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THE INFLUENCE OF BENTAZONE
ON
STOMATAL MOVEMENT

BY

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A thesis submitted to the Council of National Academic Awards in partial fulfilment for the degree of Doctor of Philosophy.

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December 1988

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Signed . . . K.J. Nichols . . . .
(Candidate)

Signed . Dr. A.H. Cobb . . . .
(Director of Studies)

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ABSTRACT

THE INFLUENCE OF BENTAZONE ON STOMATAL MOVEMENT

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KARIN J. NICHOLS B.Sc.

The herbicide, bentazone, was shown to alter stomatal movement in abaxial epidermal peels of several plant species. It was shown to have the ability to both enhance and depress stomatal movement, which appeared to be dependent on potassium concentration.

The plant species chosen for the major part of this investigation was Commelina communis L. and the stomatal responses in this species were characterised with respect to bentazone concentration, potassium concentration, light, darkness and pH. The effect of the non-toxic bentazone derivative 6-hydroxybentazone on stomata was also observed and showed that this small change in the molecule altered the activity sufficiently to prevent enhancement of stomatal opening.

A comparison was made between the effect of bentazone and IAA on stomatal movement. Both molecules were shown to be most active at pH 4 and an interaction was apparent, with respect to stomatal aperture, when the chemicals were applied together. Both bentazone and IAA were shown to be able to change the specific activity of malate dehydrogenase in abaxial epidermes. However, it was not clear whether these alterations were due to alteration of the ionic balance in the guard cells or direct action on the enzyme itself. Studies using the radioisotope ^{86}Rb, as a substitute for potassium, and some metabolic inhibitors suggested that bentazone and IAA were capable of altering potassium uptake and retention and that this may involve ATPase activity. Comparisons are made between the activities of bentazone and IAA and possible mechanisms of action are discussed.

PUBLICATIONS

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NICHOLS, K. J. and COBB, A. H. (1986). pH dependance of auxin-induced stomatal opening. Proceedings of the 5th Congress of the Federation of European Societies of Plant Physiology (Hamburg) 10, 15.

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LIST OF ABBREVIATIONS

ABA	–	Abscisic acid
AVG	–	Aminoethoxyvinyl glycine
BSA	–	Bovine serum albumin
CCCP	–	Carbonyl cyanide-m-chlorophenyl hydrazine
cpm	–	Counts per minute
2,4-D	–	2,4-dichlorophenoxyacetic acid
DCMU	–	3-(4-chlorophenyl)-1,1-dimethylurea
DES	–	Diethylstilbestrol
DNP	–	2,4-dinitrophenol
dpm	–	disintegrations per minute
IAA	–	Indol-3-yl acetic acid
IRGA	–	Infra-red gas analyser
MES	–	Morpholino ethanesulphonic acid
NAA	–	1, napthylacetic acid
NOXA	–	2, napthoxyacetic acid
OAA	–	Oxaloacetic acid
PAR	–	Photosynthetically active radiation
PEP	–	Phosphoenol pyruvate
PEPC	–	Phosphoenol pyruvate carboxylase
RUBPC	–	Ribulose-bis-phosphate carboxylase
2,4,5-T	–	2,4,5-trichlorophenoxyacetic acid

CHAPTER 1 INTRODUCTION

1.1 Significance and structure of stomata

When plants first began to colonize land, some 400 million years ago, only those with a hydrophobic waxy outer layer were able to avoid desiccation (Martin, Donkin and Stevens, 1983). This barrier however could not solve all the problems encountered by plants on dry land because although it prevented excessive water loss it would also reduce the supply of CO_2 to the plants. This problem was overcome by the development of stomata, which are small movable pores on the epidermis which provide the principal sites for gas exchange and water loss. These pores are highly sensitive to both internal and external environmental conditions and regulate the uptake of carbon dioxide and the loss of oxygen and water. They therefore exert considerable control over both photosynthesis and transpiration. The control of these two processes, in turn, will alter the rates of transport of compounds, both natural and synthetic, in both xylem and phloem and may alter the rates of uptake of pesticides such as herbicides and their subsequent movement within the plant.

Most typically stomata are found in the epidermes of leaves, green stems and modified leaves such as tendrils, although they may also be present on floral parts and on the inner and outer surfaces of pods and other fruits. Stomata are usually randomly scattered within the epidermis, however in some species such as *Saxifraga stolonifera* L. stomata are located in patches (Willmer, 1983). In most monocotyledons and in Gymnosperms stomata are located in rows (Willmer, 1983). There are also differences between species in relation to the ratio of stomata on each leaf surface. The majority of species tend to have more stomata on the lower leaf surface (abaxial) than on the upper leaf surface (adaxial), with the exception of some monocotyledonous plants, e.g. *Allium cepa* L. and *Hordeum vulgare* L., which have a fairly equal distribution between the two surfaces (Willmer, 1983). Many angiosperm tree species have no stomata on the adaxial leaf surface at all, e.g. *Corylus americana* L. and *Quercus triloba* L. (Willmer, 1983). Despite differences in distribution between species, the stomatal pores themselves are almost invariably surrounded by specialised cells known

as guard cells. There are two major types of guard cell, one type is characterised by kidney-shaped guard cells (Figure 1.1(a)) and stomata possessing these are called elliptical stomata. The other guard cell type is dumb-bell-shaped restricted to monocotyledonous species and are commonly known as graminaceous stomata (Figure 1.1(b)). Initial development of guard cells is identical in all plant groups (Martin et al, 1983). Guard cell walls are differentially thickened by cellulose microfibrils, the orientation of which is thought to be mediated by microtubules in the plasmalemma during development (Palevitz, 1981). The type of thickening varies with species and growth conditions to give guard cells of different shapes (Palevitz, 1981). In elliptical guard cells, such as those in Vicia faba L. and Allium cepa L. the shape is due to the thickening of the paradermal and ventral walls in a fan-like pattern that radiates from the pore (Figure 1.1(a); Martin et al, 1983). In the graminaceous type of guard cell the wall around the constricted middle of each cell is heavily thickened by cellulose microfibrils which are orientated axially to the pore (Figure 1.1(b); Palevitz, 1981). Apart from guard cells there may be other subsidiary cells in the stomatal complex. These cells are distinct from the epidermal cells (Figure 1.2) and may have a role in maintaining the turgor balance within the guard cell.

