic with the PC vesicle systems, were made up by titrating the free acid in D_2O with NaOD of KOD. Increments of 5 or 10 μL of these IAA solutions were added to the NMR tube with a microlitre syringe. Pr^{3+} and Mn^{2+} were added to the PC dispersions as D_2O solutions of the nitrate and chloride, respectively. KCl was added as a saturated solution in D_2O.

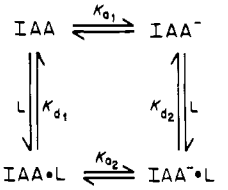
NMR. ^1H NMR spectra were recorded at 60 MHz in continuous-wave mode or at 90 MHz in Fourier-transform mode. Spectra at 60 MHz were obtained on a JEOL PMX-60 spectrometer with a sweep width of 240 Hz and a sweep rate of 250 s at a probe temperature of 32 °C in the unlocked mode. Chemical shift reproducibility was within ±0.02 ppm relative to an internal reference of dioxane.

Spectra at 90 MHz were recorded on a JEOL FX-90Q Fourier-transform spectrometer with a spectral width of 800 Hz accumulated into 8192 addresses at a probe temperature of 24 °C. Dioxane was used as an internal standard, but when Pr^{3+} or Mn^{2+} ions were added to the phospholipid preparations, an external reference of 1% (trimethylsilyl)propionic acid (tetradeuterated) (TSP) dissolved in D_2O was also used. Chemical shift reproducibility was better than ±0.005 ppm.

K_d Calculations. Values for the dissociation constants (K_d) and complex chemical shifts (Δ) for the interaction between IAA and soybean PC were determined from the phospholipid resonance positions observed with changes in IAA concentration (Sykes, 1969; Nicholson & Spotswood, 1973). For 1:1 stoichiometry, under conditions of rapid exchange of IAA between free- and bound-lipid states, the observed chemical shift (δ_{obsd}) of the monitored lipid (L) resonance is related to the change in chemical shift on complex formation (Δ) and the chemical shift of the free species (δ_{free}) by

$$\delta_{obsd} = ([HL]/[L_0])\Delta + \delta_{free}$$

where [HL] and [L_0] are the concentration of the IAA–lipid complex at equilibrium and the total concentration of lipid, respectively. Similarly, equations can be developed for stoichiometries other than 1:1. An iterative procedure was used to calculate theoretical curves for a range of K_ds and Δ's by using various simple stoichiometries (1:1, 2:1, 1:2, etc.). These curves were then compared with the experimental data. A single expression was used to describe the interaction between IAA and PC vesicles in D_2O/acetate buffer at pH 3.85 where all of the IAA was assumed to be in the un-ionized form. However, at higher pH, complex formation between IAA and phospholipid was considered in terms of both ionized and un-ionized IAA. A further equilibrium between bound un-ionized IAA and bound ionized IAA was also employed. The resulting equilibrium diagram is as follows:



where K_{a_1} and K_{a_2} are the ionization constants of IAA in free solution and in the bound state, respectively; K_{d_1} is the dissociation constant of the un-ionized IAA–phospholipid complex, and K_{d_2} is the dissociation constant of the ionized IAA–phospholipid complex.

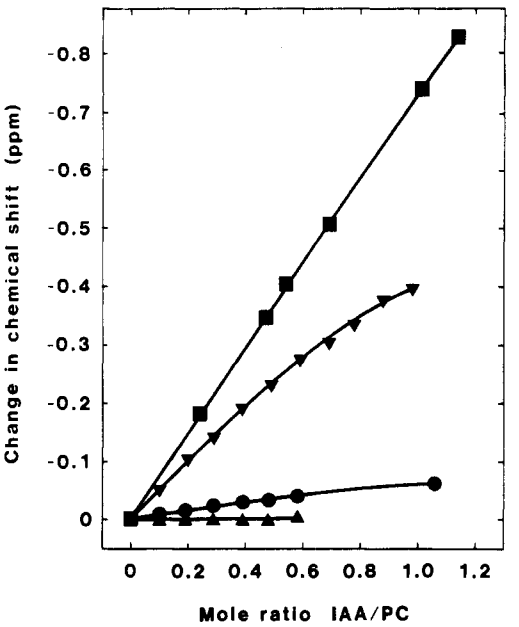


FIGURE 1: Effects of solvent on the upfield changes in ^1H chemical shift, relative to an internal 1,4-dioxane standard, of the $-N^+(CH_3)_3$ protons of PC (~65 mM) with different IAA concentrations: (■) $CDCl_3$ solution at 32 °C; (▼) vesicle preparation in D_2O/acetate buffer (pH 3.85) at 24 °C; (●) MeOH solution at 24 °C; (▲) Me_2SO-d_6 solution at 24 °C. The solid lines through the experimental points represent theoretical curves from which K_d and Δ values were obtained.

