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Ethrel enhances the degradation of artificial membrane vesicles (liposomes) by phospholipase A_2 and phospholipase C

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SUMMARY

Ethylene, a lipid-soluble gas, is known to have considerable effects on plant metabolism. It is possible that some of these metabolic effects are the result of ethylene's lipid solubility. The effect of low concentrations of 2-chloroethylphosphonic acid (ethrel), an ethylene-releasing compound, on the susceptibility of artificial membrane vesicles (liposomes) to phospholipase A_2 and phospholipase C was studied. Liposomes were found to be more susceptible to degradation by these two phospholipases in the presence of ethrel. Ethrel promoted maximal degradation of liposomes by phospholipase C at a concentration of 55 nM. Ethrel also enhanced the leakiness of liposomes to L-leucine only in the presence of phospholipase A_2. The rate of degradation of liposomes by both phospholipases was enhanced by about 20–30% in the presence of ethrel. The results may indicate that ethylene may affect the accessibility of phospholipases to their target sites within the lipid bilayer of artificial membrane vesicles.

Key-words: ethrel, ethylene, membrane degradation.

INTRODUCTION

The production of ethylene in senescent plant cells is known to be correlated with increased membrane permeability (Borochov & Woodson 1989). Ethylene production was correlated with a decrease in phospholipid content during senescence (Borochov & Woodson 1989). Exposure of pre-senescent plant tissues to ethylene results in increased membrane permeability and phospholipid loss (Suttle & Kende 1980). Suttle & Kende (1980) suggested that the increased membrane permeability induced by ethylene was the result of enhanced activity of phospholipases which degrade membranes and cause leakage. In petals the reduction in phospholipid content was suggested to be the result of increased activity of phospholipase A_2 and other phospholipases and a reduction in synthesis of phospholipids (Borochov *et al.* 1982; Paliyath *et al.* 1987). It was not clear if the increased activity of these phospholipases is a result of ethylene affecting the membranes (substrate) or the enzyme. Furthermore, plants treated with ethylene in the presence of protein-synthesis inhibitors did not exhibit loss in phospholipid content or membrane leakiness (Borochov & Woodson 1989) which indicates that protein synthesis is needed for early response to ethylene. While the activity of phospholipase C was

detected in plants (Connett & Hanke 1986) its involvement in phospholipid degradation during senescence was not shown (Paliyath *et al.* 1987).

Hypobaric pressure was used to delay this ageing phenomenon and in some cases the sensitivity of aged tissue to ethylene (Dilley 1977). Ethylene is a lipid-soluble gas (Abeles 1973) and it is possible that ethylene solubility in the cellular membranes (Abeles 1973) increases their phase transition point. The phase transition of membranes can be increased by application of hyperbaric pressure (MacDonald & Cossins 1983). Therefore, it might be that the hypobaric treatment decreased the membrane phase-transition temperature and eliminated a portion of this ageing-associated phenomenon.

The binding of ethylene to a membrane-soluble receptor (Smith & Hall 1985) at a very low Kd (6×10^{-10}M) is considered to be the first step in ethylene action. Binding seems to be responsible for most of the array of physiological and biochemical responses that have been correlated with ethylene production (Smith & Hall 1985). While all these phenomena occur at the beginning of ethylene production, the possible function of high concentrations of ethylene are not discussed usually because they are not reversible and are associated with tissue death (Borochov & Woodson 1989).

Activity of phospholipases depends on the physical state of the lipid bilayer (Mazliak 1980). The activity of phospholipases was enhanced considerably by the presence of disrupting agents that allow the enzymes access to their substrate in the lipid moiety of the membrane (Mazliak 1980). The activity of phospholipase A_2 was found to depend on the phase transition temperature of the phospholipid bilayer (Op den Kamp *et al.* 1975). It was shown that the activity of phospholipase A_2 on liposomes made from either one type of phospholipid or a mixture of different phospholipids was enhanced considerably at the phase transition temperature due to membrane disruptions (Op den Kamp *et al.* 1975).

