EFFECTS OF METABOLIC INHIBITORS ON THE TRANSLOCATION OF AUXINS

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ABBREVIATIONS

ATP-ase	Adenosine triphosphatase
DCCD	N,N-dicyclohexylcarbodiimide
DIDS	4,4-diisothiocyano-2,2'-stilbene disulfonic acid
FC	Fusicoccin
GRAM	Gramicidin
JUG	Juglone
NADH	Nicotinamide adenine dinucleotide reduced
NPA	Sodium alanap (N-1-naphtylphthalamic acid)
QUIN	Quinacrine
2,3,5-TIBA	2,3,5-triiodobenzoic acid
2,4,5-T	2,4,5-Trichlorophenoxyacetic acid
VAN	Vanadate (sodium orthovanadate)

CHAPTER I

INTRODUCTION

Auxin transport is important in the regulation of numerous developmental events in the plant. These include cell elongation, vascular differentiation, apical dominance and abscission. Thus, it is not surprising that substantial efforts have been directed towards understanding the mechanism underlying the processes of translocation.

Plants cells expend energy to maintain a pH gradient across the plasma membrane so that the wall space is acidic relative to the cytoplasm of the cell's interior. Since cell membranes are more readily permeable to uncharged molecules, undissociated, fat soluble auxin molecules readily enter the cell by diffusion. Once in the cytoplasm most of these molecules dissociate due to the higher cytoplasmic pH. This is a phenomenon which permits auxin to be concentrated within the cytoplasm. Since the membrane is relatively impermeable to auxin anions, the continued influx of undissociated auxin leads to an internal accumulation of dissociated auxin (Rubery and Sheldrake, 1973; Raven, 1975; Goldsmith et al.,1981).

There is evidence that phloem cells may have a low proton concentration (about pH 8.0 - 8.5) along with a high potassium ion and sucrose concentration (Crafts et al., 1971). This is in contrast to mesophyll and other cell types which have a lower pH gradient. Therefore, this indicates that phloem tissue may be a very effective

system for accumulation and subsequent translocation of substances through the plant.

Giaquinta (1977), suggested that the characteristics of the phloem cells were favorable for sugar uptake to be coupled to the co-transport of protons into the phloem. Evidence has been presented that sugars are accumulated into the phloem from the free space prior to translocation (Geiger et al., 1974) and that the uptake resembled an enzyme mediated process (Sovonick et al., 1974). This process appears to be related to the ATPase activity located on the plasma membrane of the sieve elements as was shown in Ricinus (Browning et al., 1980) and in a number of other species (Cronshaw, 1980). It is proposed that ATPase, a ubiquitous proton pump on membranes, provides the mechanism required for proton transport across the membrane using ATP as the immediate energy source and the pH gradient then results in sucrose loading (Mitchell, 1966). Some evidence of ATPase activity associated with proton transport was the stimulation of activity produced by nigericin in microsomal vesicles from tobacco callus (Sze, 1980). This was followed by demonstrations of electrogenicity in microsomal vesicles from tobacco callus by Sze and Churchill (1981), and pea internodes by Rasi-Caldogno et al. (1981), and demonstrations of proton transport (Dupont et al., 1982; Hager et al., 1980; Mettler et al., 1982; Stout and Cleland, 1982). Thus, to reemphasize, the primary active transport process in higher plants is thought to be an electrogenic transport of protons (Poole, 1978; Spanswick, 1981). The proton motive force generated by proton pumping provides the driving force for transport of solutes, including cations, anions, aminoacids and sugars according to Mitchell's chemiosmotic hypothesis (Mitchell, 1976; Nicholls, 1982). Electrical potential and

pH measurements of intact plant cells have suggested that electrogenic proton pumps are localized on the plasma membrane and on the tonoplast (Spanswick, 1981; MacRobbie, 1979; Raven and Smith, 1979; Aoki and Nishida 1984; Marin, 1983; Churchill et al.,1983) Proton pumps are important not only for mediating active transport of nutrients but also are directly involved in various physiological processes such as cytoplasmic pH regulation and opening and closing of stomata. The proton electrochemical gradient may also regulate cell elongation and cell wall synthesis (Cleland, 1971; Hager et al., 1971). Furthermore, ion currents, including those mediated by proton pumping are related to development of plant as well as animal cells (Jaffe, 1982).

