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Effects of phospholipid oxidation on the indole-3-acetic-acid-facilitated transport of Pr^{3+} ions into phosphatidylcholine vesicles

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The rates of indole-3-acetic-acid-facilitated movement of Pr^{3+} across phosphatidylcholine (PC) vesicular membranes increased by a factor of up to 100 as a result of a small amount of phospholipid oxidation. The activation energy for indoleacetic-acid-enhanced ion movement decreased from 24 ± 2 kcal · mol^{-1} in least-oxidized PC vesicles to 10.3 ± 2.6 kcal · mol^{-1} in the most oxidized vesicles and was accompanied by a decrease in indoleacetic acid (IAA) stoichiometry from a value of 8.3 ± 0.5 obtained with freshly prepared vesicles, i.e., rate of Pr^{3+} entry $\alpha[IAA]^{8.3}$, to 2.8 ± 0.3 for oxidized vesicles. The results indicate that very small changes in membrane components can significantly alter the functional response of the membrane to the hormone.

Introduction

The plant growth substance, indole-3-acetic acid, interacts with small unilamellar vesicles prepared from soybean phosphatidylcholine (PC) and, inter alia, promotes the movement of cations into these vesicles [1,2]. One technique demonstrating this cation movement utilizes ^{1}H-NMR in conjunction with an NMR-active cation such as Pr^{3+} or Mn^{2+}. Addition of the cation to the extravesicular solution enables the NMR signal which from the inside and outside vesicular headgroups to be differentiated [3]. Movement of cations across the membrane causes changes in the inner ^{1}H-NMR signal which can be used to measure transport rates [4,5]. The method has been applied to study Pr^{3+} transport across dipalmitoylPC vesicular membranes induced by antibiotic ionophores [4,6], bile salts [7] and the detergent Triton X-100 [8], and to the study of Mn^{2+} transport across egg PC membranes by the ionophore X-537A [5] and the mammalian hormone angiotensin II [9]. In the course of our initial indoleacetic-acid-induced transport rate studies, it was observed that large increases in the measured rates occurred with increased time of storage of the purified chloroform/methanol solution of soy PC. This material was chromatographically identical to the freshly prepared phospholipid and it became apparent that lipid oxidation was affecting the indoleacetic-acid-induced transport rates. This phenomenon had been observed by Bangham and co-workers for K^+ permeabilities of lysosomes of naturally-occurring phospholipids prepared by sonication in the presence of air [10]. Furthermore, it has been suggested that peroxidation of phospholipids in intact plant tissue may cause an

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increase in the permeability of the cell membranes [11].

Subsequent studies have shown that during senescence the permeability of liposomes prepared from bean cotyledon membranes increased [12] and that leaf senescence was correlated not only with increased membrane permeability but also with increased levels of lipid peroxidation [13]. Recent studies have shown that the products of lipid peroxidation accumulate during senescence of bean cotyledons [14] and that these compounds may contribute to membrane damage and loss of cellular integrity associated with senescence of plant tissues [14–16]. In these experiments, exposure of the tissue to ozone was shown to reproduce the effects of natural senescence in terms of inducing the formation of gel-phase lipid in the microsomal and chloroplast membrane fractions [15]. The co-existence of liquid-crystalline and gel-phase lipid has been shown to greatly increase the permeability of bilayer membranes [16]. On the other hand, the application of cytokinins confers resistance to ozone damage of leaves [17]. In this paper we report the effects of lipid oxidation on the indoleacetic-acid-induced movement of cations across soybean PC model membranes.

Materials and Methods

PC was isolated from soybean commercial grade PC (Sigma, Type II-S) and purified by column chromatography on neutral alumina and silica-gel as described previously [1,2]. Thin-layer chromatography of the phospholipid vesicles was carried out on precoated silica-gel plates (Merck) using a chloroform/methanol/acetic acid/water (50:25:7:3, v/v) solvent system. Phospholipids were visualized using molybdate reagent [18] or iodine vapour. Indoleacetic acid (Sigma) was recrystallized from dichloroethane. Pr^{3+} (praseodymium nitrate hydrate) was purchased from Fluka and ^2H_2O was purchased from the Australian Atomic Energy Commission.

