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Promotion of cation transport across phospholipid vesicular membranes by the plant hormone indole-3-acetic acid as studied by ^{1}H-NMR

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Nuclear magnetic resonance spectroscopy has been used to study the facilitated movement of Pr^{3+} across phosphatidylcholine (PC) vesicular membranes by the plant hormone indole-3-acetic acid (IAA). The rate of Pr^{3+} movement across PC membranes is strongly dependent on hormone concentration with a rate proportional to [IAA]^8 for soybean PC vesicles. This indole acetic acid stoichiometry is a function of the nature of the membrane lipid, the corresponding values for egg PC and dioleyl PC are 9.6 ± 0.5 and 10.3 ± 0.2, respectively. The rate of Pr^{3+} movement is also dependent on temperature with overall activation energies falling in the range 14–24 kcal · mol^{-1} for different lipid compositions.

Introduction

The plant hormone, indole-3-acetic acid, has been shown to interact with model membranes composed of soybean phosphatidylcholine (PC) by monitoring the changes in NMR chemical shifts of headgroup protons [1]. The interaction is selective in that closely related auxins have different interaction parameters [2]. The origins of the changes in chemical shift are not clear although it has been suggested that indole acetic acid molecules intercalate between phospholipid molecules with the indole nuclei projecting into the hydrocarbon region of the membrane and the carboxyl groups located in the headgroup region [3]. This intercalation is thought to cause changes in the time-averaged orientations of the choline headgroup. The presence of indole acetic acid carboxyl groups in the headgroup region, together with changes in headgroup packing, alter the surface charge density of the membranes monitored in the NMR by changes in Pr^{3+}-induced hyperfine shifts of headgroup protons. A further effect of indole acetic acid in these systems is the promotion of cation fluxes across the membranes. Using Mn^{2+} and Pr^{3+} to differentiate between the NMR resonances of internal and external headgroups of small unilamellar vesicles [4] it has been shown that indole acetic acid promotes the movement of these cations in an indole acetic acid concentration-dependent manner [1].

Whilst the mechanism of action of indole acetic acid as a plant hormone is not understood it has been shown that indole acetic acid promotes the release of divalent cations from soybean hypocotyl membranes in vitro [5] and the secretion of protons in hypocotyl segments in vivo [6].

The present study further explores the ability of indole acetic acid to promote cation movement across vesicular membranes, in terms of indole acetic acid concentration, lipid composition and temperature effects.

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Materials and Methods

Soybean phosphatidylcholine was isolated from the crude material (Sigma Type III) by column chromatography on alumina as previously reported [1] and further purified on a silica gel (Merck Kieselgel 60) column. The purified phospholipid appeared as a single spot on silica gel TLC plates under normal loading conditions when developed using chloroform/ methanol/ acetic acid/water (50 : 25 : 7 : 4, v/v), and when visualized using molybdate reagent. On heavy loading ($\times 10$), however, a faint spot due to lyso-PC could be observed. Dioleyl PC and dilinoleyl PC were obtained from Aranti Polar Lipids and used as received. Egg PC (Type X1-E) was obtained from Sigma and used without further purification. Indole acetic acid (Sigma) was recrystallised from dichloroethane. Praseodymium nitrate hydrate (Pr^{3+}) and ^2H_2O were purchased from Fluka (Switzerland) and AAEC (Australia), respectively.

Preparation of vesicles

A solution of the appropriate PC (typically 180–200 mg) in chloroform/ethanol (4 : 1, v/v) was evaporated to dryness under vacuum for 3 h at 35°C and then hydrated in 4 ml of sodium acetate/acetic acid (^2H_2O) buffer (200 mM, pH 3.85) under N_2. The phospholipid dispersion was then sonicated for a total sonicating period of 30 min under a blanket of N_2 in ice using a Branson B12 ultrasonicator equipped with a microtip, as previously reported [1]. After sonication, the clear vesicle dispersion was centrifuged for 30 min at $30\,000 \times g$ at 20°C (fixed angle rotor) to remove titanium particles and multilamellar liposomes. Samples for NMR analysis were withdrawn immediately after centrifugation. This procedure yields a reasonably homogeneous population of PC vesicles which have an average diameter of 280 Å. Precautions were taken to exclude atmospheric oxygen during all stages of the preparation of the vesicles and during the NMR experiments. Phospholipid concentrations were determined both gravimetrically and from their phosphorus content [7].