The above observations lead to the hypothesis that ethylene may affect the physical state of the cellular membrane phospholipid bilayer especially at relatively high physiological concentrations. If ethylene induces a disruption in the bilayer or increases the phase transition due to its lipid solubility this may be detected when monitoring the activity of phospholipases on artificial membrane vesicles (liposomes).

MATERIALS AND METHODS

Preparation of liposomes for phospholipase activity assays

For phospholipase C activity assay, 1 mg of crude, dried, soy-bean phospholipid was dissolved in 1 ml Tris buffer (100 mM, pH 7·4) and centrifuged vigorously until a milky suspension was obtained. The phospholipid suspension was sonicated on ice under a nitrogen stream until the milky appearance disappeared and the suspension was transparent.

For phospholipase A_2 activity assay, liposomes were prepared as above with addition of 1–5 U of L-amino acid oxidase (Sigma Co. St Louis, MO, USA) to the preparation buffer. The sonication of the liposome suspension encapsulated the L-amino acid oxidase inside the liposomes. All other steps were as described above. Freshly prepared liposome suspensions from each preparation were separated into two fractions, one as a control and the other as a treatment. The enhancement in activity is expressed as per cent above the control because the absolute activity differed between individual experiments.

Phospholipase C activity assay

For the phospholipase C (PLC) assay, 200 µl of the liposome suspension were added to 800 µl of assay solution (100 mM Tris, pH 7·4, 1% BSA and 1 mM $CaCl_2$) in a 1 ml cuvette in the presence or absence of the indicated concentration of ethrel.[1] Phospholipase C (0·5–2 U, Sigma Co., St Louis, MO, USA) was added to the cuvette and the turbidity change due to liposome degradation was monitored at 530 nm.

For assaying the release of the free orthophosphate which is a consequence of phospholipase C activity, 1·5 ml liposome fractions were added to 2 ml Tris buffer (100 mM, pH 7·4), 0·5 ml $CaCl_2$ (50 mM) and 1 ml phospholipase C (2 U in 1% BSA) in the presence of bubbled ethylene gas (80 ppm) or air. Two hundred µl samples were taken and added to 200 µl 10% TCA and incubated on ice for 15 min. After the incubation, the phospholipids were pelleted at 20 000 *g* and the supernatant that contained the free-phosphate head groups was kept. Fifty µl 10% $Mg(NO_3)_2$ in ethanol were added to the supernatant and the sample was dried at 120°C for 90 min. The dried powder was burned until a white crust appeared on the bottom of the tube, 0·6 ml of HCl (0·5 N) was then added and incubated for 15 min in a boiling water bath. Phosphate reagent 1·4 ml (1 N H_2SO_4, 5% $FeSO_4$ and 1% $(NH_4)_6Mo_7O_{24}\cdot H_2O$ was added to determine free phosphaten concentration. The colour was developed for 5 min at 45°C and the absorbance was monitored at 660 nm.

Phospholipase A_2 activity assay

Phospholipase A_2 (PLA) activity assay was based on a coupled reaction in which phospholipase A_2 induced permeability changes in liposomes. The leakage of the amino acid L-leucine into the liposomal lumen was detected by oxidation by L-amino acid oxidase that was encapsulated inside the liposomes and was monitored as O_2 consumption in an O_2 electrode. Two hundred µl of liposome suspension with encapsulated L-amino acid oxidase were added to 1800 µl assay solution (50 mM phosphate buffer, pH 7·4 and 2 mM $CaSO_4$) in a Clark-type oxygen electrode in the presence or absence of the indicated concentration of ethrel. Phospholipase A_2 (10 U, Sigma Co., St Louis, MO, USA) was added to the assay solution after the addition of ethrel. L-Leucine was added at the indicated concentrations and activity was measured as oxygen consumption per min for each preparation of liposomes. Each preparation was divided into two samples, one assayed in the absence of ethrel and one assayed in the presence of ethrel. A representative of 3–10 experiments on the effect of either PLC or PLA is shown for each treatment. Statistical analysis was performed by ANOVA.

