D I S C U S S I O N

DISCUSSION

Peroxidase and ATPase, which are widely distributed in plants, have been implicated with various physiological manifestations. Peroxidase has been reported to be involved in growth and development, senescence, fruit ripening and abscission. It has been used as a parameter for various stress conditions. Peroxidase activity is phytochrome mediated and is affected by plant hormones, herbicides, fungicides and heavy metals. ATPases have also been well characterized from mitochondria, chloroplasts, plasmalemma as well as from cytoplasm. The membrane bound ATPases are associated with ion absorption and transport and are controlled by cations, polar lipids, polyamines and guanidines. Apart from these ATPases are also known to be affected by toxicants, heavy metals and plant hormones.

Polyamines which occur ubiquitously, are believed to be involved in growth processes through their interaction with nucleic acids. In plants, however, they are also involved in the control of several stress related processes such as senescence, wounding and temperature and salt stress. The protective effect of polyamines in these processes may be associated with the stabilization of the membrane structure. Guanidino compounds, which are

structurally related to polyamines, are known to destabilize the membranes and to interfere with the mitochondrial metabolism, transport of ions and inhibition of growth. Recent studies have shown that the destabilization of membranes and growth inhibition produced by guanidines could be reversed by polyamines.

Since polyamines are involved in the control of some stress related phenomena with which peroxidase is implicated and guanidines interfere with ion transport which is controlled by ATPase and as both polyamines and guanidines bind with the membrane and modulate its properties, an attempt was made to investigate whether these compounds have any controlling effect on peroxidase and ATPase activity. Maize scutellum tissue was considered ideal for the study as it is associated with transport and undergoes senescence.

Preliminary studies on the developmental pattern of peroxidase and ATPase, when assayed in slices, showed that though the two enzymes increase with germination, the development was more rapid in the scutella excised and incubated, rather than in the freshly excised scutella from the seeds germinated for the same period. Increased oxygen availability during the incubation of the excised scutella may account for the early development of the enzymes during incubation.

Preincubation of scutellum slices excised from 4 day germinated seedlings with a series of diamines, polyamines and guanidines caused inhibition of peroxidase by di- and polyamines and activation by some guanidines. However, both groups of compounds caused activation of ATPase. The inhibition of peroxidase by diamines increased with the increasing chain-length upto diaminobutane (putrescine) but decreased with further increase in the chain-length. The two polyamines were more effective than the diamines. The inhibition of peroxidase by polyamines may be attributed to their binding to the negatively charged membrane phospholipid fraction. Previous studies have provided evidence that polyamines may regulate several membrane bound enzymes either by directly binding to the regulatary site of the enzyme as in the case of acetyl-choline esterase (Peter et al., 1979) or as a result of their binding to the phospholipids of the membrane leading to a change in its structure and consequently the modulation of the enzymes such as glucose-6-phosphatase (Nordlie et al., 1979) and ATPase (Peter et al., 1981).

Polyamines have been reported to inhibit ATPase from human erythrocytes and pig kidney (Peter et al., 1973). Inhibition of ATPase by polyamines has also been reported for Na^+, K^+ ATPase from developing chick brain (Heinrich-Hirsch et al., 1977). The inhibition was more pronounced

with spermidine than with spermine and it was suggested that spermidine acts as an allosteric inhibitor. Contrary to these observations Tashima et al. (1978) and Peter et al. (1981) have reported that spermine activates Na^+, K^+ ATPase and the activation is prevented by excess Na^+ or K^+.

Out of the guanidino compounds tested in the present study, arginine, agmatine, creatine and creatinine had no effect on peroxidase or ATPase but guanidino acetic acid, guanidino butyric acid, dodine and guazatine activated both peroxidase and ATPase. Octylguanidine has been reported to inhibit ATPase in higher plants (Marre, 1978), octylguanidine and dodecylguanidine have also been found to inhibit ATPase from pea and chlamydomonas chloroplast fragments (Mottley, 1978). Methyl guanidine has been found to inhibit Na^+, K^+ ATPase from rat brain microsomes (Matsumoto and Mori, 1976).