Solutions of indole acetic acid at concentrations which were approximately isotonic with the PC vesicle system, were made up by titrating the free acid with NaO^2H in ^2H_2O. Using a microlitre syringe aliquots of these solutions were pipetted into NMR tubes (5 mm outer diameter) containing 0.5 ml phospholipid vesicle preparation. Pr^{3+} was added as a ^2H_2O solution of the nitrate to give an appropriate final concentration (typically 10 mM).

NMR measurements

All ^1H measurements were performed on a JEOL FX90Q Fourier transform spectrometer equipped with a variable temperature accessory using a spectral width of 800 Hz accumulated into 8K addresses. The variable temperature device was calibrated with an accuracy of ± 1 deg. C using the chemical shift separation of ethylene glycol. Sixteen transients were routinely accumulated giving a chemical shift reproducibility better than ± 0.005 ppm using dioxane as an internal reference.

Kinetic measurements were performed automatically with the JEOL Autostacking software FAFT70-800912 where the *WAIT parameter was used to determine the time interval between successive data sets.

Analysis of NMR kinetic data

The equation describing passive diffusion of Pr^{3+} across the lipid bilayer into the interior of vesicles is

$$\frac{dC_i}{dt} = k(C_o - C_i) \tag{1}$$

where C_o is the Pr^{3+} concentration in the extravesicular medium and C_i is the average Pr^{3+} concentration inside the vesicles. Because the internal volume of the vesicles is small with respect to the total sample volume the solution to Eqn. 1 given the boundary conditions $C_i(t) = 0$ for $t = 0$, and $C_i(t) = C_o$ for $t = \infty$, is

$$2.303 \log(C_o - C_i) = kt \tag{2}$$

For carrier-facilitated diffusion, however, Eqn. 1 becomes

$$\frac{dC_i}{dt} = k(C_o - C_i)^m (I)^n \tag{3}$$

where I is the carrier concentration and m and n

reflect the stoichiometry of the transported species. m and n can be evaluated under conditions of fixed carrier and permeant ion concentrations, respectively. In our experiments a good fit to Eqn. 3 (or its derivatives) was obtained in all cases confirming the validity of its use. As will be shown later, $m = 1$, indicating that the carrier-mediated process is first order with respect to Pr^{3+} concentration. Hence, for constant indole acetic acid concentration

$$t_{1/2} = \frac{0.693}{k} \tag{4}$$

where $t_{1/2}$ is the half-life of the first-order process.

The downfield shift of the inner $-\overset{+}{N}(CH_3)_3$ resonance is a function of local Pr^{3+} concentration and $C_i(t)$ can be determined, therefore, from the difference in chemical shift between the inner (δ_i) and outer (δ_o) $-\overset{+}{N}(CH_3)_3$ protons at time t. However, since this difference ($\Delta\delta$) is not directly proportional to the Pr^{3+} concentration difference on the outside and inside of the vesicles, calibration curves were required. These were obtained by adding various concentrations of Pr^{3+} to vesicle preparations containing various concentrations of indole acetic acid and measuring $\Delta\delta$. This had to be done quickly at higher concentrations of indole acetic acid so as to negate any effects of facilitated diffusion of Pr^{3+} into the intravesicular volume. This procedure is valid in our experiments at pH 3.85 since at equilibrium when $C_i = C_o$, $\delta_i = \delta_o$. Calibration curves in the presence and absence of indole acetic acid are shown in Fig. 1. Calibration curves are identical for concentrations of indole acetic acid of 0.3 mole ratio (IAA/PC) and higher and for PCs containing different acyl chains. Small changes were observed with an increase in temperature although these were neglected in producing Arrhenius plots.