The concentration of ethylene in the gaseous phase was not determined. It was assumed that at least part (about 5–10%) of the ethrel was turned into ethylene (Warner & Leopold 1969) before and during the experiments. All assays were performed at room temperature (25°C).

Phospholipid extract (crude soy-bean extract), phospholipase A_2, phospholipase C, L-amino acid oxidase and buffers were purchased from Sigma Co., St Louis, MO, USA. All other chemicals were of analytical grade.

RESULTS AND DISCUSSION

Ethrel is a water soluble compound that releases ethylene when the pH of the solution is above 3·5 (Warner & Leopold 1969; Yang 1969). This compound has been used in

[1]It was recently shown that the susceptibility of unilamellar vesicles to phospholipases A_2, C and D was altered in the presence of membrane active peptides such as gramicidin, melittin and alamethicin (Madhusudana Rao, N. *Biochem. Biophys. Res. Comm.* **182:** 682–688).

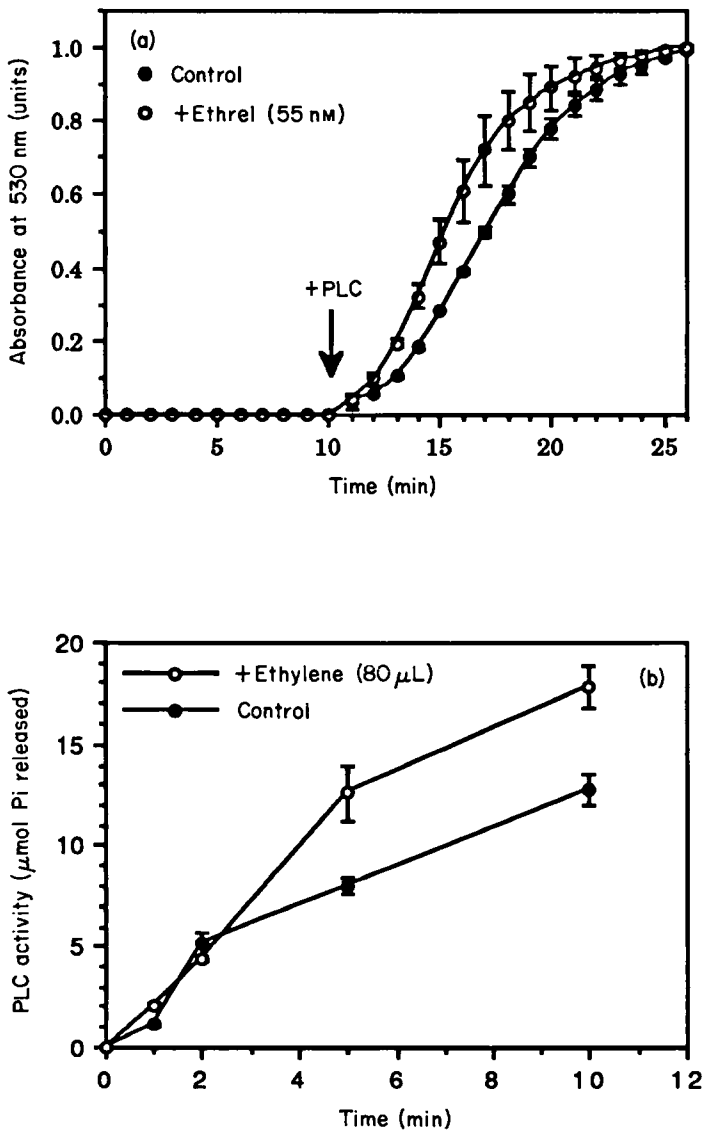

Fig. 1. Ethrel enhanced phospholipase C activity on artificial membrane vesicles. (a) Artificial membrane vesicles (liposomes) were incubated in the presence of 1 unit of phospholipase C (PLC) as described in 'Materials and Methods' with (○) or without (●) added ethrel (55 nM). Activity was monitored as increase in absorbance at 530 nm. Arrow indicates the time of addition of phospholipase C (PLC). (b) Artificial membrane vesicles (liposomes) were incubated in the presence of five units of phospholipase C (PLC) as described in 'Materials and Methods' with bubbling air (●) or 80 ppm ethylene (○). The released orthophosphate was measured in samples withdrawn at the indicated time. Each point is the mean ± SD of three separate measurements. When bars are not shown they are smaller than the symbol size.