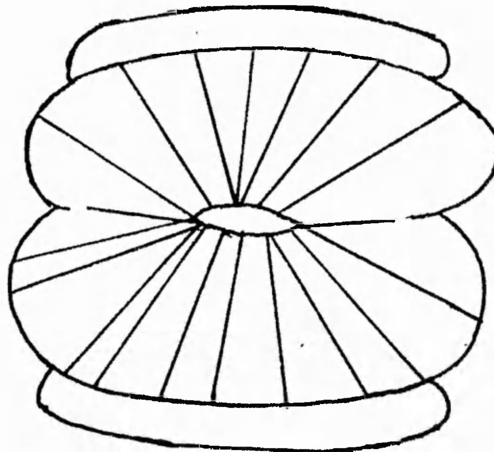
1.2 Stomatal movement

1.2.1 Mechanics of stomatal movement

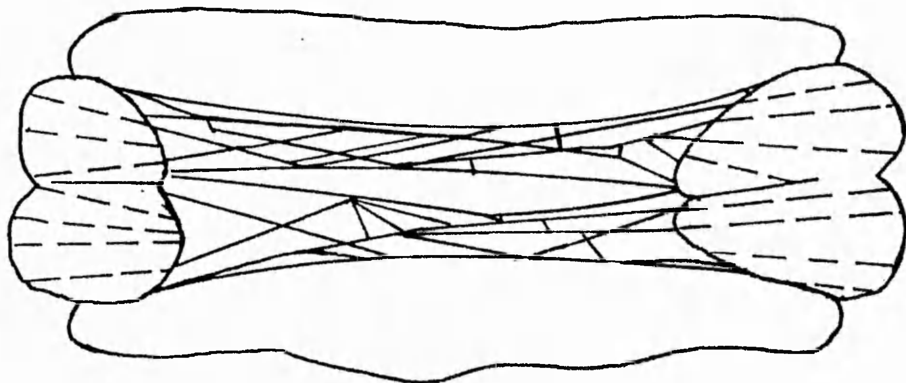
The degree of stomatal opening or closing is mediated by changes in the shape of the guard cells which surround the pore (Figure 1.3). The process of opening and closing the stomata is achieved by osmotic volume changes in the guard cells which results in differential expansion of cells as dictated by guard cell structure (Martin et al, 1983). In elliptical guard cells the radial orientation of the cellulose microfibrils allows expansion of the guard cells in a lengthwise direction (Figure 1.3(a)). Such a radial arrangement of the microfibrils will allow the cross-sectional shape to change but the cross-sectional area of the outer dimensions will remain almost constant (Willmer, 1983). Raschke (1975) suggested that opening is effected in the

Figure 1.1. STOMATAL TYPES SHOWING THE BASIC ARRANGEMENT OF CELLULOSE MICROFIBRILS

a) Elliptical Stomata



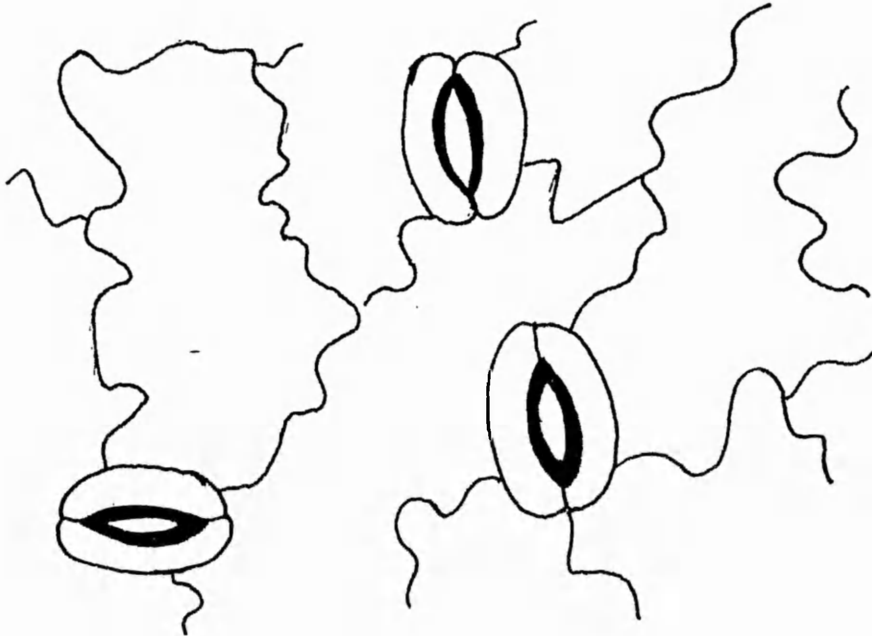
b) Graminaceous Stomata



(adapted from Palevitz 1981)