Peroxidase and ATPase were, however, not affected by any of the compounds tested from the scutella of 8 day germinated seedlings. These results indicate that with increase in period of germination from 4 to 8 days, which may result in disintegration and loss of membrane integrity (Parrish and Leopold, 1978), the effect of amines and guanidines was abolished. The compounds thus appear to alter peroxidase and ATPase activity by binding to membrane. This was further evident when homogenate instead of slices

was used. The compounds had some effect on the enzymes in the homogenate prepared from scutella excised from 4 day germinated seedlings but had no effect on the homogenate prepared from 8 day germinated seedlings. This may be attributed to the breakdown of membrane structure during homogenization. The disintegration and loss of membrane integrity of the scutellum during germination was further confirmed by lipid peroxidation which is an index of membrane integrity (Packer et al., 1967; Dhindsa et al., 1981, 1982). Lipid peroxidation was 4 fold higher in the scutella of 8 day germinated seedlings compared to the scutella of 1 day germinated seedlings.

The effect of the compounds on both the enzymes was concentration dependent. The inhibition of peroxidase by polyamines increased with increasing concentration in both slices and homogenate but the activation by guanidines increased initially with increasing concentration upto 2 mM and decreased thereafter. All the compounds activated ATPase initially upto about 2 mM and inhibited thereafter with increasing concentration. A similar biphasic response of polyamines has been reported for ATPase from human erythrocytes and pig kidney (Peter et al., 1973) and it was suggested that the inhibition was not due to the displacement of Mg for Mg-ATP. In the present study also increasing Mg concentration in the presence of spermine at higher concentration did not alter the effect of spermine.

Since polyamines and guanidines have opposite effect on peroxidase and a similar effect on ATPase, studies were carried out to elucidate the possible interaction of these compounds in modulating the two enzymes. The inhibitory effect of polyamines on peroxidase was reversed by guanidino compounds having a free guanidino group but the activating effect of guanidines was not reversed by polyamines. In case of ATPase, where both groups of compounds activate, there was no additive effect when treatment with one group of compounds was followed by the other. These results indicate that there must be some common binding sites on the membrane for polyamines and guanidines, and some additional sites for guanidines. Previous studies on betacyanin efflux from beet root discs and ion efflux from beet root and swede discs have shown that guanidines increase betacyanin efflux in the presence of spermine even though spermine alone decreased it (Srivastava and Smith, 1982a). In another study the inhibitory effect of guanidines on growth in light grown cress, barley, oats and apple cells grown in suspension culture was found to be reversed by spermidine and spermine (Srivastava and Smith, 1982b).

Sulfhydryl groups of membrane proteins appear to be important for membrane function and membrane permeability (Levitt, 1972). For instance, the non-permeant sulfhydryl reagent p-chloromercuri-benzene sulfonate inhibits membrane transport in beet leaf discs (Giaquinta, 1976). They also

affect the permeability of red blood cells (Knauf and Rothstein, 1971). The interaction between sulfhydryl groups and polyamines has also been demonstrated in beet root discs (Naik et al., 1981). The sulfhydryl reagent p-chloromercuri-benzoate (p-CMB) enhanced betacyanin efflux in beet root discs and the stabilizing effect of polyamines was abolished but the toxic effect of guanidines was potentiated by p-CMB (Srivastava and Smith, 1982a). In the present study sulfhydryl reagents increased both peroxidase and ATPase activity. The inhibitory effect of polyamines on peroxidase was reversed by sulfhydryl reagents and the activating effect of sulfhydryl reagents was reversed by polyamines. Polyamines and sulf-hydryl reagents which activated ATPase separately did not give any additive effect on ATPase when they were followed one after the other. However, sulfhydryl reagents gave an additive effect for both peroxidase and ATPase when they were followed by guanidines or vice versa. These results indicate that polyamine binding was reversed by guanidines and sulfhydryl reagents whereas guanidine binding was not reversed by polyamines but potentiated by sulfhydryl reagents. Thus, there must be some common binding sites on the membrane between polyamines and guanidines and between polyamines and sulfhydryl reagents. However, the non-reversibility of guanidine binding by polyamines or sulfhydryl reagents and the additive response between guanidines and sulfhydryl reagents would indicate the presence of additional sites for guanidines.