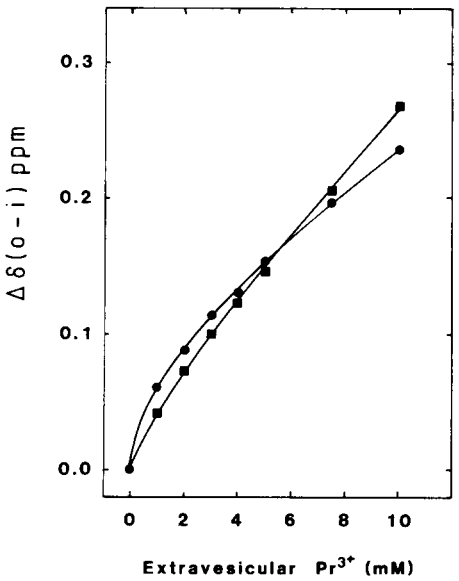

Fig. 1. Change in separation of outside and inside $-\overset{+}{N}(CH_3)_3$ ^{1}H-NMR resonances of soybean PC vesicles (65 mM, 200 mM acetate buffer (pH 3.85)) with increasing extravesicular Pr^{3+} concentration in the absence (■) and presence (●) of indole acetic acid (IAA) (0.3 molar ratio IAA/PC) at 25°C.

Results

Fig. 2a shows a typical ^{1}H-NMR spectrum of soybean PC vesicles in the presence of indole acetic acid. On addition of 10 mM Pr^{3+} to the extravesicular solution the peak associated with the $-\overset{+}{N}(CH_3)_3$ protons is split into two components (Fig. 2b); the upfield peak i and the downfield peak o are associated with the inner and outer-facing PC molecules, respectively. On standing, the upfield signal i moves gradually downfield

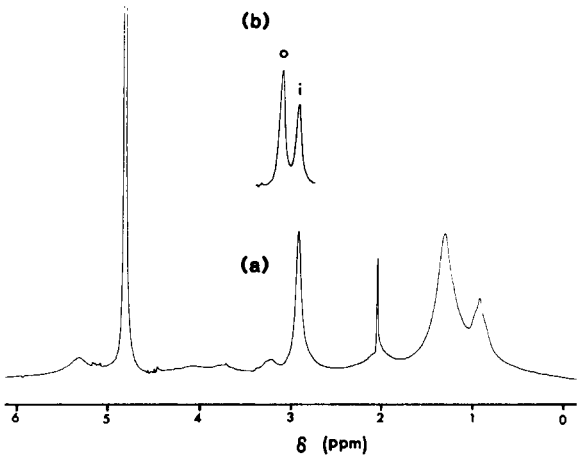

Fig. 2. Typical ^{1}H-NMR spectrum of soybean PC vesicles in the presence of indole acetic acid (0.5 molar ratio) before (a) and after (insert (b)) the addition of 10 mM Pr^{3+} to the extravesicular volume. The peaks o and i refer to the exterior and interior vesicular $-\overset{+}{N}(CH_3)_3$ ^{1}H-NMR resonances, respectively.