Figure 1.2. TYPES OF ELLIPTICAL STOMATA AND THEIR RELATION TO EPIDERMAL CELLS

a) Stomata without subsidiary cells (eg. *V. faba*)



b) Stomata with six subsidiary cells (eg. *C. communis*)

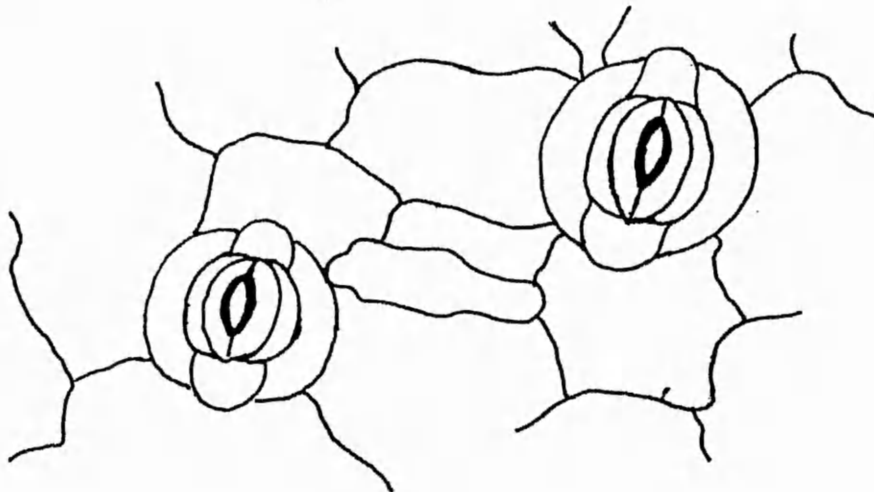
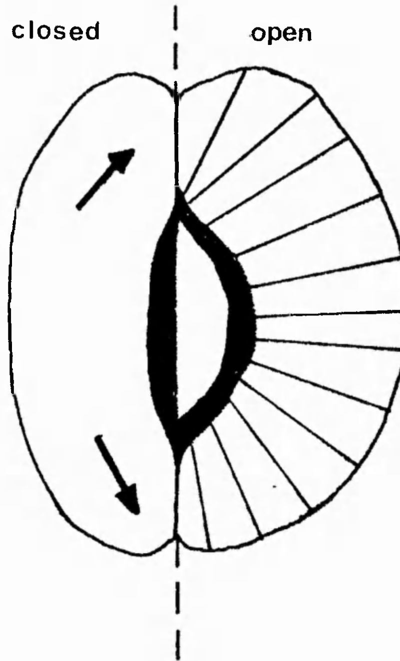


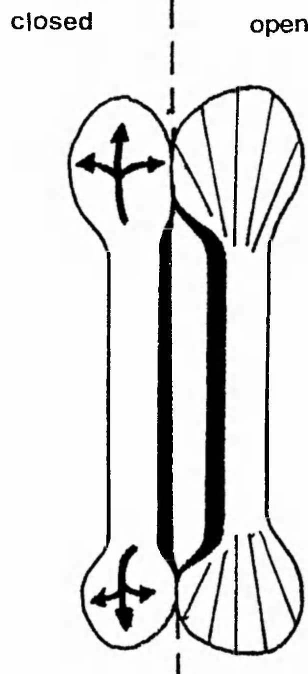
Figure 1.3. THE CHANGE IN SHAPE OF GUARD CELLS ON STOMATAL OPENING

a) Elliptical Stomata



(adapted from Martin *et al* 1983)

b) Graminaceous Stomata



(adapted from Martin *et al* 1983)

➞ - Direction of cell expansion during stomatal opening

following manner: the ends of the guard cells swell and push each other apart, and, since the length of the stomatal apparatus remains virtually constant, guard cell expansion occurs outwards into the surrounding epidermal or subsidiary cell. However, it is important to remember that the movements of guard cells are not only dependant on their own osmotic status but are modified by the turgidity of their surrounding cells (Martin et al, 1983). The degree of stomatal opening is the result of an osmotic imbalance between the guard cells and their adjacent cells (Spence, Sharpe, Powell and Rogers, 1983). This allows us to explain the ability of one guard cell to bend out even when its partner has been punctured, which was not possible under the explanation of Raschke (1975). It is also noticeable that when a cell adjacent to a guard cell is damaged, the complex is able to bend out even further to give a larger aperture as a result of the reduced pressure from the adjacent cell.

In graminaceous stomata, the central regions of each guard cell are extremely rigid, whilst the bulbous polar ends are comparatively thin walled and have radiating micellae (Figure 1.1(b)). As the volume of the guard cell pair increases the polar ends balloon-out and press against their opposite number (Figure 1.3(b); Raschke, 1975). The inflexibility of the centre of the guard cells results in a pore with parallel side (Willmer, 1983). The effects of subsidiary cells and epidermal cells on aperture are as discussed above for elliptical guard cells.

1.2.2 Proposed mechanisms of stomatal movement

Stomata are able to open and close to regulate water loss and the exchange of O_2 and CO_2. Many researchers have tried to discover the mechanism which allows this movement to occur, and to establish how it is regulated. Of the many hypotheses put forward no single one has been able to explain the mechanism and the final answer is likely to involve several of them. Almost all species, with the exception of *Paphiopedilum* sp. and *Pelargonium zonale* L. (Martin et al, 1983) possess chloroplasts within their guard cells, and with the exception of *Allium* sp.