Results

Interaction of IAA with PC in Organic Solvents. Addition of IAA to $CDCl_3$ solutions of soybean PC produced an upfield shift of the $-N^+(CH_3)_3$ proton peak as previously reported (Marker et al., 1978) whilst no detectable shifts were observed for the acyl methylene and methyl protons. Concomitant with this shift was a small increase in line width of the choline resonance, from 3.5 Hz in the absence of IAA to 4.4 Hz in the presence of a 1:1 molar ratio of IAA to PC. Figure 1 shows titration curves for incremental additions of the hormone to $CDCl_3$, MeOH-d_4, and Me_2SO-d_6 solutions of PC. Also shown is the titration curve in D_2O/acetate buffer (pH 3.85) of PC vesicles with IAA (as the sodium salt) for comparison.

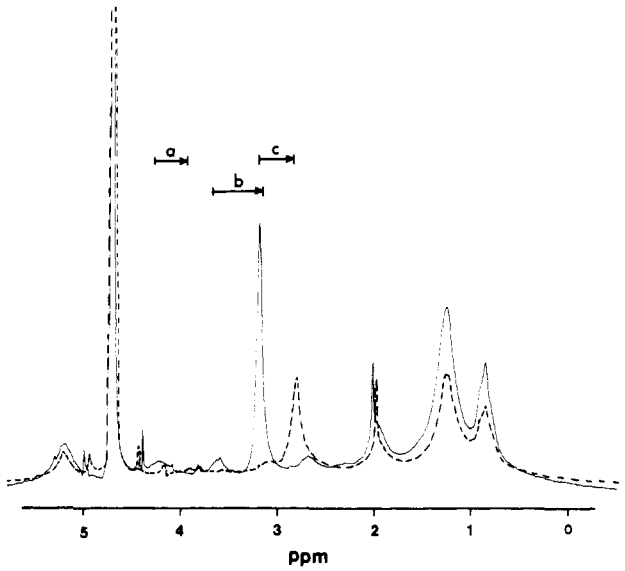


FIGURE 2: Proton NMR spectra of PC (65 mM) as vesicles in acetate buffer (pH 3.85) at 24 °C in the absence of IAA (solid line) and the presence of 65 mM IAA (broken line). Peaks that move upfield relative to an internal 1,4-dioxane standard in the presence of IAA are (a) $-O-CH_2-$, (b) $-CH_2-N-$, and (c) $-N^+(CH_3)_3$.

The shifts induced in $CDCl_3$ solution were substantially greater than those induced in MeOH-d_4 whilst negligible shift changes were observed for the $-N^+(CH_3)_3$ protons in DMSO-d_6. The shape of these curves enabled the calculation of K_ds and Δ's for the interaction between IAA and PC. Values of (6.1 ± 1.1) × 10^{-5} M^2 (K_d) and −1.579 ± 0.006 ppm (Δ) were obtained for the $CDCl_3$ system in which a stoichiometry of 2:1 (IAA to PC) best described the interaction. The corresponding values for methanol-d_4 solution are 79 ± 12 mM and −0.176 ± 0.002 ppm for a 1:1 stoichiometry.

Interaction of IAA with PC in Aqueous Systems. On addition of IAA to sonicated PC vesicle preparations (pH 3.85), an upfield shift of the trimethylammonium proton peak was produced similar to the effects observed in $CDCl_3$ but of smaller magnitude (Figure 1). Again, no chemical shift changes were observed for the acyl methylene and methyl protons. From this curve, a K_d of 4.7 ± 0.6 mM and Δ −0.528 ± 0.005 ppm were obtained for the interaction (1:1 stoichiometry).