Preparation of vesicles

PC (290–300 mg) in chloroform/ethanol (4 : 1, v/v) was evaporated to dryness under vacuum for 2 h at 35°C and then resuspended in 5.5–6.0 ml of sodium acetate/acetic acid (^2H_2O) buffer (0.2 M, pH 3.85) by vigorous shaking. The phospholipid dispersion was sonicated with a Branson B12 ultrasonicator, equipped with a microtip, at a power setting of 5–6. The sonicating tube was placed in an ice bath and a gentle stream of N_2 was directed into it. The total sonicating period was 45–60 min, with 5 min periods of sonication followed by 5 min pauses to allow dissipation of heat. After sonication the vesicle preparation was centrifuged for 30 min at $30000 \times g$ (18°, fixed angle rotor) to remove titanium particles. The phospholipid concentration of the vesicle preparations was determined by phosphorus assay [19]. Solutions of indoleacetic acid in ^2H_2O (325 mM), as the sodium salt, were made up by titrating the free acid with NaO^2H (pH of the resulting solution, 8.5.). Aliquots of the indoleacetic acid solution were pipetted into NMR tubes (5 mm o.d.) containing 0.5 ml of vesicle preparation so as to give the appropriate indoleacetic acid/PC mole ratio (final pH of the vesicle preparation 3.85–4.11). Pr^{3+} was added as a ^2H_2O solution of the nitrate to give a final concentration of 10 mM.

Autoxidation of PC vesicle preparations

The vesicle preparations were allowed to stand in stoppered tubes at room temperature under nitrogen for up to 7 days. Alternatively, aliquots were incubated in stoppered tubes in a shaking water bath at 40°C for 20–60 h. The use of a nitrogen atmosphere over the vesicle preparations rather than air was preferred since the time-course for autoxidation was significantly slower in N_2 and could be better controlled. Phospholipid composition of the vesicle preparation was checked before and after oxidation by thin-layer chromatography. Freshly prepared vesicles showed only one spot on normal loading, with an R_F of 0.37, corresponding to PC. On standing for several days at room temperature, however, three additional spots with R_F values of 0.15, 0.07 and 0.0, corresponding to lysoPC and two unidentified oxidation products, were observed. At the highest level of oxidation measured (oxidation index 0.61) the level of lysoPC was estimated at approx. 2% with the levels of the two oxidation products each approx. 5%. These values are comparable to those observed during the air-initiated autoxidation of egg PC vesicles [20]. The extent of autoxidation

induced by the treatments was measured by ultraviolet spectroscopy [21]. Aliquots of the vesicle preparations (15 μl) were dissolved in 3 ml of methanol and an ultraviolet spectrum from 190 to 320 nm was measured on a Perkin Elmer λ5 Spectrophotometer. The oxidation index was taken as the ratio of the absorbance at about 230 nm, due to conjugated dienes, to the absorbance at 206 ± 210 nm, due to phospholipid and buffer carbonyl groups [21].

It is of importance to note that freshly isolated, untreated PC vesicle preparations also demonstrated a finite oxidation index, though always of a considerably lower magnitude (about 0.17) than oxidized preparations. It is not known whether this oxidation index represents a measure of fatty acid oxidation which is always present in the soybean phospholipid, or is occasioned by the extraction technique, or is ultraviolet absorbance due to unoxidized components.

Fatty acid analysis

Aliquots of the PC vesicle preparation were pipetted into 5 ml of 5% H_2SO_4 in methanol and, following the addition of methyl heptadecanoate as an internal standard, the samples were heated at 70°C for 3 h. The fatty acid methyl esters were extracted with petroleum ether and quantitated by GC on a column of 15% DEGS at 175°C.

NMR measurements

The ^{1}H-NMR measurements were performed on a JEOL FX90Q Fourier transform spectrometer equipped with a variable temperature accessory. A spectral width of 800 Hz was accumulated into 8K addresses. Dioxane was used as an internal reference for measurement of chemical shift separation. The movement of Pr^{3+} into the PC vesicles was monitored by obtaining spectra at suitable intervals following the addition of indoleacetic acid. These data were obtained automatically by use of the JEOL Autostacking software FAFT70-800912 in which the *WAIT parameter determines the time interval between successive spectra. The kinetic data obtained in this way were analysed as described previously [2].