(Schnabl and Zeiger, 1977), these chloroplasts contain starch. It was this starch which yielded the first hypothesis on the mechanism of stomatal movement, the 'starch-sugar' hypothesis of Lloyd (1908). This hypothesis was based on the observation that starch appeared to breakdown in the light when stomata opened and reappear on stomatal closure. Lloyd (1908) believed that the main cause of stomatal opening was an enzymatic digestion of starch to osmotically active sugars. These sugars therefore drew water into the cell in order to reinstate the osmotic balance. The water drawn into the cell increased the volume of fluids within the cell causing the guard cell to swell, initiating guard cell movement and increasing stomatal aperture. Stomatal closure in darkness was attributed to the removal of the osmotically active sugars by resynthesis of starch (Lloyd, 1908), thus altering the osmotic balance so that the concentration of sugars outside the guard cell was greater than that within and causing a net flow of water out of the guard cell. This therefore led to the guard cell returning to its original size and stomatal closure. Similar observations to Lloyd were made by Sayre (1926) who studied the stomatal physiology of Rumex patienta L. Further support was given to the 'starch-sugar' hypothesis by Alvim (1949) who found that there was a reduction in the stomatal response to light, buffers and high temperature in Zebrina spp. if plants had been 'starved', by long periods of darkness, to reduce the starch content of the plastids. For many years the starch-sugar hypothesis remained the most feasible explanation of stomatal opening, although it could not explain the movement of stomata whose guard cells possessed no chloroplasts or possessed chloroplasts which did not contain starch.

In 1967, however, Fujino found that when stomata opened on epidermal peels of Commelina communis L. large amounts of potassium were accumulated in the guard cells. He also noticed that the pH of the guard cells increased with opening in C. communis and Allium spp., although the significance of this was not understood for several years. Fujino (1967) carried out experiments with ATP and found that the utilization of ATP in

the light was increased by the addition of 10^{-3}M $CaCl_2$ and that in the presence of such a high level of $CaCl_2$ stomatal opening was reduced. He concluded that stomatal movement involved active potassium transport and that an ATPase was responsible for the excretion of potassium out of the guard cell. Since these observations it has been shown that potassium uptake is involved in the stomatal opening of many species including C. communis (Penny and Bowling, 1974; Jarvis and Mansfield, 1980; MacRobbie and Lettau, 1980), V. faba (Fischer, 1968; Pallaghy and Fisher, 1974; Schnabl, 1978), Zea mays L. (Pallaghy, 1971; Raschke and Fellows, 1971), Stachytapheta indica L. (Pemadasa, 1975), A. cepa (Schnabl et al, 1977; Schnabl, 1978), Mimosa pudica (Dayanandan and Kaufman, 1975), Ophioglossum engelmanni (Dayanandan et al, 1975), Ginkgo bilboa (Dayanandan et al, 1975) and Pinus sylvestris (Dayanandan et al, 1975). It was therefore established that potassium uptake into guard cells played a major role in the stomatal opening of most species. However, it was evident to workers that if potassium was being accumulated in vast quantities a balancing ion had to exist to maintain the charge balance across the membrane. This balance could be maintained either by a negative ion being imported alongside potassium or a positively charged ion being excreted while potassium was being accumulated. By far the most favoured choice for the balancing ion was chloride, however in V. faba only 30% of the potassium taken up was balanced by chloride ions (Fischer, 1968; Pallaghy et al, 1974). In C. communis, experiments with chloride-selective electrodes indicated active transport of chloride ions during both stomatal opening and closure which appeared energetically independant of potassium transport (Penny, Kelday and Bowling, 1971). MacRobbie (1982) also found that when chloride was available it was often the major balancing ion in C. communis, however, it did not account for the total balance. Similarly, in Z. mays, chloride was taken up but only at half the rate of potassium ions (Raschke et al, 1971). The only species where the charges of the potassium ions seemed fully compensated for by chloride ions was A. cepa, and thus it was suggested that in the majority of

plant species the role of starch was to deliver organic counter ions for imported potassium ions, therefore reducing the need for chloride uptake (Schnabl et al, 1977). It was therefore clear that although chloride had a role to play in stomatal movement other ions were also involved. In 1973 Raschke and Humble carried out experiments on the stomata in epidermal peels of V. faba using pH electrodes and discovered that as the stomata opened the media became more acidic. They concluded that no uptake of anions was required by opening stomata as the guard cells could release protons to balance the charges. Outlaw (1983) demonstrated that guard cell potassium uptake from the apoplast could be mediated by a proton-extruding ATPase situated on the guard cell plasmalemma. One of the most feasible sources of these protons are the organic acids such as malate and citrate which can be detected in guard cells. Indeed, the majority of CO_2 fixed by guard cells (about 60%) was found as malate (Birkenhead and Willmer, 1984). Most of the CO_2 fixation by guard cells is mediated by phosphoenol pyruvate carboxylase (PEPC, 4.1.1.31) however there is a low level of ribulose-1,5-bis phosphate carboxylase (RUBPC E.C.4.1.1.39) present which may fix very small levels of CO_2 (Willmer, Pallas and Black, 1973). In protoplast studies where the guard cell protoplasts swell in response to stimuli which would normally cause stomatal opening, only PEPC has been shown to be functional (Schnabl, 1981). It is therefore possible that PEPC fixation of CO_2 is the process which provides a high percentage of the malate, the concentration of which many workers have been able to correlate with stomatal aperture (Pearson, 1973; Pearson and Milthorpe, 1974; Outlaw and Lowry, 1977; Schnabl, 1980a; Reddy, Rao and Raghavendra, 1983). Pearson (1973) was also able to calculate that the differences in minimum and maximum malate concentrations were sufficient to balance potassium changes found to occur by other authors on the opening of V. faba stomata. Radio label studies have also shown that about 22% of the malate can be fixed into guard cell starch on stomatal closure (Dittrich and Raschke, 1977; Schnabl, 1980b), and Mansfield (1983) states that there is little doubt that the starch in the guard cells of many