until it merges with the downfield signal in a time-dependent manner as shown in Fig. 3. In the absence of indole acetic acid the soybean PC spectrum does not noticeably change over a period of 36 h. Since we have shown that vesicle lysis is not the reason for these changes [1] we conclude that indole acetic acid facilitates the movement of Pr^{3+} across PC bilayers into the interior of vesicles. Similar results were obtained with vesicles made up with PCs containing other acyl chains although the rate constants determined for these systems vary considerably. (It is noted that the interaction between indole acetic acid and these PC systems as determined by indole acetic acid-induced changes in headgroup ^1H resonance positions [1] are identical (Jones, G.P. and Paleg, L.G., unpublished data)). These are presented in Table I. Dilinoleyl PC bilayers show the largest indole acetic acid-facilitated movement of Pr^{3+} at 25°C with a rate constant of $(8.4 \pm 0.5) \cdot 10^{-3}$ min^{-1}, compared with the less unsaturated dioleyl PC membranes which have a rate constant of $(1.9 \pm 0.6) \cdot 10^{-4}$ min^{-1}. Nevertheless, the rate constant for highly unsaturated soybean PC bilayers ($(4.9 \pm 0.4) \cdot 10^{-3}$ min^{-1}) is only slightly larger than that for the more saturated egg PC vesicles ($(1.2 \pm 0.6) \cdot 10^{-3}$ min^{-1}). In the absence of indole acetic acid all these systems show negligible rates of Pr^{3+} movement under the same conditions. Indeed no discernible differences were observed for dioleyl PC and egg PC bilayers over a period of 9 days indicating that the self diffusion rates for Pr^{3+} movement across these membranes are substantially smaller than $5 \cdot 10^{-5}$ min^{-1}. Self diffusion rates measured over a period of 5 days for soybean PC and dilinoleyl PC are approximately $1 \cdot 10^{-4}$ min^{-1} at 25°C. However, because the latter systems are more prone to auto-oxidation, which leads to substantially faster rates (Jones, G.P. and Paleg, L.G., unpublished data), it is not known if these values reflect the true self diffusion rates.

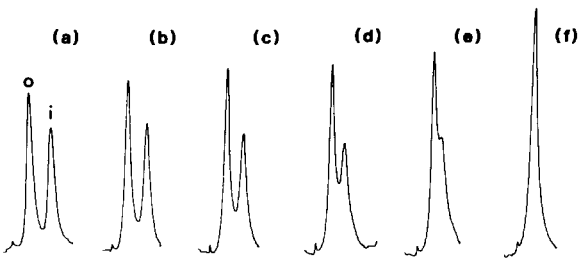

Fig. 3. Time-dependent nature of the NMR spectrum of $-N(CH_3)_3$ protons of soybean PC vesicles containing 10 mM Pr^{3+} in the extravesicular volume at 45°C. The spectra show the result of movement of Pr^{3+} into the intravesicular volume after (a) 1.5, (b) 3.5, (c) 6.0, (d) 11.0, (e) 21.0 and (f) 245 min caused by indole acetic acid (0.5 molar ratio).

TABLE I

RATES OF INDOLE ACETIC ACID-FACILITATED TRANSPORT OF Pr^{3+} IONS ACROSS PHOSPHATIDYLCHOLINE VESICULAR MEMBRANES

The phospholipid concentration is approx. 65 mM in 200 mM acetate buffer (pH 3.85). Molar ratio IAA/PC is 0.5.

	Rate constant at 25°C (min^{-1})	Activation energy ($kcal \cdot mol^{-1}$)
Soybean phosphatidylcholine	$4.9 \cdot 10^{-3}$	24
Egg phosphatidylcholine	$1.2 \cdot 10^{-3}$	16
Dioleylphosphatidylcholine	$1.9 \cdot 10^{-4}$	15
Dilinoleylphosphatidylcholine	$8.4 \cdot 10^{-3}$	14

Rate constants have been determined as a function of both indole acetic acid and Pr^{3+} concentrations. The rate of facilitated diffusion of Pr^{3+} is dependent on the indole acetic acid concentration as shown in Fig. 4 for vesicles made with soybean PC, egg PC and dioleyl PC. Plots of log(rate constant) against log[IAA] are shown in Fig. 5. The slopes of these lines are 8.3 ± 0.5 for soybean PC, 9.6 ± 0.5 for dioleyl PC and 10.3 ± 0.2 for egg PC.

However, a plot of log initial rate against log Pr^{3+} concentration, with an indole acetic acid concentration of 0.5 mole ratio for soybean preparations, gives a slope of 1.0 ± 0.2 (Fig. 6) indicating that the movement of a single Pr^{3+} ion across the membrane involves an appreciable number of indole acetic acid molecules.