The use of a Fourier-transform spectrometer in these experiments also enabled other proton signals in the system to be observed. Figure 2 shows the ^1H spectrum of ca. 65 mM PC in acetate buffer (pH 3.85) together with the corresponding spectrum in the presence of a 1:1 mole ratio of IAA. Assignments of the PC resonances were taken from Birdsall et al. (1972). As can be seen, other resonances also move upfield with the addition of IAA. Most notable amongst these are the ca. −0.5 ppm shift of $-CH_2N^+$ protons and the approximately −0.28 ppm shift of the choline $O-CH_2-$ peak. [In the present case, this peak is obscured by some of the glycerol protons, but it has been shown to move upfield in acyl choline compounds (Jones & Paleg, 1984), and it is assumed that the upfield movement of the broad peak in the present example is due to movement of the choline peak.] The remaining acyl methylene protons do not show shift changes with added IAA. The $-CH_2COOH$ protons of IAA, which resonate at 3.60 ppm in the absence of PC, are not observed. These results for incremental addition of IAA to PC in acetate buffer are shown in Figure 3, and the K_d values, calculated for each of the shifted proton peaks, are given in Table I. The agreement between these results is satisfying. Figure 3 also shows the

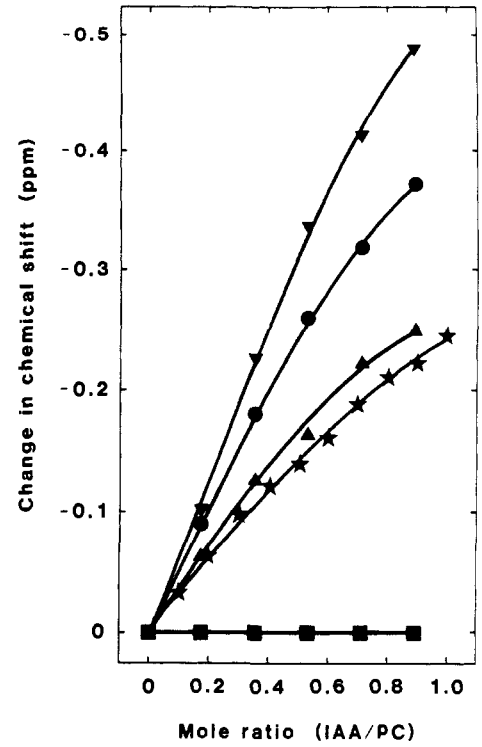


FIGURE 3: Changes in proton chemical shifts of PC (65 mM) as vesicles in acetate buffer (pH 3.85) with different concentrations of IAA. Peak positions were measured relative to an internal 1,4-dioxane standard: (●) $-N^+(CH_3)_3$; (▼) $-CH_2N$; (▲) $O-CH_2$ (choline); (■) glycerol, acyl methylene, acyl methyl, and olefinic protons. The solid lines through the experimental points [with the exception of (■)] represent theoretical curves from which K_d and Δ values of Table I were obtained. IAA-induced PC $-N^+(CH_3)_3$ proton shift changes for a lower concentration of PC (8.98 mM) (solid stars) are also included.

Table I: Dissociation Constants (K_d) and Calculated Complex Shifts (Δ) for 1:1 Molar Interaction between IAA and PC (65 mM) in Acetate Buffer (pH 3.85)

	K_d (mM)	Δ (ppm)
$-N^+(CH_3)_3$	5.1 ± 0.7	−0.532 ± 0.008
$N-CH_2-$	3.5 ± 0.5	−0.641 ± 0.006
$-CH_2-O$	4.4 ± 0.6	−0.339 ± 0.007

titration curve of 8.92 mM PC (in acetate buffer, pH 3.85) with IAA. A K_d of 4.2 ± 0.7 mM and Δ of −0.512 ± 0.008 ppm were calculated from these data.

Titration of PC vesicles with K^+IAA gave results identical with those with Na^+IAA, indicating that monovalent cations do not affect the interaction. Furthermore, no differences were observed when the titrations were carried out in the presence of KCl at concentrations in the range 0–380 mM (data not shown).

Addition of IAA to PC vesicle preparations caused a monotonic increase in turbidity. Whilst this was slight at lower concentrations of IAA (<0.7 mole ratio IAA to PC at PC concentrations of ca. 65 mM in acetate buffer, pH 3.85), at higher concentrations the turbidity increased rapidly, and at a mole ratio of approximately 1.1:1 (PC concentration ca. 65 mM), precipitation was observed. At an appreciably lower PC concentration (ca. 10 mM), in the same buffer, a mole ratio of 3.2:1 was attained before precipitation occurred. Also observed in the titration experiments was a decrease in intensity of the $-N^+(CH_3)_3$ proton peak and the peaks associated with the acyl protons, with respect to the internal standard. This reduction in intensity was accompanied by a small but insubstantial line broadening. These effects could be due to