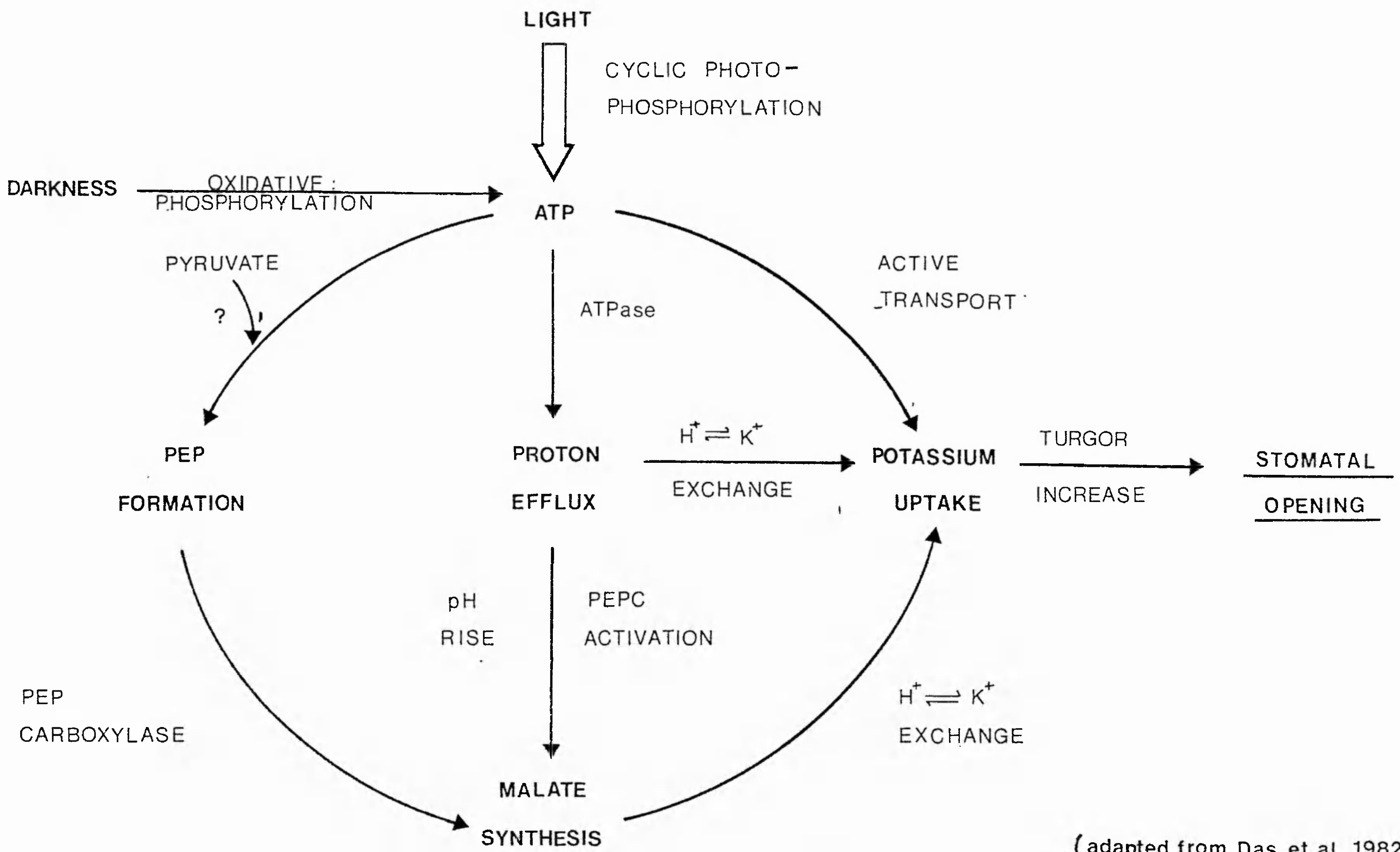
species is the source of carbon skeletons for malate production. Stomatal movement therefore involves many interrelated processes including: potassium uptake, chloride uptake, proton efflux, starch breakdown, ATPases and malate production, (Figure 1.4) and controls exerted at any of these points are likely to alter stomatal function.

1.2.3 Methods of measuring changes in stomatal aperture

There are several methods of measuring stomatal movement, some of which give direct aperture measurements and others which allow estimates of stomatal aperture to be made from epidermal or leaf conductance values.

1.2.3.1 Methods of determining stomatal aperture on intact leaves: Direct microscopic measurements on intact leaves are difficult because of the reflection of light from waxy leaf surfaces and, in the case of thick leaves, minimal amounts of light being transmitted through the leaf (Meidner, 1981). Some data was obtained by Stalfelt (1959) who immersed the leaf in liquid paraffin to obtain clearer images, and reduce the influence of changes in CO_2 concentration and loss of H_2O vapour which would alter stomatal measurement. More recently measurements have been obtained using sophisticated digital image-processing equipment coupled to a microscope (Omasa, Hashimoto and Aiga, 1983; Omasa and Onoe, 1984). These measurements are more detailed than those obtained by earlier workers and have the advantage of not damaging the leaves. However, the methodology is complex and requires a great deal of expensive equipment and thus can only be used in a laboratory situation. As a result of the many difficulties in direct measurement the majority of observations on stomatal movement on intact leaves are carried out employing conductance methods using porometers. Porometric investigations do not destroy the leaves, although they may temporarily alter leaf functions (Meidner, 1981). The main advantage of porometers is that they provide quantitative measurements