numerous studies (352 since 1984; source Agricola) as a source of ethylene (Roustan *et al.* 1990; Vera & Conejero 1990). The effect of ethrel was studied on artificial membranes.

The addition of the indicated concentrations of ethrel had no effect on the pH of the incubation solutions as determined at the end of the experiment.

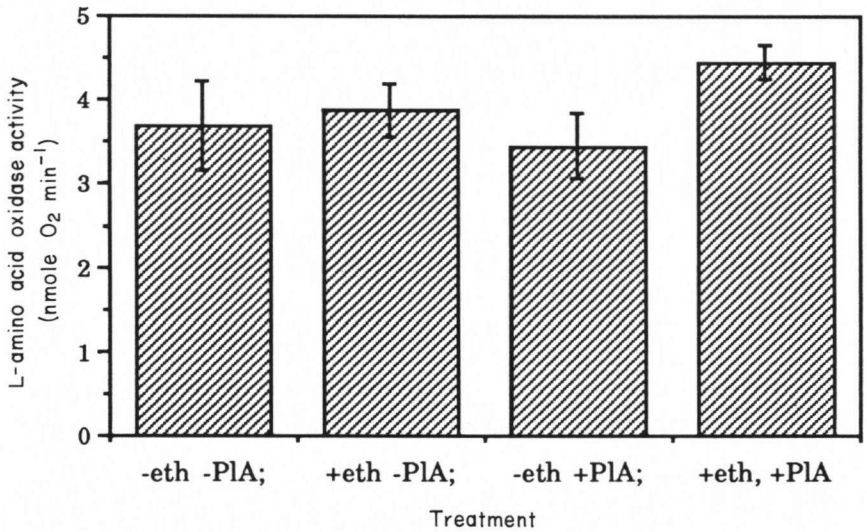

Fig. 2. Ethrel-induced enhancement of L-amino acid oxidase activity that is encapsulated inside liposomes in the presence or absence of phospholipase A_2 (PlA). L-Amino acid oxidase activity was measured as described in 'Materials and Methods', in 1·5 mM leucine, in the presence (+eth) or absence (−eth) of 82·5 nM ethrel and in the presence (+PlA) or absence (−PlA) of 10 U of phospholipase A_2. Activity is expressed in nmoles O_2 $(min)^{-1}$ of three measurements ± SD. Statistical analysis using ANOVA showed that the only statistically significant change was when the liposomes were treated with ethrel and phospholipase A_2 concurrently (+eth+PlA).

The disruption of liposome structure by phospholipase C activity was montitored as an increase in light scattering of the liposome solution at 530 nm (Fig. 1a). No disruption of liposome structure was observed when ethrel was added to the liposome solution in the absence of phospholipase C. However, the presence of ethrel in the incubation solution increased the rate of degradation of liposomes by phospholipase C (Fig. 1a). Maximal enhancement of ethrel effect on the activity of phospholipase C on artificial liposomes was observed at about 55 nm (data not shown).