There are two types of mechanisms that have been postulated to drive proton transport and result in pH gradients: 1) proton pumping dependent upon the hydrolysis of ATP catalyzed by membrane ATPases (Hodges, 1973; Poole, 1978; Spanswick, 1981; Bennett and Sze, 1984; Chen and Sze, 1984; Churchill and Sze, 1983; Scherer, 1984; Sze, 1983), and 2) proton pumping driven by a plasma membrane NADH oxidation system (Crane and Low, 1976; Ivankina and Novak, 1980; Lin,1982, 1984; Thom and Maretzki, 1985).

There is evidence that two types of H+ ATPases are associated with plant membranes. One type is found in fractions enriched in tonoplast membranes, and the other type is associated mainly with the plasma membrane. Characterization of these ATPases was done by using the differential sensitivity to vanadate and nitrate (Sze, 1982; Churchill and Sze, 1983; Sze and Churchill, 1983; Aoki and Nishida, 1984; Marin, 1983; Bennett et al., 1984; Rasi-Caldogno et al., 1985). The vanadate sensitive ATPase is, for convenience, referred to as plasma membrane

enzyme, though this type of ATPase may also be associated with the golgi apparatus (Churchill et al., 1983) and secretory vesicles of oat cells (Binari and Racusen, 1983). The vanadate-insensitive and nitrate sensitive ATPase is referred to as a tonoplast enzyme. Unlike the tonoplast type ATPase, the plasma membrane ATPase activity is relatively insensitive to anions, and is stimulated by alkali cations. Thus, the implications are that the establishment or control of pH gradients in plant cells are maintained by ATPases or NADH oxidation on the plasma membrane and/or tonoplast membranes.

The evidence indicates that tonoplast and plasma membrane ATPases and possibly NADH oxidases are involved in the maintenance of pH and electrical gradients that may be involved in the control of long-distance auxin translocation. Thus, it should be possible to determine the site and possibly the types of mechanisms of proton excretion that are involved by utilizing the differential sensitivity of these mechanisms to metabolic inhibitors (see Appendix). For example, DCCD is an inhibitor of ATPase proton excretion mechanisms on both the plasma and tonoplast membranes (see review by Sze,1984) as well as proton excretion channels utilizing NADH oxidase (Lin, 1984) and at least two of the cytochrome mediated electron transport systems (Preschek,1984; Prochaska et al., 1981; Esposti et al., 1983; Casey et al., 1980). Thus, this inhibitor should promote an increase of pH in the cell wall area and a decrease of pH in the cytoplasm. This effect should result in a decrease in auxin uptake and an increase in auxin efflux. On the other hand, vanadate and DES are inhibitors of plasma membrane ATPases (Sze, 1984). These inhibitors should increase the pH of the cell wall area and should inhibit the uptake of auxins. DIDS is an inhibitor of

tonoplast ATPase (Sze, 1984) and should decrease the pH of the cytoplasm. This effect should stimulate efflux and vein unloading from tissues such as the sieve tubes. Quinacrine and juglone are inhibitors which interact with NADH oxidase mediated proton excretion mechanisms. Quinacrine was shown to inhibit potassium uptake in protoplasts (Lin, 1982), to inhibit NADH oxidase (Lin, 1984) and to be a specific inhibitor of transmembrane oxidoreductase (Crane and Low, 1976). Quinacrine would be expected to inhibit proton excretion at the plasma membrane and, thus, would inhibit auxin uptake if this mechanism is extensively involved in maintaining the pH gradient. Juglone was shown to be an acceptor of electrons during the oxidation of NADH by NADH oxidase (Crane and Low, 1976) and, thus, may result in the inhibition of proton excretion that is associated with NADH oxidation. This inhibitor would possibly inhibit auxin uptake. Fusicoccin is a phytotoxin which is known to enhance proton excretion and intensify the electropotential gradients (Cleland et al., 1977; Marre, 1979) presumably because of malate synthesis and acidification of the cytoplasm (Bertl and Felle, 1985). Because of the acidification of the cell wall area, fusicoccin should enhance auxin uptake. Because of acidification of the cytoplasm and the increase of the negative charge in the cytoplasm, fusicoccin should cause a large enhancement of auxin efflux. Gramicidin is an agent which collapses electropotential gradients (Bennett and Spanswick, 1983), and should counteract the effects of agents such as fusicoccin, which produce hyperpolarization of electrical gradients. Nickel sulfate inhibits the activity of phosphoenolpyruvate carboxylase and the synthesis of malate (Morgutti et al., 1984). This enzyme is believed to be a component of the pH stat