Both peroxidase and ATPase have been reported to be present in bound as well as in the soluble fractions. The membrane-bound peroxidase may be bound ionically or covalently and the ionically bound enzyme can be solubilized by Ca^{++} (Haard, 1973). Studies on the subcellular fractions of maize scutellum showed that polyamines inhibit peroxidase and activate ATPase in all the particulate fractions to varying degrees, whereas guanidines such as guanidino acetic acid and dodine activate both peroxidase and ATPase, while the other guanidines have no effect. None of the compounds tested, however, had any effect on the enzymes present in cytosolic fraction indicating that these compounds can modulate only the bound enzymes and not the enzyme present in the cytosolic fraction.

Ionically bound peroxidase could be solubilized by Ca^{++} and ATPase could be solubilized by triton and deoxycholate from mitochondrial or membrane fractions. The solubilized enzyme was not affected by the compounds indicating that these compounds can modulate only the bound enzymes and lost their effect once the enzymes were solubilized.

Attempts made at reconstitution of the enzymes solubilized from the particulate fraction were successful only with ionically bound peroxidase where it was found that about 50% of the enzyme solubilized from the membrane fraction

could be again attached to the membrane and after attachment it again responded to the modulating effects of polyamines and guanidines. Further, the reconstitution of the enzyme bound to the membrane could not only be achieved from the enzyme which was previously detached from the membrane but also with the enzyme present in the cytosolic fraction. This observation suggests that the peroxidase enzyme bound ionically is probably not different from the cytosolic enzyme. The cytosolic enzyme when attached to the membrane could also be modulated by the compounds. This observation appears to be of great significance since this type of modulation may have an important role in the enzyme regulation. It was also observed that attachment of compounds to the membrane fraction from which the peroxidase was previously detached did not alter its subsequent binding showing that the groups involved in attaching the compounds to the membrane were not the ones involved in attaching the enzyme to the membrane. Thus, the levels of polyamines, guanidines and Ca^{++} may play a role in regulating the peroxidase activity in the tissue. The mechasism of Ca^{++} regulation may be to control the enzyme bound to the membrane while the polyamines and guanidines have a more direct role in modulating the activity of enzyme associated to the membrane.

Inhibition of peroxidase and activation of ATPase by polyamines and the activation of both the enzymes by guanidines was found to alter the Km of the enzymes for the substrate indicating that the compounds modulate the membrane bound enzyme by affecting their affinity for the substrate.

The modulation of bound peroxidase and ATPase by the compounds was not specific to scutellum tissue only, since a similar response was obtained with subcellular fractions obtained from several other tissues suggesting that modulation of the bound enzymes by these compounds is a general phenomenon.

Phytochrome mediates a vast range of developmental responses in plants (Smith, 1970, 1974, 1975, 1976; Mohr,1974; Shrophire and Mohr, 1982). Though,a great deal of progress has been made in understanding the mechanism of light perception and in describing the final morphogeneic manifestation very little is known about the process that links light perception to its manifestations. Mohr (1966) proposed a hypothesis that phytochrome regulates development by controlling gene expression at the genome level. However, the effect of phytochrome on gene expression implies a rather slow response, since the synthesis of a new enzyme necessarily takes a finite time and some rapid effects of light mediated by the phytochrome could not be explained on the basis of this.