The rate of indole acetic acid-facilitated transmembrane movement of Pr^{3+} has been found to vary significantly with temperature (Fig. 7). Arrhenius plots were constructed from rates measured between 25°C and 55°C for soybean PC vesicles at three different mole ratios of indole acetic acid, viz. 0.4, 0.5 and 0.6 (IAA/PC). The activation energies for the transport process, 24 ± 2 $kcal \cdot mol^{-1}$, are the same for the three different

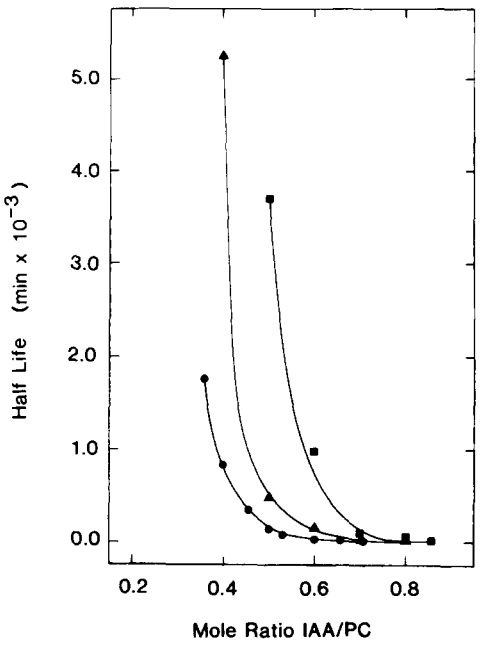

Fig. 4. Dependence of Pr^{3+} transport rate (at 25°C) on indole acetic acid concentration across vesicular membranes composed of (●) soybean PC, (▲) egg PC and (■) dioleyl PC. The vesicle preparations (65 mM) were made up in acetate buffer 200 mM at pH 3.85. Pr^{3+} concentration in all experiments was 10 mM.

indole acetic acid concentrations indicating that the activation energy for Pr^{3+} movement across the membranes is not a function of the IAA con-

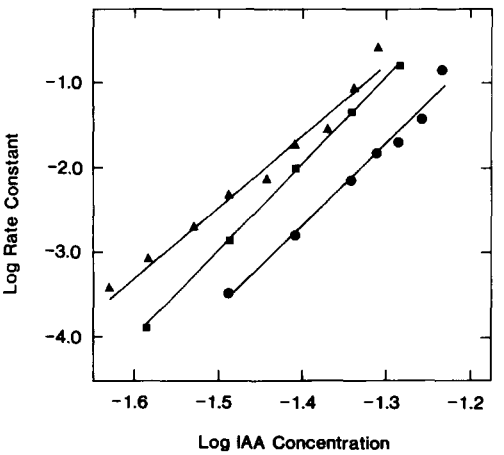

Fig. 5. Dependence of Pr^{3+} transport rate (at 25°C) on indole acetic acid concentration. Plots of log rate constant against log[IAA] are shown for vesicles (65 mM) of soybean PC (▲), egg PC (■) and dioleyl PC (●).

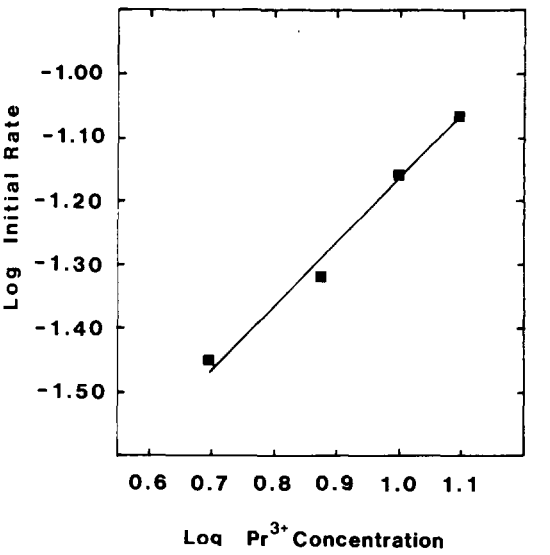

Fig. 6. The dependence of Pr^{3+} transport rate across soybean PC vesicular membranes (65 mM, acetate buffer (pH 3.85)) on Pr^{3+} concentration at 35°C in the presence of indole acetic acid (0.5 molar ratio). Initial rates were obtained from plots of intravesicular Pr^{3+} concentration against time and have units of $mM \cdot min^{-1}$. Pr^{3+} concentration is in mM.