Results

When 10 mM Pr^{3+} is added to a soybean PC vesicle preparation the ^{1}H-NMR signal of the $-\overset{+}{N}(CH_3)_3$ protons in split into two peaks. The upfield peak is associated with the PC molecules on the inner side of the vesicle bilayer, while the downfield peak is the signal from the PC molecules on the outer surface of the vesicle. The outer-facing PC molecules interact with the Pr^{3+} in the extravesicular solution resulting in a downfield shift of the $-\overset{+}{N}(CH_3)_3$ signal [3]. As Pr^{3+} ions move into the vesicles the upfield signal moves gradually downfield until it merges with the downfield signal. This is a time-dependent process and it has been used to measure the rate of movement of Pr^{3+} ions into the PC vesicles [2,4,6–8].

In the absence of indoleacetic-acid, self-diffusion of Pr^{3+} into PC vesicles is a very slow process. A self-diffusion rate constant of approx. $1 \cdot 10^{-4}$ min^{-1}, measured over a period of 5 days, has been obtained for soybean PC vesicles [2]. However, accurate determinations of such rates are prone to serious error because the polyunsaturated fatty acids in soybean PC are susceptible to autoxidation, which probably influences the rates of Pr^{3+} entry. In order to explore this behaviour, soybean PC vesicles which had an initial oxidation index of approx. 0.17, were allowed to undergo autoxidation at room temperature for 7 days. At the end of the period the oxidation index had risen to 0.53, at which time 10 mM Pr^{3+} was added. During the next 21 h, at 25°C, the measured self-diffusion rate was $3.2 \cdot 10^{-5}$ min^{-1}. This increased to $1.2 \cdot 10^{-4}$ min^{-1} over the next 27 h. Since the earlier measurements had shown that the oxidation index of the PC vesicle preparation increased on standing, it can be inferred that the observed increase in self-diffusion rates was attributable to increased oxidation. However, the data presented above show that even relatively highly autoxidized soybean PC vesicles (oxidation index 0.53) provide an effective barrier to the entry of Pr^{3+}.

Since the addition of indoleacetic acid to soybean PC vesicles has already been shown to enhance the movement of Pr^{3+} ions across vesicular membranes [2], we examined the effects of autoxidation on this process by allowing PC ves-

icle preparations to undergo autoxidation at room temperature over a 7 day period. At appropriate intervals aliquots were removed for measurements of the oxidation index by ultraviolet spectroscopy and for ^{1}H-NMR-based measurement of the rate of indoleacetic-acid-facilitated Pr^{3+} ion transport at fixed concentrations of indoleacetic acid and Pr^{3+}. It is noted here that added indoleacetic acid and/or Pr^{3+} do not increase but, in fact, slightly decrease the rate of autoxidation of PC membranes. A plot of rate constant against oxidation index (Fig. 1) indicated that the rate constant increased exponentially with increasing levels of autoxidation. This was confirmed by the linear relationship ($r^2 = 0.96$) between log rate constant and oxidation index (Fig. 1, inset). The rate of indoleacetic-acid–stimulated Pr^{3+} movement across the vesicle bilayer was 85-times greater in a sample that was autoxidized (oxidation index 0.5) than the rate obtained with a freshly prepared sample (oxidation index 0.17). The effects of lipid oxidation on the relationship between rate constants and indoleacetic acid concentration were examined by incubating PC vesicles at 40°C for different lengths of time so that various levels of oxidation were obtained. The rate constants were determined from ^{1}H-NMR measurements in the