The range of the enhancement of liposome degradation rate by phospholipase C activity caused by ethrel was between 11 and 80% for different experiments ($n=10$ experiments). The median of the distribution of per cent enhancement of liposome degradation rate by phospholipase C was between 10 and 30% ($n=8$) and the mean (24%) was significantly different from the control (at $P=0{\cdot}05$).

Similar enhancement of phospholipase C activity (28%) was observed when the activity was measured as release of free orthophosphate (Fig. 1b). Bubbling 80 ppm of gaseous ethylene increased the release of orthophosphate from $17{\cdot}6 \pm 1{\cdot}6$ μmole Pi/10 min, in the presence of bubbling air to $22{\cdot}4 \pm 0{\cdot}6$ μmole Pi/10 min ($P=0{\cdot}1$).

It was proposed that phospholipase A_2 activity was correlated with permeability changes of phospholipid bilayers (Faragher *et al.* 1987), thus, the effect of ethrel on the permeability of liposomes to L-leucine was investigated in the presence or absence of phospholipase A_2. L-Leucine leakage into the liposomal lumen was detected by its oxidation by encapsulated L-amino acid oxidase inside the liposomes. The presence of ethrel concurrently with phospholipase A_2 enhanced the permeability of crude soy-bean liposomes to leucine by 20% (Fig. 2).

It might be argued that phospholipase A_2 alone enhanced the activity of the L-amino acid oxidase that was used to detect leakage of L-leucine into the internal liposome lumen.

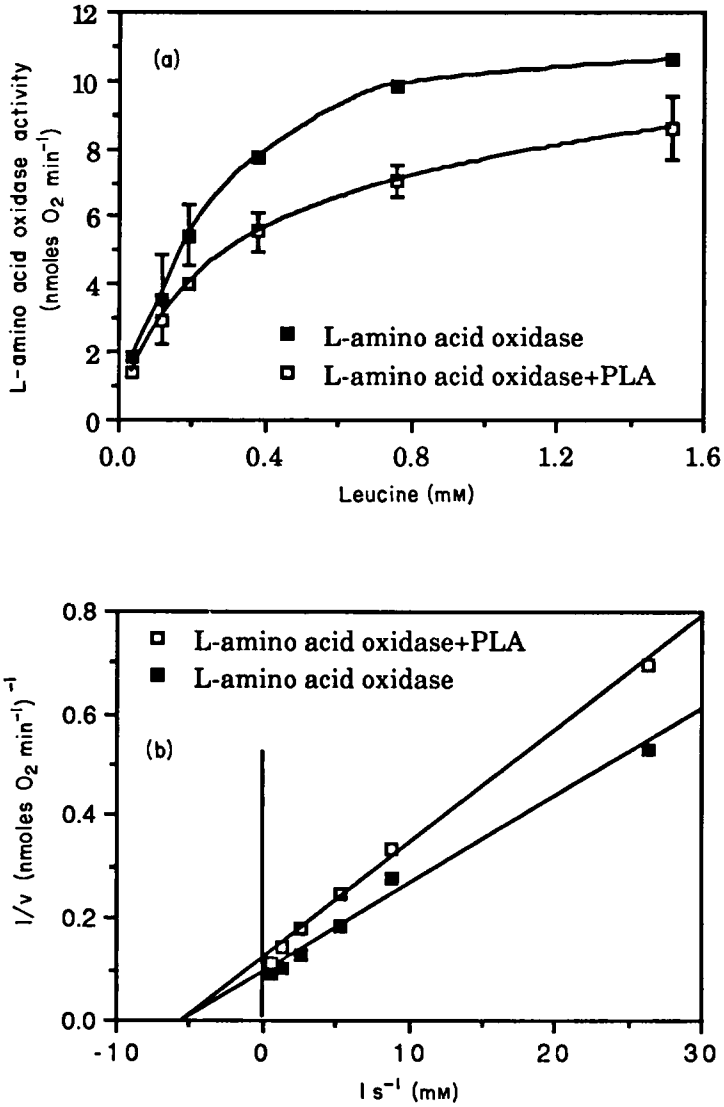

Fig. 3. Phospholipase A_2 effect on L-amino acid oxidase activity. L-amino acid oxidase activity was assayed as described in 'Materials and Methods', without the preparation of liposomes in the presence (□) or absence (■) of phospholipase A_2. Phospholipase A_2 (10 U) was added to the reaction medium and activity was assayed at various L-leucine concentration. When bars are not shown they are smaller than the symbol size.