Hendricks and Borthwick (1967) postulated that phytochrome may act by controlling the membrane permeability and thus allowing for the rapid effects achieved. It is now believed that phytochrome operating initially through a change in the properties of certain critical membranes may cause the release of some substances which interact with the mechanism controlling gene expression. This may account for both the rapid effects on membrane permeability as well as the long term changes in the development.

Phytochrome control of enzyme activity was first reported by Marcus (1960) and since then a large number of enzymes have been reported to be regulated by phytochrome (Smith, 1974, 1975, 1976; Schopfer, 1977) including peroxidase (Schopfer and Plachy, 1973; Sharma et al., 1976; DeGreef et al., 1977; Huault and Klein, 1980; Penel et al., 1980) and ATPase (Jose, 1977; Brain and Susan, 1981; Serlin et al., 1984). Recent studies have shown that polyamine levels and their biosynthetic enzymes are also regulated by phytochrome (Dai and Galston, 1981; Goren et al., 1982a,1982b).

In the present study the membrane bound peroxidase and ATPase were found to be modulated by polyamines and guanidines as a result of their binding to the membrane and changing the affinity of the enzymes for their substrates.

Phytochrome has been reported to be bound to membranes (Mackenzie and Briggs, 1978) though recent reports also suggest that they may get redistributed on the membranes after light perception (Cedel and Roux, 1980; Watson and Smith, 1982; Verbelen et al., 1982). These studies would indicate a possible interrelationship between polyamines, guanidines and phytochrome in controlling peroxidase and ATPase activity from maize scutellum.

Studies carried out on the phytochrome induction of peroxidase and ATPase in maize scutellum tissue showed that both the enzymes are phytochrome mediated since red light treatment enhanced their activity which could be abolished by far-red light. The phytochrome response was also found to be an age dependent phenomenon since the response increased initially with period of germination and was abolished later with further increase in germination. Age dependence of phytochrome response has been reported in other studies also (Sharma et al., 1977). Studies on the interaction of polyamines and guanidines with phytochrome showed that the red light induced activation of peroxidase but not of ATPase was reversed by polyamines. The reversal of light modulating effect by polyamines was evident more when the tissue was exposed to compounds before exposure to light rather than when the two were given simultaneously or when light exposure was given prior to the treatment with

compounds. This may be due to a slow intake of polyamines in the intact scutellum tissue. The question, why polyamines were able to reverse the light mediated activation of peroxidase and not of ATPase is not clear yet. This may be due to the differential nature of binding to the membranes. The guanidino compounds activate both peroxidase and ATPase even in the dark control group and red light which activates both the enzymes could not give any additive response when it was given along with the guanidino compounds suggesting that a common mechanism may be operating in the activation of these enzymes by red light and guanidino compounds. This was further confirmed in the studies where the far-red light was found to reverse the red light as well as the guanidine mediated activation of both the enzymes.

Bassim and Pecket (1975) have shown that phytochrome mediated anthocyanin biosynthesis could be controlled by membrane stabilizers like calcium chloride and cholesterol and the red light effect could be mimicked by membrane destabilizers like n-propanol (Pecket and Bassim, 1974a; Whitelam and Johnson, 1981) and kinetin (Pecket and Bassim, 1974b) and the increase brought about by membrane destabilizers could be reversed by immediate far-red light treatment (Whitelam and Johnson, 1981) or calcium chloride treatment (Pecket and Bassim, 1974b). These observations suggest that red light acts as a membrane destabilizer whose effect can be

mimicked by destabilizers and reversed by membrane stabilizers. Far-red light acts as a membrane stabilizer as it could reverse the destabilization caused by red light as well as membrane destabilizers. The results of the present study also show that the activation of the two enzymes by red light and guanidines may be associated with the destabilization of membrane which is reversed by far-red light. The red light mediated activation of peroxidase, though not of ATPase, was reversed by polyamines showing that atleast in case of peroxidase polyamines appear to act by stabilizing the membrane and thereby abolishing the red light effect. This data in general seems to agree with the earlier reports that polyamines stabilize the membranes (Naik and Srivastava, 1978, 1981; Popovic et al., 1979; Naik et al., 1980; Galston et al., 1980; Altman, 1982b; Srivastava and Smith, 1982a) whereas guanidines destabilize them (Gomez-Lepe et al., 1979; Srivastava and Smith, 1982a, 1982b). From these studies it appears that polyamines and red light act in opposing manner thus antagonizing the phytochrome response whereas guanidines mimic it.