Figure 1.4. A SUMMARY OF THE CONTEMPORARY OUTLOOK OF EVENTS LEADING TO STOMATAL OPENING



(adapted from Das *et al* 1982)

of the collective conductance of several thousand stomata together, however, any epidermal or cuticular conductance is also included in the measurement (Meidner, 1981). There are two major types of porometers, mass flow porometers and transpiration (diffusion) porometers, although there are many adaptations of each model. Mass flow porometers measure the mass flow of a gas mixture. usually air, through the leaf, whilst transpiration porometers measure the rate of diffusion of water vapour out of the leaf into a known external atmospheric humidity (Meidner, 1981). In practice only leaves with stomata on both adaxial and abaxial surfaces can be used with mass flow porometers (Meidner, 1981), and unless leaf discs are used there is an unknown variable area of stomata outside the attachment which are joined by the mesophyll. These can offer resistance to air flow depending on the state of stomatal opening. However, if suitable leaves are used mass flow porometry can give valid records of stomatal movements in response to stimuli and also provide an insight into interactions between stimuli and their effects on stomata, providing the results are suitably corrected (Meidner, 1981). The transpiration porometer of most value is the steady-state transpiration porometer (Meidner, 1981). In this apparatus the air flow over the leaf is adjusted so that its vapour density remains constant at a predetermined level (Beardsdell, Jarvis and Davidson, 1972). This prevents alteration of stomatal aperture caused by a build-up of water vapour or CO_2 while the measurements are being taken. Water vapour loss can also be measured by water-IRGA's, these are infra-red gas analysers which are extremely sensitive to changes in water vapour concentrations. For these to be used effectively the leaf has to be sealed in an airtight chamber so that the water vapour changes can only be attributed to diffusion out of the leaf. The gas around the leaf is sampled regularly and the water vapour release calculated from readings obtained from

the calibrated IRGA scale. The advantage of using this system is that several leaves can be monitored at the same time by switching the sampling from chamber to chamber. Also other gas analysers such as CO_2 IRGA's can be included to measure changes in the gases around the leaf, and this allows processes such as photosynthesis and transpiration to be measured simultaneously. However, it should be noticed that none of these indirect measurements can totally exclude interference from epidermal and cuticular diffusion of water vapour.

1.2.3.2 Methods of determining stomatal aperture from the leaf epidermis: The majority of studies on stomatal movement in past years have used epidermal peels. One advantage of using these is that mesophyll effects are removed and the responses observed are due to epidermal processes alone. However, this technique is limited to a few species which peel easily and cleanly, and in which peeling does not cause extensive damage. Willmer and Mansfield (1968) stated that if consistent responses to environmental factors such as light and CO_2 can be regarded as indicating normal stomatal function, only *Commelina* spp. are suitable, although *V. faba* peels also give fairly consistent results. Apart from functional epidermal peels, peeling methods have been used to attempt to show the aperture of plants under field conditions. Lloyd (1908) peeled the leaves of plants in the field and fixed them in absolute alcohol, however, it has since been shown that this yields very unreliable results (Meidner, 1981). Peels have also been used as a means of isolating guard cells by both micro-surgery (Outlaw *et al*, 1977) and low pH treatment (Squire and Mansfield, 1972), and as a starting material for guard cell protoplast production, reducing mesophyll protoplast contamination considerably (Fitzsimons and Weyers, 1983).

The final method of measuring stomatal aperture is by micro-relief techniques. In these a compound such as collodion or paraffin wax is applied to the leaf surface and as it sets it moulds itself into the surface shapes. This can then be removed providing a permanent record of the state of stomatal opening. However, this method is not suitable for all species and can fail to show changes in the relevant pore dimensions. For example, if a stoma possesses a ridged outer lip on the guard cell this will show up in detail and will often mask the actual pore. In most cases the measurements of the distance between ridges do not compare to pore size and can lead to false aperture determinations. This method may be of use for plant classification but it has not contributed markedly to the study of plant physiology (Meidner, 1981).

1.3 Influence of the environment on stomatal movement

1.3.1 Light

Stomata are known to respond to light, and this response may differ with light intensity, wavelength and species. At low light intensities stomatal opening is most sensitive to blue light of 400-450 nm (Hsiao, Allaway and Evans, 1973; Mansfield, 1983). This would suggest that at low light intensities photosynthesis is not the underlying process in stomatal movement as photosynthesis would also occur in red light. At higher quantum flux densities uptake of potassium and opening of stomata also respond to red light (600-680 nm) and slightly to green light with a minimum at 540-560 nm (Hsiao *et al*, 1973; Lurie, 1978). This pattern of wavelengths may indicate a possible involvement of photosynthesis as photosynthetic pigments absorb in both the red and blue regions of the spectrum, but hardly at all in the green. Blue light stimulation of stomatal opening has been demonstrated in several species, including *Phaseolus vulgaris* L. (Goring, Koshuchowa, Munnich and Dretrich, 1984), *C. communis* (Roth-Bejerano and Itai, 1981; Morison and Jarvis, 1983;

Inoue, Noguchi and Kubo, 1985), V. faba (Lurie, 1978; Shimazaki, Iino and Zeiger, 1986) and Paphiopedilum (Sheriff, 1979), and may be of particular value in dawn opening where the light intensity is low. The blue light effect is independant of CO_2 (Sheriff, 1979; Mansfield, 1983; Roth-Bejerano, Nejidat and Itai, 1985). In some species from the Gramineae and Cyperaceae the response to blue light appears different from that found in other species (Johnsson, Issaias, Brogardh, Johnsson, 1976). In these plant families there appeared to be a two phase response to blue light, an initial rapid opening response (within 2 minutes) followed by a slower opening response (after 5-10 minutes). The first response was CO_2-insensitive and the second CO_2-sensitive. It was suggested that the rapid response was due to special anatomy and/or function in stomata of plants from these two genera. All species showed the same slow response to red light (Johnsson et al, 1976).