mechanism involved in the regulation of the cytoplasmic pH. Blockage of this enzyme should lead to an increase of the pH of the cytoplasm which, in turn, would decrease the activity of plasma membrane ATPase (Romani et al., 1985) and increase the pH of the cell wall area. The net effect of nickel sulfate might be to decrease auxin uptake and decrease auxin efflux if this inhibitor causes the expected effects on pH. TIBA and NPA are chemical agents which are thought to interact in polar auxin transport as first shown by Kuse (1953) and Morgan and Soding (1958). It is generally believed that these agents act by blocking carrier-mediated auxin anion efflux (see review by Kaldewey, 1984). If auxin efflux is blocked, these agents might affect vascular translocation of auxins by inhibiting vein unloading of tissues such as sieve elements. Thus, the overall effect of TIBA and NPA might be to promote symplastic translocation of auxins in the vascular system even though polar transport of auxins in other tissues is usually inhibited by TIBA and NPA.

There are many physiological processes that are affected by endogenous auxins and, furthermore, there are many auxins-like compounds that are used in agricultural practices. Consequently, there are many reasons why it is important to study auxin translocation. Therefore, this investigation was conducted to ellucidate the mechanisms that may be involved in auxin translocation.

The objectives were to determine what membrane types and what proton excretion mechanisms may be involved in pH changes and auxin translocation. To achieve the objectives, auxins with different pK values were selected to determine if pH gradients were involved in auxin translocation. Metabolic inhibitors and other compounds that showed

differential inhibition on proton excretion mechanisms were selected and used in order to determine the membrane location and which type of proton excretion mechanisms was involved in auxin translocation.

CHAPTER II

MATERIALS AND METHODS

Bush bean (Phaseolus vulgaris L. cv Stringless Greenpod) seeds were germinated in perlite moistened with half-strength Hoagland's nutrient solution (Hoagland and Arnon, 1938) for 5 days at 32°C, under continuous fluorescent light of 90 $\mu E/m^2s$. The seedlings were transferred to amber glass jars containing 400 ml of aerated, half-strength, Hoagland's nutrient solution and grown for 4 or 5 days in a growth chamber with a photoperiod of 14 hours, 33°C days and 10 hours 29°C nights with a relative humidity ranging from 20 to 30%.

The effects of inhibitors of ATPase activity and the NADH-oxidation system for proton excretion, and other compounds such as fusicoccin, juglone, sodium alanap, 2,3,5-triiodobenzoic acid, nickel sulfate and gramicidin on auxin translocation were determined. Nine or ten day old seedlings were injected with 1 µl amounts of the inhibitors along with radioactive auxins, except for sodium orthovanadate and juglone which were injected separately in the cotyledonary node. During injection, a 1-µl syringe needle was forced down the center of the stem about 1 cm below the cotyledonary node where the chemical were deposited. In those cases where chemical treatments were made by two separate injections, the second injection was made by inserting the syringe in the opening made by the first injection.

The treatments were made using ^{14}C-carboxyl labeled 2,4,5-trichlorophenoxyacetic acid (2,4,5-T, 54 Ci/M, Amersham), and indole-3-acetic acid (IAA, 48 and 61 Ci/M, Amersham). These auxins were dissolved in 1 µl of 95% ethanol. Before treatment, the auxins were diluted in half with the following inhibitors : 4,4-diisothiocyano-2, 2'-stilbene disulfonic acid (DIDS, Sigma Chemical Co.) dissolved in 50% ethanol, N,N'-dicyclohexylcarbodiimide (DCCD, Sigma Chemical Co.) dissolved in 95% ethanol, quinacrine (QUIN, Sigma Chemical Co.) dissolved in water, diethylstilbestrol (DES, Sigma Chemical Co.) dissolved in 95% ethanol and sodium orthovanadate (VAN, Fisher Scientific Co.) dissolved in water. Other compounds selected were: juglone (JUG, Sigma Chemical Co.) dissolved in 95% ethanol, fusicoccin (FC, Italchemia S.A.) dissolved in 95% ethanol, sodium alanap (NPA, Uniroyal Chemical) dissolved in water, gramicidin (GRAM, Sigma Chemical Co.) dissolved in 95% ethanol, 2,3,5-triiodobenzoic acid (TIBA, Eastman Organic Chemical) dissolved in acetone and nickel sulfate ($NiSO_4$, Fisher Chemical Co.) dissolved in water.