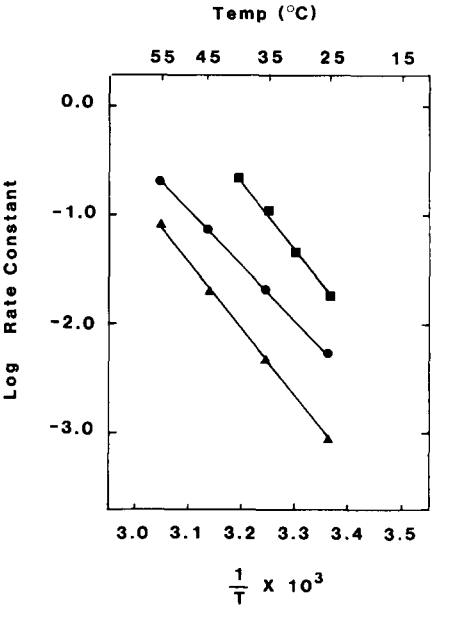

Fig. 7. Dependence of Pr^{3+} transport rate across soybean PC vesicular membranes (65 mM) on temperature in the presence of indole acetic acid at a molar ratio of 0.6 (■), 0.5 (●), 0.4 (▲).

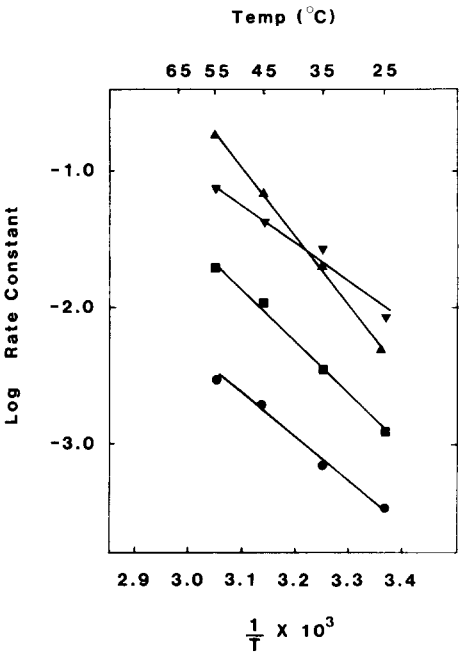

Fig. 8. Variation in Pr^{3+} transport rate across soybean PC (▲), dilinoleyl PC (▼), egg PC (■) and dioleyl PC (●) vesicular membranes with temperature. The phospholipid concentration in each measurement was 65 mM with an indole acetic acid concentration of 0.5 molar ratio.

centration. When egg PC was used in our system the activation energy obtained for Pr^{3+} transport was 16 kcal · mol^{-1} (Fig. 8, Table I). Values obtained for dioleyl PC and dilinoleyl PC were 15 and 14 kcal · mol^{-1}, respectively (Table I).