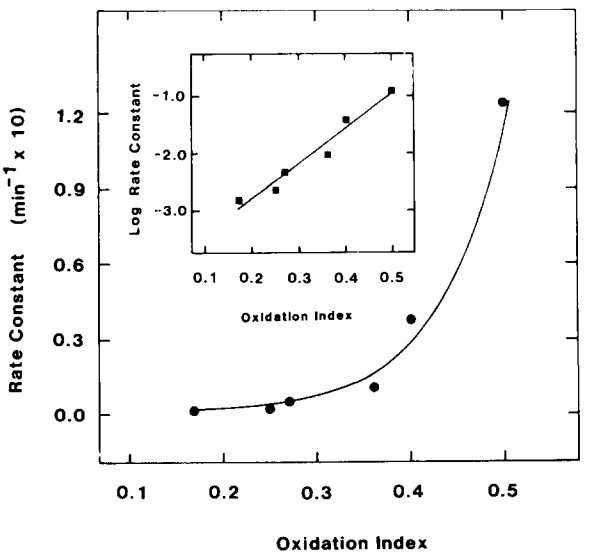

Fig. 1. Dependence of Pr^{3+} transport rate (at 25°C) on the degree of autoxidation of soybean PC vesicles (55.7 mM in acetate buffer (pH 3.85)). Indoleacetic acid/PC molar ratio was 0.58 in all cases.

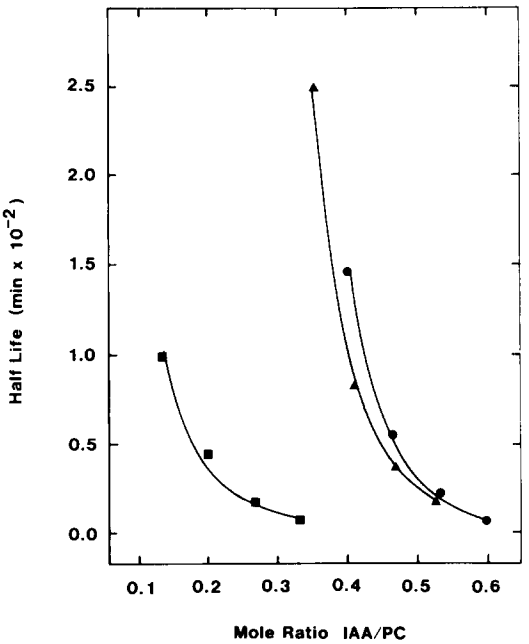

Fig. 2. Dependence of half life of the Pr^{3+} transport process (at 25°C) on the mole ratio of indoleacetic acid (IAA)/PC. Plots are shown for vesicles with oxidation indices of 0.43 (●), 0.53 (▲) and 0.58 (■). The phospholipid concentration of the vesicles with an oxidation index of 0.53 was 55.6 mM, while the vesicles with oxidation indices of 0.43 and 0.58 had a phospholipid concentration of 48.9 mM.

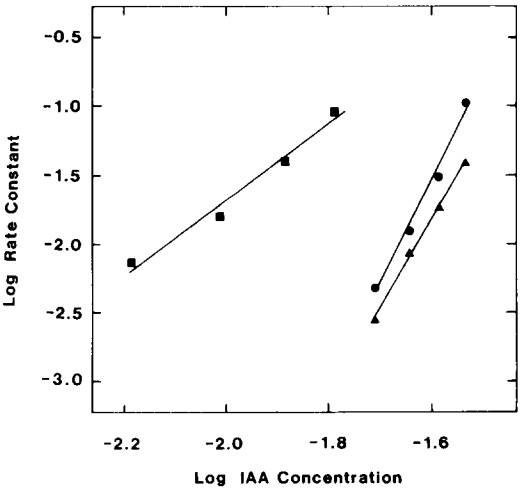

Fig. 3. Dependence of Pr^{3+} transport rate (at 25°C) on indoleacetic acid (IAA) concentration. Plots of log rate constant against log indoleacetic acid concentration for vesicles with oxidation indices of 0.43 (●), 0.53 (▲) and 0.58 (■) are shown. The phospholipid concentration was 48.9 mM (oxidation indices of 0.43 and 0.58) or 55.6 mM (oxidation index of 0.53).

TABLE I

THE EFFECT OF AUTOXIDATION ON THE RATE CONSTANTS AND ACTIVATION ENERGIES FOR THE INDOLEACETIC-ACID-FACILITATED TRANSPORT OF Pr^{3+} ACROSS PC VESICULAR MEMBRANES

The phospholipid concentration of the vesicles with oxidation index 0.52 was 53.6 mM, while the vesicles with oxidation index 0.42 and 0.61 had a phospholipid concentration of 52 mM. The indoleacetic acid/PC mol ratio was 0.4 in all cases.