Eight replications of each treatment were used in this study. After treatment the plants were returned to the growth chamber for 4 hours before harvesting. The plants were harvested and then divided into the following six plant parts: young shoots, including all tissue above the primary leaves including petioles; epicotyl; treated area including all tissue 0.5 cm above the cotyledonary node down to 2.5 cm below the cotyledonary node; hypocotyl; and roots. The plant parts were freeze-dried, weighted and the primary leaves were homogenized in 10 ml of 95% ethanol and the other plant parts were homogenized in 5 ml of 95% ethanol. Two-tenths ml of the primary leaves homogenate and 0.5 ml

aliquots of each of the other plant part homogenates were taken and assayed for radioactivity by liquid scintillation counting with appropriate correction for quenching. At the time of harvesting, also 5 ml of nutrient solution was removed, freeze-dried and assayed for radioactivity.

The data obtained were analyzed by calculating the percent of the recovered ^{14}C which moved out of the treated area. This value is believed to represent vein loading. Vein unloading was calculated as the ^{14}C found below the treated area divided into the ^{14}C found in the primary leaves.

Statistical analyses were standard F tests, Duncan's multiple range test (Duncan, 1955), and LSD tests.

CHAPTER III

RESULTS

Comparison of the Effect of DCCD and DIDS on the Translocation of 2,4,5-T-1-^{14}C and IAA-1-^{14}C

DCCD was previously shown to affect the long-distance translocation of auxins (Corbett, 1977). In some experiments of this study the effects of DIDS were compared to the effects of DCCD. DCCD, which is known to inhibit all proton excretion channels, inhibited vein loading and enhanced vein unloading of 2,4,5-T-1-^{14}C and IAA-1-^{14}C although the effects for IAA were greater for vein unloading and less for vein loading than for 2,4,5-T (Table I). DIDS, the inhibitor which inhibits only tonoplast ATPases, significantly enhanced the vein unloading and slightly enhanced vein loading of 2,4,5-T. This inhibitor did not significantly affect the translocation of IAA. DCCD significantly enhanced the recovery of ^{14}C in some treatments.

The data in table VII show the actual distribution of 2,4,5-T, and IAA in the entire plant as affected by DIDS treatment.

Effects of DES, Quinacrine, Vanadate and Juglone on the Translocation of 2,4,5-T-1-^{14}C

The effects of a number of other ATPase inhibitors on the translocation of auxins were determined. DES, the inhibitor of plasma membrane ATPase activity, inhibited vein loading and had no effect on vein unloading. However, vanadate, which has also been described as an inhibitor of plasma membrane ATPase activity, had no effect on vein loading or unloading (Table II).

The effects of other inhibitors which may be active on proton excretion mediated by NADH oxidase and cytochrome electron transport systems were also determined. The effect of quinacrine, an inhibitor of NADH oxidase promoted proton excretion, was to inhibit vein loading of 2,4,5-T-1-^{14}C but there was no effect on vein unloading (Table II). In some experiments (Table III) quinacrine did not produce a significant decrease in vein loading. In addition, juglone, which has been shown to interact in cytochrome mediated electron transport, was very effective in altering auxin translocation. Juglone drastically enhanced vein unloading and had very little effect on vein loading (Table III). Juglone was even more effective in enhancing vein unloading when it was applied in the presence of quinacrine (Table III). In some experiments quinacrine slightly decreased vein loading of 2,4,5-T-1-^{14}C. While quinacrine applied with DES caused a slightly greater decrease than DES applied alone. In contrast, quinacrine did not enhance the activity of vanadate (Table II).