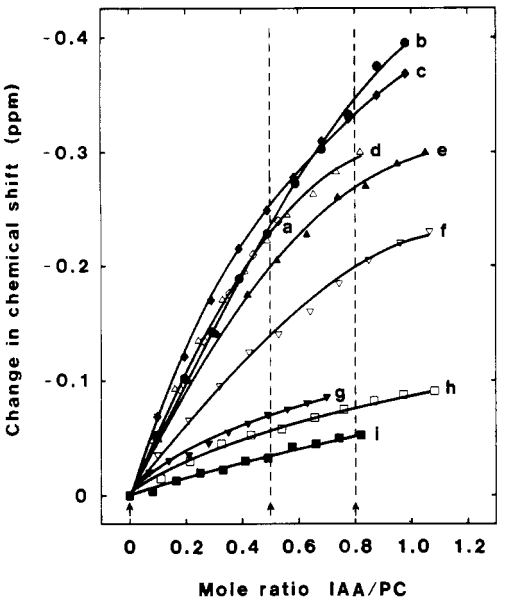


FIGURE 4: Changes in chemical shift, relative to an internal 1,4-dioxane standard, of $-N^+(CH_3)_3$ protons of PC as vesicle preparations at ~65 mM with added IAA in different buffers (200 mM): (a) citrate (pH 3.45–3.54); (b) acetate (pH 3.85–4.15); (c) citrate (pH 4.80–5.01); (d) citrate (pH 5.31–5.48); (e) maleate (pH 5.30–5.58); (f) maleate (pH 6.00–6.12); (g) Tris-HCl (pH 7.10–7.42); (h) Tris-HCl (pH 7.88–7.90); (i) Tris-HCl (pH 9.55–9.68). Solid lines through the experimental points in acetate buffer (curve b, pH 3.85) and Tris-HCl buffer (curve i, pH 9.55) represent theoretical curves from which K_d and Δ values were obtained. Vertical broken lines at mole ratios of 0.5 and 0.8 IAA to PC indicate the values presented in Figure 5.

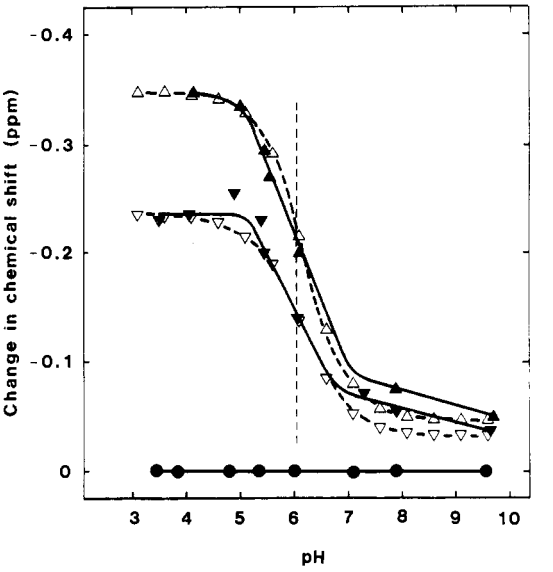


FIGURE 5: Changes in chemical shift of PC $-N^+(CH_3)_3$ protons as a function of pH. Values were obtained from the data presented in Figure 4. Changes in chemical shift with pH (solid lines) are shown for the three different mole ratios of IAA to PC (indicated in Figure 4 by arrows): (▲) 0.8; (▼) 0.5; (●) 0.0. Calculated values (see text) are shown as open symbols and broken curves. The vertical broken line at pH 6.05 indicates the approximate point of inflexion of the chemical shift curves.

IAA–PC complex formation or, at higher IAA concentrations, to IAA–induced fusion of small vesicles.

pH Effects. Titration curves generated by the incremental addition of the Na^+ salt of IAA to PC vesicle preparations in various buffers are shown in Figure 4. The curves demonstrate a monotonic decline in the ability of IAA to affect the $-N^+(CH_3)_3$ chemical shift with increasing pH. The values obtained in acetate buffer (curve b, pH 3.85) and in Tris-HCl buffer (curve i, pH 9.55) were used to calculate K_d and Δ values representing, respectively, the interaction of un-ionized IAA with PC and ionized IAA with PC. At low pH (3.85–4.15), the K_d was 4.7 ± 0.6 mM and the Δ was −0.528 ± 0.005 ppm while at high pH (9.55–9.68) the K_d was 33.0 ± 2.0 mM and the Δ was −0.111 ± 0.002 ppm.

A greater stability of the system to increased amounts of added IAA was also noted with increasing pH. For example, turbidity and precipitation were observed at an IAA–PC mole ratio of approximately 0.6 in citrate buffer at pH 3.45, whereas in Tris-HCl buffer (pH 9.55) very little increase in turbidity was observed at a mole ratio of 1.0.