Oxidation index of PC vesicles	Rate constant at 25°C (min^{-1})	Activation energy ($kcal \cdot mol^{-1}$)
0.42	$1.6 \cdot 10^{-3}$	23.4 ± 0.5 [a]
0.52	$7.4 \cdot 10^{-3}$	20.0 ± 3.3
0.61	$7.9 \cdot 10^{-2}$	10.3 ± 2.6

[a] Mean ± 95% confidence interval.

presence of different indoleacetic acid concentrations. Fig. 2 shows plots of half-life of Pr^{3+} entry into the vesicles against mole ratio of indoleacetic acid/PC for three different levels of oxidation. Graphs of log rate constant against log indoleacetic acid concentration at the three levels of oxidation (Fig. 3) gave straight lines with slopes of 7.5 ± 0.6 (oxidation index 0.43), 6.5 ± 0.3 (oxidation index 0.53) and 2.8 ± 0.3 (oxidation index 0.58).

The indoleacetic-acid-enhanced Pr^{3+} transport process is temperature-dependent and the effects of phospholipid oxidation on this temperature-dependence are illustrated in Fig. 4. Arrhenius plots were constructed from rates measured between 5°C and 55°C, using 0.4 mole ratio indoleacetic acid/PC, for PC vesicles which were at three different levels of oxidation. The activation energy decreased from 23.4 $kcal \cdot mol^{-1}$ for vesicles with

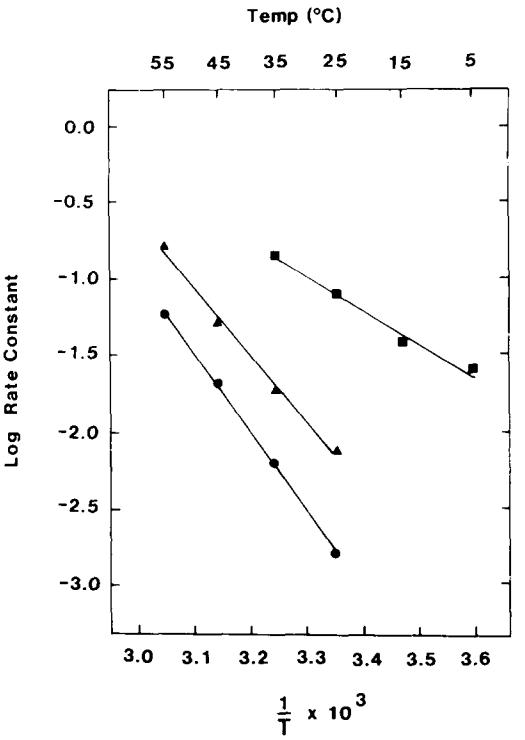

Fig. 4. Dependence of rate constants for Pr^{3+} transport into PC vesicles on temperature. Plots of log rate constant against $1/T$ for vesicles with oxidation indices of 0.42 (●), 0.52 (▲) and 0.61 (■) are shown. The phospholipid concentration was 52.0 mM (oxidation indices of 0.42 and 0.61) or 53.6 mM (oxidation index of 0.52). Indoleacetic acid/PC molar ratio was 0.4 in all cases.

an oxidation index of 0.42 to 20.0 $kcal \cdot mol^{-1}$ for vesicles with an oxidation index of 0.52. There is a further dramatic decrease in the activation energy to 10.3 $kcal \cdot mol^{-1}$ on increasing the oxidation of the vesicles to an oxidation index of 0.61 (Table

TABLE II

FATTY ACID COMPOSITION OF PC VESICLES AT DIFFERENT STAGES OF AUTOXIDATION

Oxidation index	Rate constant [a] (min^{-1})	nmol of fatty acid/μmol P					
		16:0	18:0	18:1	18:2	18:3	total
0.23	$1.95 \cdot 10^{-3}$	284 ± 11 [b]	59 ± 2	123 ± 5	1274 ± 47	146 ± 6	1886 ± 70
0.34	$1.46 \cdot 10^{-2}$	302 ± 5	64 ± 1	127 ± 1	1262 ± 5	143 ± 1	1897 ± 9
0.41	$5.49 \cdot 10^{-2}$	300 ± 7	61 ± 1	127 ± 2	1254 ± 21	132 ± 3	1874 ± 33
0.53	$1.88 \cdot 10^{-1}$	297 ± 5	60 ± 1	125 ± 1	1202 ± 14	122 ± 1	1806 ± 21

[a] The phospholipid concentration was 55.8 mM and the IAA/PC molar ratio was 0.58.
[b] Mean ± S.E.