The Effect of Fusicoccin, IAA, and Gramicidin on the Translocation of IAA-1-^{14}C

Fusicoccin is a phytotoxin that causes hyperpolarization of the membrane electrical potential and causes proton excretion from the cytoplasm to the cell wall space. In addition, IAA also causes proton excretion and hyperpolarization of the plasmamembrane. Plants were treated with fusicoccin and an excess of IAA and their effects on IAA-1-^{14}C were determined (Table IV). Fusicoccin at 20 µg per plant did not significantly affect IAA-1-^{14}C translocation. Twenty µg of IAA also did not significantly affect the translocation of the 0.5 µg of IAA-1-^{14}C but, again, there was a strong tendency toward enhanced vein unloading. When fusicoccin and excess IAA were applied together there was a large increase in vein unloading and a slight decrease in vein loading. Gramicidin, a chemical agent which was shown to collapse electrical potential gradients, slightly decreased vein loading and had no effect on vein unloading. However, when gramicidin was applied with fusicoccin and excess IAA, it completely reversed the effects of fusicoccin and excess IAA on IAA-1-^{14}C vein unloading. When only excess IAA was applied, gramicidin did not reverse the effects of excess IAA on vein unloading of IAA-1-^{14}C but appeared to enhance the effect.

Effects of TIBA and NPA on the Translocation of IAA-1-^{14}C

TIBA and NPA are specific inhibitors of polar transport and are postulated to act by blocking carrier mediated auxin efflux. The

implications are that these inhibitors could also block efflux and, thus, inhibit vein unloading in the vascular translocation of auxins. The effects of TIBA and NPA on auxin translocation are shown in table V. Both NPA and TIBA drastically enhanced vein unloading rather than inhibiting vein unloading as might have been expected. These inhibitors had no effect on vein loading.

Nickel Sulfate effects on 2,4,5-T-1-^{14}C Translocation

Nickel sulfate is a compound that inhibits the activity of the enzyme phosphoenolpyruvate carboxylase and the synthesis of malate. This would result in an increase of the cytoplasmic pH and might stimulate the accumulation auxin in the cytoplasm. The effects of nickel sulfate on auxin translocation are shown in table VI. There was very little effect of nickel sulfate on auxin translocation. Vein loading was slightly decreased but there was no significant effect on vein unloading of the auxin 2,4,5-T-1-^{14}C.

TABLE I

THE EFFECT OF VARYING CONCENTRATIONS OF DCCD AND DIDS ON THE TRANSLOCATION OF 0.5 µg of 2,4,5-T-1-^{14}C AND 0.5 µg of IAA-1-^{14}C

Treatment	% ^{14}C Recovered	Percent of the Control: Vein Loading	Percent of the Control: Vein Unloading
2,4,5-T-1^{14}C			
0 µg DCCD	65a	100a	100a
1 µg DCCD	72a	83b	148a
4 µg DCCD	86b	48c	239b
20 µg DCCD	78a	21d	333c
0 µg DIDS	72a	100a	100a
1 µg DIDS	71a	109ab	190ab
4 µg DIDS	78a	116ab	229b
20 µg DIDS	71a	123b	353c
IAA-1-^{14}C			
0 µg DCCD	77a	100a	100a
1 µg DCCD	71a	100a	259b
6 µg DCCD	76a	102a	606c
20 µg DCCD	90b	62b	629cd
0 µg DIDS	83a	100a	100a
1 µg DIDS	87a	114a	106a
4 µg DIDS	86a	104a	119a
20 µg DIDS	90b	108a	63a

Values followed by the same letter are not significantly different at the 0.05 level as determined by LSD test.

TABLE II

THE EFFECT OF DES, VANADATE AND QUINACRINE ON THE TRANSLOCATION OF 0.5 µg of 2,4,5-T-1-^{14}C

Treatment	% ^{14}C Recovered	Percent of the Control	
		Vein Loading	Vein Unloading
Control	87c	100c	100a
20 µg DES	86c	60a	87a
20 µg DES + 20 µg QUIN	79b	54a	117a
20 µg QUIN	79b	73b	119a
20 µg VAN	71a	92c	112a
20 µg VAN + 20 µg QUIN	70a	76b	80a

Values followed by the same letter are not significantly different at the 0.05 level as determined by LSD test.

TABLE III

THE EFFECT OF JUGLONE AND QUINACRINE ON THE TRANSLOCATION OF 0.5 µg of 2,4,5-T-1-^{14}C

Treatment	% ^{14}C Recovered	Percent of the Control	
		Vein Loading	Vein Unloading
Control	95a	100a	100a
20 µg JUG	89a	85a	669b
20 µg QUIN	89a	91a	116a
20 µg QUIN + 20 µg JUG	89a	85a	1001c

Values followed by the same letter are not significantly different at the 0.05 level as determined by LSD test.