The way in which stomata sense light and the mechanism of the response is poorly understood. Initially, it was thought that the chloroplasts played a major role in sensing light and that the response was mediated by photosynthesis. However, this cannot be the whole answer as guard cells containing no chloroplasts, and stomata on plants with a chlorophyll deficiency induced by SAN 9789, still respond normally to blue light, although the response to red light is weakened (Karlsson, Hoglund and Klockare, 1983). The stomatal response to red light is easier to correlate with photosynthesis as it is reduced by chlorophyll deficiency (Karlsson et al, 1983) and by the addition of the photosystem II inhibitor DCMU, (3-(4-Chlorophenyl)-1,1-dimethyl urea; Lurie, 1978; Inoue et al, 1985). As blue light responses are unaffected by either treatment it is unlikely that the effect of blue light is due to a change in thylakoid function. Blue light may have its effect by acting at a specific blue light receptor site and at least three blue light receptors are thought to exist in plant membranes (Briggs and Iino, 1983). These three receptors have been distinguished on the basis of action spectra and other criteria. One is tentatively identified

as a flavoprotein-cytochrome complex and probably located in the plasma membrane. The second class may be a flavin or carotenoid, and the third group can only be distinguished by an action spectrum which shows a single sharp peak near 475 nm and seems unlikely to be either flavin or carotenoid (Briggs et al, 1983). However, much more work is required in the area of blue light responses and receptors.

Another possible light sensor may be phytochrome as far-red light can reverse red light-induced stomatal opening (Roth-Bejerano et al, 1981; Goring et al, 1984) and the treatment of tissue with filipin, a chemical which interferes with phytochrome binding at the membrane, reduces stomatal opening (Roth-Bejerano et al, 1985). If phytochrome is the light sensor it is proposed that blue light has its effect due to its fluorescence in the red region, and that the effect of inhibitors is to increase the fluorescence in the far-red region, therefore reducing stomatal opening (Roth-Bejerano et al, 1985). However, it should be noted that blue light can cause stomatal opening in the absence of chlorophyll (Karlsson et al, 1983) and in the absence of chloroplasts (Sheriff, 1979) where there is little blue light-induced fluorescence in the red region. Thus, phytochrome on its own does not provide a satisfactory answer. It may be that photosynthesis, phytochrome and specific blue light receptors all have a role to play in light-induced stomatal movement.

1.3.2 Carbon-dioxide (CO_2)

The effect of CO_2 on stomata is governed by its concentration and can influence the response of stomata to other factors. High concentrations of CO_2 tend to cause stomatal closure (Meidner and Heath, 1959; Pallas, 1965; Pallaghy, 1971; Travis and Mansfield, 1979; Blackman and Davies, 1984; Morison, 1985) and low CO_2 concentrations stimulate stomatal opening (Meidner et al, 1959; Zelitch, 1965; Pallaghy, 1971; Morison, 1985). There is no real understanding of the way in which CO_2 concentration may control stomatal movement. It may be a metabolic effect as PEPC is present in the guard cells and therefore

CO_2 can be fixed into oxalo acetate which could be a source of the malate required for opening. However, if this were the case it would be expected that increasing CO_2 concentration would cause opening and not closure (Mansfield, 1983). In the presence of fusicoccin, a fungal toxin which induces stomatal opening (see 1.5.1 for further details), CO_2 had the reverse of the usual effect, i.e. increasing CO_2 concentration caused opening (Travis _et al_, 1979; Mansfield, 1983). Fusicoccin also allowed the malate level to increase to a level greater than that found under low CO_2 conditions (Travis _et al_, 1979). It is therefore thought that there may be two mechanisms of CO_2 action, one of which is dominant under natural conditions and masks the other. The first is the major action of CO_2 on potassium accumulation in guard cells which is inhibited by increasing CO_2 concentration, and the second is the ability of CO_2 to improve the supply of malate (Travis _et al_, 1979). Mansfield (1983) proposed that fusicoccin-stimulated proton efflux could override the effect of CO_2 on potassium accumulation and therefore placed demands on the capacity of the guard cell to form malate in order to balance the incoming potassium. Therefore, in the presence of fusicoccin, increasing CO_2 concentration leads to increased opening indicating that the primary effect of CO_2 is on ion transport mechanisms. This is further supported by the fact that CO_2 has a weaker effect if potassium ions are replaced by sodium ions (Jarvis and Mansfield, 1980). CO_2 responses are observed in species which do not possess chloroplasts and therefore photosynthesis is not a pre-requisite of stomatal sensitivity to CO_2 (Sheriff, 1979). The effects of CO_2 can be reduced by cytokinins (Blackman _et al_, 1984), IAA (Snaith and Mansfield, 1982; Eamus and Wilson, 1984), and low temperature (Eamus _et al_, 1984), however, in most cases the addition of ABA restores sensitivity (Snaith _et al_, 1982; Blackman et al, 1984).