I). Changes in fatty acid composition brought about by oxidation were also measured and are presented in Table II. An increase in oxidation index from 0.23 to 0.53 resulted in a 100-fold increase in the rate of indoleacetic-acid-facilitated Pr^{3+} movement, although overall fatty acid composition did not change significantly on a mol% basis except at the highest oxidation level examined, where losses of linoleic acid (6%) and linolenic acid (16%) were observed.

Discussion

The autoxidation of soybean PC vesicles causes an increase in the permeability of these vesicles to Pr^{3+} ions on the presence of indoleacetic acid. The mechanism of autoxidation of lipids containing polyunsaturated fatty acids is reasonably well understood [22]. Thus, the autoxidation of linoleic acid is initiated by the abstraction of hydrogen from the methylene group linking two double bonds. This results in the formation of a stable conjugated diene free radical which reacts with oxygen to form a hydroperoxide radical which can, in turn, abstract a hydrogen from the methylene group of another molecule of linoleic acid to produce a conjugated diene hydroperoxide and another conjugated diene free radical. Thus, once initiated, the autoxidation process can be self-propagating. The oxidation of phosphatidylcholine-containing polyunsaturated fatty acids has been studied both in homogeneous solution and in liposome preparations [23–27]. The rate of autoxidation is significantly greater in vesicle preparations than it is in homogeneous solution, probably because the highly ordered bilayer structure facilitates the interaction of the reactive species formed [26]. In addition, the rate of oxidation of phospholipid vesicles increases with an increase in temperature [20] and this was also confirmed by our results in which a PC vesicle preparation left at room temperature was found to have a lower oxidation index after 160 h than a similar preparation incubated at 40°C had after 34 h.

Wu et al. [27] have reported that the major products formed during the autoxidation of soybean PC liposomes at 40°C are the 9- and 13-hydroperoxyoctadecadienoates formed by the free radical oxidation of linoleic acid, which comprises 67% of the total fatty acids in soybean PC. It was observed (Table II) that an increase in the oxidation index from 0.23 to 0.53 resulted in a 6% loss of linoleic acid and a 16% loss of linolenic acid. From the values in Table II, and assuming that the lost fatty acids are completely coverted to the corresponding hydroperoxides, 72 nmol of hydroperoxide derived from linoleic acid and 24 nmol of hydroperoxide derived from linolenic acid would be formed per μmol of PC. These estimates are corroborated by another calculation in which the increase in ultraviolet absorption at about 230 nm on increasing the oxidation index from 0.23 to 0.53 was used to estimate the concentration of hydroperoxide in the vesicle preparation. Using a molar extinction coefficient of $2.52 \cdot 10^4$ for linoleic acid hydroperoxides [28], an estimate of 72 nmol of hydroperoxide per μmol phospholipid was obtained. The most autoxidized PC vesicle preparation used in our experiments (oxidation index 0.61) would contain 116 nmol of hydroperoxides/μmol of phospholipid based on a similar calculation. Thus, in this preparation, the ratio of oxidized to non-oxidized acyl chains may be as high as 5 mol%.

The incorporation of hydrophilic hydroperoxide groups into the acyl chain region of the membrane may assist the movement of hydrophilic solutes and ions across the membrane in that the membrane may appear less hydrophobic to the permeating cation; this is consistent with a reduced activation energy for the transport process. In addition, the presence of hydroperoxide groups in the membrane would be energetically unfavourable and may tend to destabilize adjacent regions of the membrane, a process which may be at least partially reduced by the formation of domains of hydroperoxide-containing lipids. It has been postulated, as well, that permeability at the borders of lipid domains is greater than permeability within the domains [29].