TABLE IV

THE EFFECT OF FUSICOCCIN, IAA, AND GRAMICIDIN ON THE TRANSLOCATION OF 0.5 µg of IAA-1-^{14}C

Treatment	% ^{14}C Recovered	Percent of the Control	
		Vein Loading	Vein Unloading
Control	55bc	100b	100a
20 µg FC	44ab	79ab	109a
20 µg IAA	45ab	83ab	264b
20 µg GRAM	44ab	71a	91a
20 µg IAA + 20 µg FC	42a	76a	391c
20 µg IAA + 20 µg GRAM	63c	82ab	500d
20 µg FC + 20 µg GRAM	60c	76a	45a
20 µg IAA, of GRAM and FC	45ab	88ab	118a

Values followed by the same letter are not significantly different at the 0.05 level as determined by LSD test.

TABLE V

THE EFFECT OF TIBA AND NPA ON THE TRANSLOCATION OF 0.5 µg of IAA-1-^{14}C

Treatment	% ^{14}C Recovered	Percent of the Control	
		Vein Loading	Vein Unloading
Experiment 1			
Control	75a	100a	100a
20 µg TIBA	72a	114a	620b
Experiment 2			
Control	72a	100a	100a
20 µg NPA	83b	94a	900b

Values followed by the same letter are not significantly different at the 0.05 level as determined by LSD test.

TABLE VI

THE EFFECT OF DIFFERENT CONCENTRATIONS OF NICKEL SULFATE ON THE TRANSLOCATION OF 0.5 µg of 2,4,5-T-1-^{14}C

Treatment	% ^{14}C Recovered	Percent of the Control	
		Vein Loading	Vein Unloading
0 µg $NiSO_4$	61a	100a	100a
1 µg $NiSO_4$	53a	69b	120a
4 µg $NiSO_4$	77a	101a	145a
20 µg $NiSO_4$	75a	102a	167a

Values followed by the same letter are not significantly different at the 0.05 level as determined by LSD test.

TABLE VII

THE EFFECT OF VARYING CONCENTRATIONS OF DIDS ON THE TRANSLOCATION OF 0.5 µg of 2,4,5-T-1-^{14}C AND 0.5 µg of IAA-1-^{14}C

Treatment	Young Shoot	Epi-cotyl	Pri. Leaf	Treat. Area	Hypo-cotyl	Root	Nutr sol.
2,4,5-T-1^{14}C							
0 µg DIDS	.24	9.44	6.98	34.48	8.91	.41	.61
1 µg DIDS	.40	16.45	10.46	37.29	6.02	.17	.06
4 µg DIDS	.47	18.00	13.54	38.63	6.89	.15	.06
20 µg DIDS	.22	14.36	17.05	32.95	5.76	.08	.12
IAA-1-^{14}C							
0 µg DIDS	1.51	3.65	4.59	39.37	20.70	12.52	.20
1 µg DIDS	1.26	3.82	5.40	35.18	21.07	19.96	.38
4 µg DIDS	1.54	3.90	5.83	39.32	21.04	14.23	.30
20 µg DIDS	1.92	4.11	4.14	40.42	21.70	19.37	.23

CHAPTER IV

DISCUSSION AND CONCLUSION

The use of metabolic inhibitors is important for studies dealing with proton excretion mechanisms such as ATPase activity and NADH oxidase mediated proton excretion which are thought to be involved in the establishment of pH gradients and electrical potential gradients. These factors may, in turn, be involved in the control of long distance auxin translocation. Specific inhibitors could help to distinguish what proton excretion mechanism and what membrane type is involved in the pH and electrical gradients and long distance auxin translocation. Much research has been conducted on the effect of differentially sensitive metabolic inhibitors on the transport of organic compounds such as auxins, protons and ions in membrane vesicles, tissue culture cells and plant segments. However, no research has been conducted on the behavior of these inhibitors on auxin translocation in the intact plant.