The pH dependence of the interaction is also shown by plotting chemical shift changes against pH for given mole ratios of IAA–PC (the PC concentrations in the various buffers were approximately equal). Three such curves, at mole ratios of 0.8, 0.5, and 0.0, are shown in Figure 5. The latter curve illustrates the lack of effect of pH alone on the resonance position of the $-N^+(CH_3)_3$ signal. The data show the major features of acid/base titration curves, and it can be concluded that the point of inflexion of the curves represents the approximate ionization constant of IAA when bound in the complex. The derived pK_a of 6.1 compares with the published value of 4.55 for IAA in aqueous solution (Albaum & Kaiser, 1937).

As suggested earlier, if the pH 3.85 titration curve (in acetate buffer) represents the interaction of un-ionized IAA with PC and that at pH 9.55 (in Tris-buffer) represents the interaction of ionized IAA with PC, the four equilibria described earlier can be assigned values. From these values, the chemical shift changes expected at any mole ratio of IAA–PC and at any pH can be calculated in an analogous manner to the calculation of K_ds and Δ's. Two such curves are also illustrated in Figure 5 (broken lines) at the same mole ratios that have been used to present the experimental data. The agreement is good although not perfect. It is not known whether the differences are due to errors in calculating K_ds and Δ's or whether the pH dependence of the interaction is due to other factors in addition to the ionization of IAA.

Effects of Pr^{3+} and Mn^{2+}. On addition of 15 mM Pr^{3+} to a PC vesicle preparations at 64.5 mM in acetate buffer (pH 3.85), the $-N^+(CH_3)_3$ proton peak was split into two components, the major peak at 3.44 ppm and a minor peak at 3.12 ppm, with respect to the external TSP reference (Figure 6). Bystrov et al. (1971) first showed that the peak splitting was due to the inability of the Pr^{3+} shift reagent to penetrate the phospholipid bilayer, and therefore, only the head groups of the outer layer of the vesicle wall were affected. The outer layer $-N^+(CH_3)_3$ protons were shifted downfield by Pr^{3+} whilst the signal due to the inner groups was unaffected. The integrated intensities of these peaks for both unfractionated and fractionated (by differential centrifugation) vesicle preparations in the present work gave outside to inside ratios for the $-N^+(CH_3)_3$ groups of 1.7 ± 0.1 for both acetate (pH 3.85) and Tris (pH 7.1) buffer systems. Vesicles prepared in 50 mM KCl, however, give outside to inside ratios of 2.0 ± 0.1 and 2.2 ± 0.1 for unfractionated and fractionated vesicles, respectively.

On addition of IAA to vesicle preparations after addition of Pr^{3+}, both the inside and outside $-N^+(CH_3)_3$ proton peaks moved upfield. The signal from the internal standard remained at a constant position as did the acyl protons with respect to the external TSP reference. The observed shifts for the $-N^+(CH_3)_3$ protons are shown in Figure 6 and are compared with the values for IAA–PC in the absence of Pr^{3+} (broken line). Initially, the signal from the outer PC molecules moves

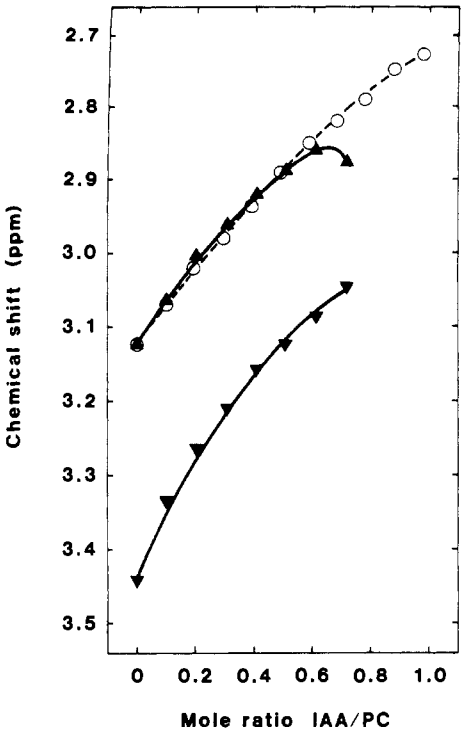


FIGURE 6: Changes in chemical shift of $-N^+(CH_3)_3$ protons of PC (61 mM) as vesicles in acetate buffer (pH 3.85) with different concentrations of IAA in the presence of 15 mM Pr^{3+}. Chemical shifts were measured with respect to an external TSP reference. (▼) Shift changes associated with the outward-facing head groups; (▲) shift changes associated with inward-facing head groups. The solid lines represent lines of best fit to the data. The broken line (O) indicates the chemical shift changes observed in the absence of Pr^{3+}.