At room temperature for brief periods, low levels of membrane oxidation would be expected, even with highly unsaturated soybean PC, and, thus, few domains of hydroperoxide-containing phospholipid would also be expected. However, as the level of oxidation increases it is envisaged that such domains in the membrane may become widespread. Certainly, in highly oxidized cotyle-

don membranes similar domains can be detected with wide-angle X-ray diffraction techniques [15]. It is in the formation and/or stabilization of the boundary regions between the oxidized and non-oxidized phospholipid molecules in the membrane that indoleacetic acid may exert its effect, thus enhancing membrane permeability to cations.

An alternate mechanism by which indoleacetic acid might induce the movement of cations across phospholipid membranes is by the formation of inverted micellar carriers. It is plausible that several molecules of indoleacetic acid could associate with a single Pr^{3+} ion to form a hydrophobic species which could traverse the membrane. Such carriers have been suggested to explain the bile-salt-facilitated movement of Pr^{3+} across dipalmitoylPC membranes [7]. At the present time, however, we have found no evidence to indicate that indoleacetic acid forms cation-containing inverted micelles in non-polar solvents (Jones, G.P. and Paleg, L.G., unpublished data).

The activation energy of the rate determining step for the indoleacetic-acid-induced translocation of Pr^{3+} across the membrane decreases with increased oxidation of the membrane. The reduced activation energy may arise directly from the incorporation of hydrophilic groups in the membrane's hydrocarbon interior. Clearly, a cation moving in a more hydrophilic environment in the membrane interior will be energetically less unfavourable than one moving in a more hydrophobic environment, whether or not the ion is moving as a carrier complex or through some transient pore in the membrane. This assumes, of course, that the movement of ions across the hydrocarbon region of the membrane is the rate-limiting step [6].

Another facet of membrane oxidation is that the indoleacetic acid stoichiometry for indoleacetic-acid-induced cation translocation decreases with increases in oxidation. At an oxidation index of 0.17 the indoleacetic acid stoichiometry is 8.3, whereas at an oxidation index of 0.58 this stoichiometry falls to 2.8. Whilst both a micellar carrier system and a transient pore mechanism can be invoked to plausibly explain this reduction in indoleacetic acid stoichiometry, it is interesting to note that the lower indoleacetic acid stoichiometries brought about by lipid chain oxidation can also arise by a change in headgroup type. With freshly prepared soybean phosphatidylinositol membranes having a low oxidation index (0.11) indoleacetic acid causes the transbilayer movement of Mn^{2+} where the indoleacetic acid stoichiometry for the rate-determining step is 2.6 (Jones, G.P., Misso, N.L.A. and Paleg, L.G., unpublished data).

The results presented in this paper indicate that phospholipid oxidation modifies the indoleacetic-acid-facilitated transport of Pr^{3+} ions across PC vesicular membranes in a number of ways. The effect of a given concentration of indoleacetic acid is amplified 100-times (the rate of Pr^{3+} movement is about 10^2-times higher) in PC vesicles which have undergone some autoxidation. With autoxidized PC vesicles the rate of Pr^{3+} transport is still dependent on indoleacetic acid concentration, but only 2–3 molecules of indoleacetic acid are involved in the transport of a single Pr^{3+} ion as compared to 7 or 8 molecules of indoleacetic acid for transport through least-oxidized PC vesicles. The activation energy for the transport process is much smaller in the case of oxidized PC vesicles, but the entropy of activation is negative. An important aspect of these findings is that the change in indoleacetic acid stoichiometry, the indoleacetic-acid-induced decrease in activation energy of ion transport and the indoleacetic-acid-induced increase in rate of ion movement are all brought about by small changes in membrane lipid composition. Less than a 5% change in total lipid content (including a 16% decrease in linolenic acid) is associated with these effects, suggesting that a powerful way to modulate hormonal effects in vivo would be through the alteration of oxidation levels in the hydrocarbon moiety of membrane phospholipids.

Changes in indoleacetic acid concentration also cause large changes in ion fluxes through model membranes [2]. Since it is probable that indoleacetic-acid-induced permeability changes are of importance in biological systems, the combination of changes in indoleacetic acid concentration and oxidation of phospholipid fatty acids (occurring in situtations such as, for example, senescence, in which phospholipid peroxidation is known to occur) could produce or control significant developmental events like abscission.

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