Any effect of ethrel and/or phospholipase A_2 on L-amino acid oxidase in solution would have manifested as a change in kinetic parameters such as K_m or V_{max}. Permeability changes of the liposome would have manifested as an increase in V_{max} only of the encapsulated enzyme.

The effect of phospholipase A_2 in the presence or absence of ethrel on the K_m and V_{max} of L-amino acid oxidase that was either encapsulated in liposomes or free in solution was evaluated. Phospholipase A_2 had no effect on the K_m of L-amino acid oxidase when there

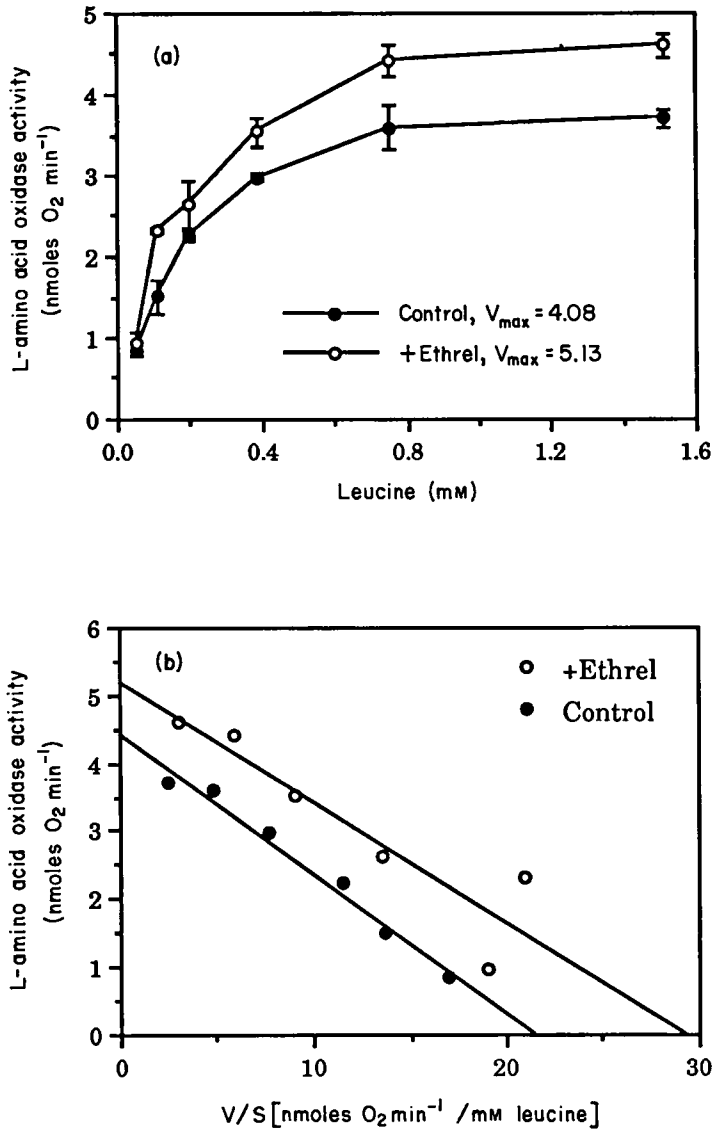

Fig. 4. Activity of L-amino acid oxidase encapsulated in liposomes in the presence of external phospholipase A_2. Encapsulated L-amino acid oxidase in liposomes was assayed as described in 'Materials and Methods', in the presence (○) or absence (●) of 82·5 nM ethrel. Phospholipase A_2 (10 U) and ethrel were added to reaction medium with liposomes containing L-amino acid oxidase and activity was assayed at various leucine concentrations. Each point is the mean ± SD of three separate experiments. When bars are not shown they are smaller than the symbol size.