upfield more quickly than it does in the absence of Pr^{3+} whilst the inside signal behaves normally. At higher IAA ratios, however, the inside peak moves more slowly and eventually begins to move downfield (precipitation at higher concentrations of IAA prevented us from observing a coalescence of the signals). Since the behavior of inside and outside head groups is not dissimilar with added IAA [only one peak for the $-N^+(CH_3)_3$ protons is observed during titration with IAA in the absence of Pr^{3+}], the observations suggested that either Pr^{3+} had entered the intact vesicles or, less likely (see below), that the vesicles had ruptured.

Vesicle preparations exclude Pr^{3+} for at least several days in the absence of IAA. When a single addition of IAA, at an IAA to PC mole ratio of approximately 0.5, was made to vesicle preparations in acetate buffer to which had been added 10 mM Pr^{3+}, two peaks at −0.762 and −0.550 ppm (with respect to an internal dioxane reference), due to the inside and outside $-N^+(CH_3)_3$ resonances, were observed (Figure 7). Monitored over a period of hours, however, the upfield (inside) peak gradually moved downfield until it merged with the downfield (outside) peak, indicating an IAA–induced time dependancy for the entry of Pr^{3+} into the vesicles. During this process, the peaks remained symmetrical, and no line broadening or reduction in intensity of the resonances (when measured against the internal standard) was observed. In the absence of IAA, no change in the position of the resonances over the same period was noted.

The addition of further 5 mM Pr^{3+} to the vesicle preparation after the merging of the inside and outside $-N^+(CH_3)_3$ resonances again resulted in two peaks associated with this group. One component was shifted downfield whilst the second component remained stationary. Over a period of hours, the upfield peak moved downfield until it again merged with the

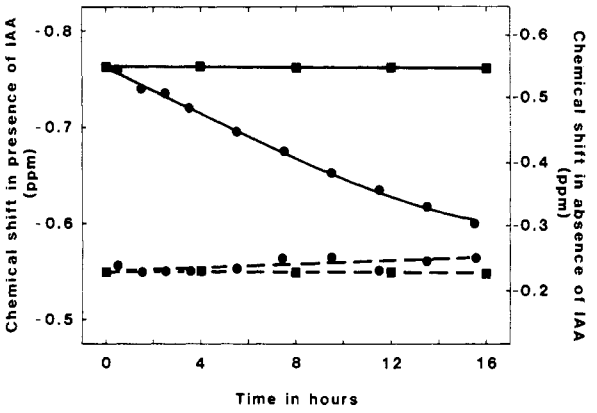


FIGURE 7: Time dependency of chemical shifts of $-N^+(CH_3)_3$ protons of PC as vesicle preparations in the presence of 10 mM Pr^{3+}. Vesicles were made up (PC at 61 mM) in acetate buffer (pH 3.85) to which was added IAA to give an IAA to PC mole ratio of 0.5. Peak positions were determined relative to an internal 1,4-dioxane reference. Solid lines indicate chemical shift changes in the inward-facing head groups, and broken lines indicate chemical shift changes of outward-going head groups. Values obtained (■) in the absence of IAA and (●) in the presence of IAA.

downfield peak, clearly demonstrating that the vesicles remain intact in the presence of IAA and that IAA facilitates the movement of Pr^{3+} through the bilayer.

The addition of 5 mM Mn^{2+} to vesicle preparations in acetate buffer (pH 3.85) produced a significant reduction in intensity of the $-N^+(CH_3)_3$ while the acyl proton peaks remained unaffected. Peak positions relative to the external TSP reference were unaffected. The intensity reduction has been ascribed to paramagnetic broadening of the signal of the outer $-N^+(CH_3)_3$ groups in the vesicle bilayer whilst the inner groups are unaffected (Bystrov et al., 1971). On this basis, the integrated intensities of the $-N^+(CH_3)_3$ signal before and after addition of Mn^{2+} gave an outer to inner ratio of 1.7 ± 0.1, which is in excellent agreement with the value obtained with Pr^{3+}.