DCCD is known to be an inhibitor of tonoplast and plasma membrane ATPase-mediated proton excretion (see review by Sze, 1984), and NADH oxidase-mediated proton excretion on the plasmalemma (Lin, 1982). This implies that DCCD should be a very efficient inhibitor of the build up of proton gradients and, thus, should decrease the cytoplasmic pH and increase the cell wall area pH. If auxin uptake and efflux is limited to the diffusion of unionized molecules through the plasma membrane, the effects of DCCD should be the inhibition of auxin uptake and the

enhancement of auxin efflux. The Henderson-Hasselbach equation predicts that the percentage change in unionized auxin that occurs with a given pH change should be the same for each auxin regardless of the pK value of the auxin. Thus, if the change in vein loading and unloading brought about by DCCD is solely the result of differences in the availability of unionized auxin due to a specific pH change, the percentage change in auxin vein loading and unloading in response to DCCD should be the same for each auxin regardless of its pK value. However, the effect of DCCD was found not to be equal for 2,4,5-T and IAA. DCCD inhibited the vein loading of 2,4,5-T more than IAA and increased the vein unloading of IAA more than 2,4,5-T. Thus the effects of DCCD must be due to some factor other than to the simple availability of unionized auxin. One interpretation of the DCCD effects is that they relate to the optimum pH for binding and carrier-mediated transport in and out of cells such as the phloem sieve elements. Using this interpretation, it could be predicted that the optimum pH on the cell wall side of the plasma membrane for carrier activity would be lower for 2,4,5-T and higher for IAA. DCCD treatment would presumably increase the pH on the cell wall side of the plasma membrane to a level above the optimum for transport activity of the low pK auxin 2,4,5-T but would increase the pH to a level which would not be less favorable for IAA since low concentrations of DCCD did not reduce vein loading of this auxin. This suggests that the pH of the cell wall side of the membrane is normally slightly below or equal to the pH level required for optimum carrier-mediated IAA uptake. The effect of DCCD on vein unloading may be explained in a similar manner. DCCD treatment presumably would decrease the pH of the cytoplasm so that the transport

activity of the carrier would be more favorable for the transport of the high pK auxin IAA and would also increase the transport activity of the carrier for the low pK auxin 2,4,5-T but not to as great an extent as for IAA. Previous work also showed a possible effect of DCCD on carrier mediated transport of IAA (Rubery et al.,1982). This implies that DCCD may have caused significant changes in pH gradients as well as in carrier transport activity which could have resulted in the large as well as differential changes in the vein loading and unloading of the two auxins.

DIDS is an inhibitor which only inhibits tonoplast ATPase (Sze, 1984) and would be expected to decrease cyoplasmic pH and not affect the cell wall pH. However, low cytoplasmic pH has been shown to enhance plasma membrane ATPase and this may, in turn, decrease the cell wall pH. This implies that DIDS treatment would possibly enhance vein loading and vein unloading. In this experiment, DIDS had a tendency to enhance vein loading and also to increase significantly vein unloading of 2,4,5-T which is the predicted results. In contrast, DIDS did not show the expected result for IAA vein loading and unloading. Therefore, it will be necessary to conduct more research with this inhibitor and other auxins before it will be possible to answer these questions.

DES is an inhibitor of ATPase on the plasma membrane (Balke and Hodges, 1979) and should affect the pH of the cell wall area. It was expected that DES would increase the pH in the cell wall area which would result in an inhibitory effect on vein loading. DES applied alone inhibited vein loading and showed an additive effect on vein loading when applied with quinacrine. Quinacrine when applied alone also inhibited vein loading. This additive effect of DES and quinacrine

suggests that the two inhibitors are acting on separate mechanisms. Possibly DES inhibited plasma membrane ATPase and quinacrine inhibited the NADH oxidase-mediated proton excretion. Quinacrine was shown to be an inhibitor of the NADH oxidase system in the plasma membrane that mediated proton excretion (Lin, 1984; Thom and Maretzki, 1985), and also a specific inhibitor of a NADH transmembrane oxidoreductase (Crane and Low, 1976).

Vanadate is reported to be a potent inhibitor of plasma membrane ATPase activity in animal cells (Cantley et al., 1977), fungi (Bowman and Slayman, 1979) and in higher plants (Cocucci et al., 1980; O'Neill et al., 1984). Vanadate did not have any effect on the translocation of 2,4,5-T in intact bean plants. The information available on the vanadate inhibitory effect on plasma membrana ATPase comes from studies made on membrane vesicles or protoplast and plant segments. Thus, it appears possible that in intact bean plants vanadate may not have been transported to the site of action and this could account for the lack of an effect of vanadate on auxin translocation in these studies. Vanadate, even when applied with quinacrine, showed no effect on auxin translocation and, thus, could not have been inactive simply because proton excretion was being mediated by the NADH oxidase system.