was no compartmentalization between them (Fig. 3) but the presence of phospholipase A_2 in the solution with L-amino acid oxidase is inhibitory to later enzyme. The average inhibition by PLA_2 was 32 ± 8% ($n = 3$). The ammonium sulphate in the phospholipase A_2 mixture (as purchased from Sigma) may be the reason for the decrease in V_{max} of L-amino acid oxidase. The average K_m of in-solution L-amino acid oxidase towards

leucine was 166 ± 14 μM ($n = 3$ experiments). Ethrel (82·5 nM) in solution had no effect on L-amino acid oxidase activity (not shown). As the encapsulated enzyme was protected from this inhibition, this leads to an apparent emphasis of the compartmentalized enzyme. Multilammelar vesicles that were produced in a rectangular bath have a diameter of 450–550 nm, therefore the amount of encapsulated enzyme might be greater by 0·3% than might be expected for the normal 100 nm diameter liposomes; it is estimated that the encapsulated L-amino acid oxidase was about 1–5% of the total starting material. Although the relative concentration of encapsulated L-amino acid oxidase was lower than the observed increased activity, the above explanations together can account for the observed increase in L-amino acid oxidase activity (as seen in Fig. 4).

An increase in V_{max} of the encapsulated L-amino acid oxidase would indicate that more L-amino acid oxidase is available to oxidize the L-leucine in the external solution. The addition of ethrel increased the V_{max} of L-amino acid oxidase that was enclosed inside the liposomes by 26% (Fig. 4) (the range for eight experiments was 16–50% enhancement, and average enhancement was 30% above the control) without affecting the K_m (Fig. 4). The phospholipase A_2-induced increased permeability enhancement by ethrel was very similar to the enhancement, by ethrel, of the activity of phospholipase C. The average K_m of the encapsulated L-amino acid oxidase was 147 ± 22 μM leucine ($n = 4$) and 156 ± 8 μM leucine ($n = 4$) respectively in the absence or presence of ethrel this is very similar to the K_m of this enzyme in solution (see above). It was concluded that the enhancement of encapsulated L-amino acid oxidase activity was not due to the effect of phospholipase A_2 or ethrel on this enzyme, but to a permeability increase that is caused by phospholipase A_2 activity in the presence of ethrel.

The results are indicative of a wounding effect of ethrel on phospholipid bilayer which seems to be correlated to ethylene release as ethrel breaks down. It is possible that ethrel interacts with the phospholipid bilayer and renders it more susceptible to degradation by various phospholipases. If this is so then the use of this compound to introduce ethylene to plants and plant tissues *in vitro* may lead to erroneous results or interpretations.

These results may also support the observation that in ethylene-producing tissues the phospholipid content decreased without a concomitant increase in in-vitro activity of acyl phospholipases (Suttle & Kende 1980). It might be that the ethylene produced during the late stages of senescence at high levels or during wounding increases the susceptibility of the cellular membranes to degradation by phospholipases which will result in increased collapse of the phospholipid bilayer. However, this hypothesis does not exclude the possiblity that there are other specific biochemical and physiological processes that are induced by ethylene binding to specific receptor(s) (Smith & Hall 1985). The response of the artificial membranes was to concentrations 10–100-fold higher (55–82·5 nM ethrel) than the K_d (0·6–4 nM) for the receptors to ethylene (Smith & Hall 1985). Therefore, it may be that the results described here occur *in vivo* at the late stages of senescence when ethylene concentrations are high.

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