On addition of IAA to this system, an upfield shift of the trimethylammonium peak was observed, identical with that in the absence of Mn^{2+}, concomitant with a progressive reduction in intensity of the peak. At an IAA to PC mole ratio of approximately 0.5, the signal disappeared completely. On the other hand, when 5 mM Mn^{2+} was added to a vesicle system containing 50 mole % IAA, a signal due to the inner $-N^+(CH_3)_3$ groups was observed, which reduced in intensity in a time-dependant manner (Figure 8) analogous to the system with added Pr^{3+}. This indicates, again, that in the presence of IAA the lipid bilayer becomes permeable to polyvalent cations.

Discussion

IAA has been shown to interact with plant phosphatidylcholine in $CDCl_3$ and as vesicles in D_2O. The interaction in $CDCl_3$ is best described by a stoichiometry of 2:1 (IAA to PC) with a K_d of 6.1×10^{-5} M^2 and a complex shift of −1.58 ppm. These values agree well with those determined previously at a similar concentration of PC (Paleg et al., 1973) but not with the values determined for the $CDCl_3$ interaction at lower concentrations of PC (Marker et al., 1978). PC has been shown to form inverted micelles in chloroform at concentrations greater than $\sim 10^{-2}$ M (Haque et al., 1972), and the amount of PC in micellar form is a function of total PC concentration. At concentrations greater than 4 or 5 times the critical micelle concentration, the molecules are predominantly in micelles [see Tanford (1973)]. The dependence of the interaction on PC concentration in the range 30–65 mM

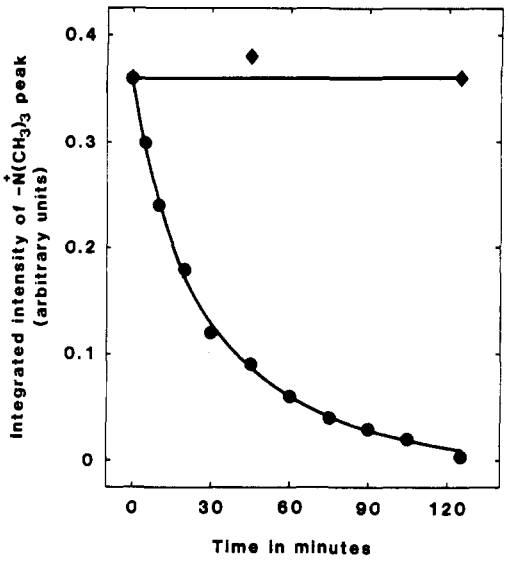


FIGURE 8: Time dependency of Mn^{2+} entry into PC (64 mM) vesicles made up in acetate buffer (pH 3.85). IAA was added to give an IAA to PC mole ratio of 0.5. Mn^{2+} at 5 mM was then added, and integrated peak intensities of the $-N^+(CH_3)_3$ protons were determined relative to an internal 1,4-dioxane reference. Peak intensities in the absence of IAA (♦) have been normalized to the initial value of the curve in the presence of IAA.

in $CDCl_3$ indicates that the interaction of IAA with PC is a function of micelle concentration rather than total lipid concentration. In addition, lecithin is known to form only small weak aggregates in lower alcohols like methanol (Elworthy & McIntosh, 1961) and is expected to be completely disperse in Me_2SO solutions. The weak interaction parameters observed in these solvents reinforce the concept that structuring of the PC molecules into ordered aggregates is necessary for the interaction.

The formation of single-walled vesicles by ultrasonication of plant PC in D_2O and the properties of such vesicles are consistent with results obtained with PC derived from other sources. The observations from gel-exclusion chromatography and inside to outside ratios of head groups of fractionated and unfractionated vesicles determined by NMR with Pr^{3+} and Mn^{2+} show almost totally homogeneous populations of vesicles in our system, emphasizing the similarity with other systems. The mean vesicle diameter that we have observed, however, appears to be a function of the type of medium in which the phospholipid is dispersed. These PC vesicle preparations interact with IAA in a manner that is best described by a 1:1 stoichiometry, and the interaction is strongly pH dependent. Since the range of pH used in these investigations precludes changes in ionization of the phosphate group or the trimethylammonium group of PC, the interaction appears to depend upon the ionization state of IAA. The interaction is stronger with un-ionized IAA in terms of both a smaller K_d value and a larger Δ.

The effect of pH on the formation of the IAA–PC complex may be of significance when considering biological implications of the interaction. It seems reasonable to assume that the presence or absence of an effect of IAA on PC-containing membranes would be as much dependent on intracellular pH as on the absolute and relative concentrations of the interactants themselves. In fact, several plant systems have been reported in which a simple lowering of the pH has triggered the onset of growth (Cleland, 1973; Vanderhoef & Stahl, 1975; Mentze et al., 1977).