Juglone was shown to act as an electron acceptor for NADH dehydrogenase in plasma membranes (Crane and Low, 1976). Thus, it could possibly interact in the NADH oxidase system for proton transport at the plasma membrane and would be expected to result in an increase of cell wall area pH and inhibition of auxin vein unloading. In this investigation, juglone caused a significant enhancement of vein unloading or 2,4,5-T efflux, but had no effect on vein loading or

uptake of 2,4,5-T. This suggests that juglone may have caused acidification of the cytoplasm or hyperpolarization of the plasma membrane. Juglone and quinacrine appear to interact in vein unloading since their effects on vein unloading were synergistic.

Fusicoccin induces proton excretion (Marre, 1980), and also caused a decrease in the cytoplasmic pH and an increase in the hyperpolarization of the plasma membrane (Bertl and Felle, 1985). In the present investigation, fusicoccin had a tendency to enhance vein unloading. Vein loading was not greatly affected. This effect implies that fusicoccin may have lowered the pH of the cytoplasm and possibly also hyperpolarized the plasma membrane and either of these effects could have caused auxin vein unloading. It should be possible to distinguish between effects of cytoplasm acidification and hyperpolarization of the membrane by the uses of specific inhibitors. Gramicidin is an agent that was shown to collapse electrical potential gradients (Bennett, and Spanswick, 1983) and, thus, should reverse the effects of fusicoccin if its effects on unloading were due to hyperpolarization. Gramicidin reversed the effects of fusicoccin on unloading and this was especially true when fusicoccin was applied in the presence of high auxin concentrations. Thus, fusicoccin appears to have large effects on auxin translocation by affecting the electropotential gradient across the plasma membranes. However, gramicidin did not reverse the IAA effects. It is possible that the IAA effect on vein unloading is mediated by some mechanism other than hyperpolarization of the plasma membrane, possibly by causing acidification of the cytoplasm.

TIBA (Kuse, 1953) and NPA (Morgan and Soding, 1958) were first reported to be inhibitors of polar auxin transport and it is generally

believed that they also block carrier mediated auxin efflux (see review by Kaldewey, 1984). For example, in crown gall cells, TIBA stimulated the net rate of IAA uptake but had less effect on 2,4-D, NAA and ABA (Rubery, 1980). This implies that TIBA and NPA should inhibit vein unloading of IAA. However, these inhibitors stimulated vein unloading rather than inhibiting vein unloading as was expected. The cause of these unexpected results is unknown at the present. However, it should be pointed out that these results were obtained using intact plants. Most work examining the effects of TIBA and NPA on carrier-mediated auxin efflux was obtained using tissue cultured cells, excised plant parts and membrane vesicles. It should be pointed out that these inhibitors probably have their effect on vein unloading by causing efflux at the phloem sieve elements. Sieve elements are different from tissue culture cells and most cells in excised plant segments since sieve elements do not have vacuoles and, so, may respond differently.

Nickel sulfate inhibits the activity of phosphoenol pyruvate carboxylase and the synthesis of malate (Morgutti et al., 1984). It is possible that the blockage of this enzyme would lead to an increase of the cytoplasmic pH which would decrease auxin efflux. An increase in cytoplamic pH could possibly result in a decrease of the activity of plasma membrane ATPase since it has been reported that the plasma membrane ATPase activity may be regulated to a certain extent by the pH of the cytoplasm (Romani et al., 1985). This decrease in ATPase activity could then increase the pH of the cell wall area and, thus, decrease vein loading. This implies that nickel sulfate might inhibit auxin vein loading and unloading. However, it was found that treatment with low concentrations of nickel sulfate slightly decreased vein

loading of 2,4,5-T but there was no other large effects of nickel sulfate on auxin translocation. The explanation for this result is unknown.

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APPENDIX

APPENDIX

DIFFERENTIAL ACTIVITY OF METABOLIC INHIBITORS ON PROTON EXCRETION MECHANISMS OF MEMBRANES

Inhibitors	ATPase on Plasmalemma	ATPase on Tonoplast	NADH oxidase Plasmalemma
DCCD	+	+	+
DIDS	-	+	-
DES	+	-	-
Quinacrine	-	-	+
Juglone	-	-	+
Vanadate	+	-	-

+ Inhibition
- No inhibition

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