Discussion

Indole acetic acid causes changes in the permeability of phosphatidylcholine membranes to Pr^{3+}. These changes are dependent on the nature of the lipid and on indole acetic acid concentration. The rate of Pr^{3+} movement across soybean PC membranes is a function of the eighth power of the indole acetic acid concentration (i.e. rate $\propto$ $[IAA]^8$) (Fig. 5) indicating that the co-operativity of eight molecules of indole acetic acid is required to transport a single Pr^{3+} ion. The large number of indole acetic acid molecules involved in the co-operative effect suggests that specific complexes of the type formed by cation ionophores such as X-537A and A23187 [8], which have been shown to transport Pr^{3+} across PC membranes [9], are not formed by indole acetic acid. It is also doubtful that the indole acetic acid-mediated transport of Pr^{3+} is due to the formation of channels in the membrane such as those formed by gramicidin [10], or amphotericin [11] where the stoichiometries found for the transport process are similar to those observed in the present study. If Pr^{3+} entered the interior of the vesicles by such a mechanism an increase would be seen in height of the downfield peak at the expense of the upfield peak [12], as observed in the movement of Pr^{3+} across egg PC membranes caused by phytanic acid and related compounds [13]. This is clearly not observed in the present systems and the results favour the formation of a carrier complex. A possible mechanism for the facilitated movement of ions and solutes across PC membranes is via the formation of inverted micelles of carrier molecules in the lipid bilayer. This has been proposed as the mechanism whereby bile salts, such as deoxycholate, transport Pr^{3+} across dipalmitoyl PC bilayers [14]. The deoxycholate molecules are considered to partition into the lipid portion of the membrane where inverted micelles containing the transported ion are formed. The composite micelle is considered to be the ion carrier. Whilst indole acetic acid partitions into the membrane [1], we have no information on the formation of inverted indole acetic acid micelles in the presence or absence of cations. Nevertheless, the variation in the indole acetic acid stoichiometry for the transport process in different PC systems could result from solvent effects on the size of micelle formed, the solvent in these examples being the acyl chains of the phospholipid.

A further plausible role for indole acetic acid in the transport of Pr^{3+} may be in altering the phospholipid packing in the membrane. Non-bilayer lipid phases in membranes have been postulated as cation translocaters [15], and it seems possible that indole acetic acid could enhance or stabilize these regions in the bilayer. The stoichiometry of indole acetic acid involved in the transport process would, therefore, reflect the number of molecules of indole acetic acid required to stabilize a region in the membrane which constitutes the cation transporter. It has been shown that PC itself is an effective cation barrier, whereas phospholipids such as phosphatidic acid and

cardiolipin have activities comparable with those of the ionophore X-537A in translocating Ca^{2+} across lipophilic solvent boundaries [16].

On the other hand the postulated indole acetic acid-stabilized lamellar/non-lamellar boundaries would differ in character from the phase boundaries observed at or close to the main transition temperature of the membrane. Such boundaries, which are thought to be stabilised by Triton X-100 [14], result in pore formation and cause gated cation movement resulting in an all-or-nothing effect [12].

Changes in membrane lipid composition cause changes in transport rates and in activation energies of the indole acetic acid-mediated transport process. These do not appear to be related to differences in membrane fluidity, however, since egg PC, dioleyl PC and dilinoleyl PC all have approximately the same values but differ appreciably in the level of unsaturation and, consequently, in membrane fluidity. Conversely, soybean PC, which contains approximately 63% linoleyl and approximately 10% linolenoyl ($C_{18:3}$) chains has an activation energy of 24 kcal · mol^{-1} (Table I). Membranes composed of mixed lipid systems have complex phase properties [17] and it is doubtful that the differences in activation energies which we observe can be accounted for simply by summing the contributions from individual lipids.

The complex phase behavior of lipids in biological membranes and the resulting diversity of properties imparted to the membrane at the boundaries between these phases may allow an explanation of the unusual dose-response properties of plant hormones in vivo. Studies have shown that plant growth substances can induce responses in a concentration range extending four to six orders of magnitude. This compares with the expected and observed dose-response curves for small ligands interacting with single, high-affinity receptors, in which the effective ligand concentration extends over a maximum of two orders of magnitude [18]. In this respect, the action of indole acetic acid in promoting differential cation movement across PC membranes composed of different lipids may be relevant.

The large stoichiometry of indole acetic acid in the transport process implies that, under favorable circumstances, small changes in indole acetic acid concentration can bring about large changes in ion fluxes through the membrane. Such changes would be of biological importance and may provide an insight into the role of indole acetic acid as a plant hormone.

Acknowledgements

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