Also apparent is a change in the physical properties of IAA as a function of binding to the PC molecules. The pK_a of the carboxyl group is alkaline shifted by approximately 1.5 units. Similar changes have been found when the ionophore X-537A is incorporated into dipalmitoyl-PC (Degani, 1978); an alkaline shift of approximately 1.3 was observed. A small shift in pyranine, when it binds to asolectin vesicles, has also been reported (the small shift in pK_a in that case was associated with weak binding; Clement & Gould, 1981).

It has been demonstrated that IAA alters Pr^{3+} and Mn^{2+} ion movement through the phospholipid bilayers. Ion carriers such as the monocarboxylic acid ionophores of the nigericin type (e.g., X-537A and A23187) are known to move polyvalent cations across membranes (Fernández & Cerbón, 1973). Valinomycin has also been shown to mediate the transport of K^+ into small unilamellar soybean phospholipid vesicles (Clement & Gould, 1981). Such carriers, however, are far more complex in their chemical structure than IAA, and it seems doubtful that IAA would form a sufficiently hydrophobic complex with these cations to enable them to be transported through the membrane.

However, since IAA does enhance the movement of cations across the phospholipid bilayer, the increase in conductance across egg PC black lipid membranes associated with an increase in K^+ concentration in the presence of IAA, observed by Zimmerman et al. (1977), is to be anticipated. Such changes are, probably, not only a function of K^+ concentration but more fundamentally a function of IAA concentration. In particular, the present results make it probable that IAA alters the movement of K^+ rather than K^+ altering the location of the IAA molecules. The hormone-facilitated movement of ions through the membrane may also have physiological significance since Mn^{2+} transport in tissues is most important, as is the general control of permeability.

The mechanism of the proton chemical shift changes induced by IAA is not clear at the present time although diamagnetic shielding effects due to aromatic ring currents in the indole system have been proposed to account for the chemical shift effects induced in acetylcholine by IAA and other aromatic molecules (Minch et al., 1979). The calculated Δ values for $-N^+(CH_3)_3$, $-CH_2N^+$, and $O-CH_2-$ protons in the acetylcholine system have the same relative order as in the present study although their magnitudes are about 5 times larger. Such a proposal has also been advanced to account for the proton chemical shifts observed in the interaction between IAA and PC in deuteriochloroform (Marker et al., 1978). However, there is evidence from X-ray diffraction (Coster et al., 1981) and deuterium NMR studies (Boulanger et al., 1981) of PC systems that shows that aromatic molecules of similar complexity to IAA are preferentially situated in the lipophilic region of phospholipid bilayers with the hydrophilic portions of the molecules projecting into the head group region. In this connection, it is noted that IAA does not cause splitting of the $-N^+(CH_3)_3$ peak and that this peak moves in an IAA concentration dependant manner. Hence, both inside and outside head groups are equally affected by IAA, indicating that IAA is able to move rapidly across the lipid bilayer and underlining the lipid solubility of IAA.

Finally, it must be pointed out that the use of high concentrations of IAA raises questions about the possible biological relevance of the approach. One of the drawbacks of NMR spectroscopy is the requirement for relatively large amounts of material; for rapidity of experimentation, ease of data observation, and interpretation of results, high concentrations of both IAA and PC were used in the present work. Nonetheless, in a determined attempt to explore this point, a number of assumptions were used to develop circumstances

more characteristic of a biological situation. On the basis of an arbitrary spherical cell 20 μm in diameter, with 20% of its total plasma membrane surface composed of PC, one molecule of which inhabits a surface area of 64 Å^2, calculations established that the PC concentration of the cell (ignoring any PC that might be found in other membranes) was somewhat greater than 10^{-5} M. The change in chemical shift of the PC $-N^+(CH_3)_3$ that would be observed at a concentration of 10^{-4} M IAA and PC was calculated to be 0.011 ppm (from our K_d and Δ data at pH 3.85). This was confirmed experimentally with an observed value of 0.026 ± 0.01 ppm. The biological implications of a shift change of this magnitude are unknown, as are the effects of a weak but extensive interaction between the hormone and a particular membrane or of a high local concentration of IAA with respect to a particular membrane.

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Registry No. IAA, 87-51-4; IAA^-, 1596-90-3; Pr, 7440-10-0; Mn, 7439-96-5; citric acid, 77-92-9; acetic acid, 64-19-7; maleic acid, 110-16-7; Tris-HCl, 1185-53-1.

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