The studies on the effect of polyamines and guanidines on the bound enzymes with scutellum slices have shown that polyamines inhibit peroxidase and activate ATPase whereas guanidines activate both the enzymes. These results were obtained when the slices were incubated for 1 hr with the

compounds before assaying. However, the studies on the phytochrome mediation of the two enzymes, where the whole scutella along with the embryo was incubated with these compounds, showed that polyamines have no activating or inhibitory effect on the two enzymes in the dark control group. Guanidines, however, still showed activation though at a decreased level. The negative response of polyamines in the whole scutellum may be either due to their breakdown in the scutellum tissue itself or because of their transport to the growing embryo. However, no amine oxidase activity could be detected in the scutellum tissue though the embryo had a significant activity. These results would indicate that the negative response of polyamines was not due to their breakdown in the scutellum tissue but primarily as a result of their transport to the embryo. Polyamines, though did not have any effect in the dark control group but could reverse the effect of red light on peroxidase activity. A further evidence for this was also noticed from studies where the scutella were incubated with or without embryo. In the group where the embryo was not present when the scutellum tissue was exposed to light and compounds, the red light had no activating effect on peroxidase or ATPase but the inhibitory or activating effects of polyamines were evident. These results suggest that in the scutellum tissue without embryo since the polyamines accumulate they gave inhibition

or activation but when the embryo was present they were transported to the embryo where they were broken down. Also the phytochrome response appears to be perceived not by the scutellum tissue but by the embryo which then transmits the light perception to the scutellum tissue to elicit its response on the two enzymes. Previous studies (Penel and Greppin, 1975; Penel et al., 1980) also have shown that the photoconversion of phytochrome in one part of the plant immediately modified the peroxidase activity in other part of the plant which is not irradiated implying a fast transmission of some signal from irradiated parts to the non irradiated parts.

The above mentioned studies with phytochrome were carried out on the slices prepared from the scutella exposed to light and compounds which measures only the membrane bound enzymes. In order to know if the phytochrome, like polyamines and guanidines, affects only the bound enzymes or it can affect the cytosolic enzyme also, the subcellular fractions were prepared from the scutellum tissue after exposure to lights and compounds and the results showed that though red light could affect the bound as well as the cytosolic enzymes but the compounds could affect only the membrane bound enzymes and had no effect on the enzymes present in the cytosolic fraction. This further confirms the earlier results that only the membrane bound enzymes could be modulated by polyamines and guanidines.

Plant hormones are known to affect peroxidase (Gaspar et al.,1975; Birecka et al., 1976; Dubucqu, 1976; Henry and Jorden, 1977; Legrand and Dabois, 1978; Burner, 1978; Dendsay and Sacher, 1982) and ATPase (Kasama and Yamaki, 1973; Scherer and Morre, 1978; Erdei et al., 1979; Henry and Ricard, 1979; Cocucci and Cocucci, 1979; Scherer, 1981; Elliott, 1982). In the present study preincubation of scutellum slices with different hormones like indole acetic acid, gibberellic acid, kinetin and etherel showed no effect on either peroxidase or ATPase except etherel which inhibited peroxidase activity. Guanidines could only partially reverse the inhibitory effect of etherel but etherel could completely reverse the activating effect of guanidines. The inhibitory effect of etherel on peroxidase activity may be associated with its metal adsorption property (Burg and Burg, 1963) or its membrane attachment property (Lyons and Pratt, 1964).