CO_2 appears to be "sensed" by the inner surface of the guard cells, since if stomata are closed they do not open when external CO_2 is reduced, however, if CO_2-free air is forced through the intercellular spaces of a leaf with almost closed

stomata, opening is readily induced (Mansfield, 1983). It has also been shown that other gases such as allene (C_3H_4), nitrous oxide (N_2O) or ethylene do not compete for a possible CO_2 receptor site (Pallaghy and Raschke, 1972). It therefore seems likely that CO_2 has its effect on membrane permeability, although neither the mechanism nor receptor site have been satisfactorily identified.

1.3.3 Humidity and water stress

Stomata play a major role in controlling the water status within the plant, and they respond to both internal and external water potentials. In general, stomata close when plants are water stressed so that water is conserved. This closure is rapid, and it was originally thought that it was induced by a build up of ABA under stress conditions (Sheriff, 1979; Mansfield, 1983), which was either a result of ABA production in the guard cells or release of stored ABA from guard cell chloroplasts (Sheriff, 1979). However, the response to water stress is rapid, occurring in a matter of minutes, and it is now considered that the initial decrease in stomatal aperture is due to either low leaf turgor or the lowering of the leaf water potential, primarily through the adjustment of osmotic potential (Das and Raghavendra, 1982; Mansfield, 1983), although ABA may be required to maintain closure. Plants which have been preconditioned to water stress by several short periods of water shortage respond more quickly and reduce the risk of serious water deficits within the plant (Brown, Jordan and Thomas, 1976; Davies, 1978). This drought conditioning may come about by increased levels of ABA being present in the guard cells (Mansfield, 1983) or may be due to adjustment of the osmotic potential within the guard cell (Brown _et al_, 1976; Levy, 1983).

Humidity also has a major role to play in maintaining the water balance within the plant by exerting a control over the stomata. When humidity is low, thus favouring water loss along a concentration gradient, the stomata close to prevent desiccation of the plant (Das _et al_, 1982; Mansfield, 1983; Ball and Farquhar, 1984) and open when humidity is high

(Sheriff, 1979; Das et al, 1982; Mansfield, 1983). The effect of humidity on stomata may be direct or indirect. The stomata of several species respond directly to humidity in a "feed-forward" manner, i.e. local water deficits develop in the stomatal apparatus which induces stomatal closure before deficits occur in the remainder of the leaf (Sheriff, 1979; Das et al, 1982). In these species, the exact mechanism is not identified but the remarkable degree of isolation between the stomatal apparatus and the rest of the leaf allows water potential gradients to develop (Sheriff, 1979; Das et al, 1982). In the majority of species, however, it is suggested that decreasing humidity leads to increased evaporation from the epidermis and that the stomata respond to the consequent fall in water potential (Sheriff, 1974; Sheriff, 1979; Mansfield, 1983). However, Raschke and Kuhl (1969) failed to find a significant humidity response in Z. mays and concluded that guard cell movement was coupled to water supply, and only indirectly to atmospheric moisture by negative feedback of transpiration on the water potential in the water conducting system.

1.3.4 Temperature

In many species, such as, Glycine max L., Triticum aestivum L., P. vulgaris, Gossypium hirsutium L. and Eucalyptus regnans F. Muell., stomatal apertures increase with temperature up to 36°C, although in two cool-climate species Pisum sativum L. and V. faba the optimum aperture is observed between 27°C and 30°C, and at higher temperatures aperture decreases slightly (Hofstra and Hesketh, 1969). As temperature increases over the range 15°C to 40°C the speed of stomatal opening can also be seen to increase (Hofstra et al, 1969). At temperatures between 15°C and 35°C, CO_2 assimilation can be shown to be proportional to stomatal aperture, indicating that CO_2 has a controlling influence. At temperatures above 35°C, aperture continues to rise but the stomata lose their ability to respond to CO_2 and any correlation between CO_2 assimilation and aperture is also removed (Raschke, 1970; Spence, Sharpe, Powell and Wu, 1984). The influence of temperature on ion-stimulated stomatal opening

is similar to that on other processes that are dependant on metabolism (Willmer and Mansfield, 1970). It may, however, be argued that the response to temperature reflects stresses on the plant. In plants kept under high temperatures, water supply may become limiting due to increased evaporation, and this may lead to the observed closure at temperatures greater than 40°C. At such temperature photorespiration may also occur, providing light and O_2 levels are relatively high, causing increases in intercellular CO_2 concentration which again leads to stomatal closure (Zetlitch, 1965; Sheriff, 1979). The response of stomata to temperature, therefore, does not appear to be direct, but is likely to be due to changes in metabolic rate, water status and intercellular CO_2.

1.4 Influence of endogenous plant growth regulators on stomatal movement

1.4.1 Abscisic acid (ABA)

Under normal conditions, ABA causes stomatal closure in many species, including epidermal fragments of *Z. mays* (Rodriguez and Davies, 1982; Blackman and Davies, 1983), epidermal peels of *C. communis* (Jarvis *et al*, 1980; MacRobbie, 1981; Wilson, 1981; Pemadasa, 1982; Blackman *et al*, 1983), epidermal peels of *Commelina benghalensis* L. (Das, Rao and Raghavendra, 1976), epidermal peels of *Tridax procumbens* L. (Das *et al*, 1976), epidermal peels of *V. faba* (Horton, 1971; Davies, 1978; Gepstein, Jacobs and Tiaz, 1982/83; Grantz, Ho, Uknes, Cheesman and Boyer, 1985), *P. vulgaris* leaves (Eamus *et al*, 1984), *Oryza sativa* L. leaves (Henson, 1983), *Xanthium pennsylvanicum*. Wall. leaves (Jones and Mansfield, 1970) and in epidermal peels of *A. cepa* (Schnabl, 1978). However, at low temperatures (around 5°C) this ABA effect is reversed and ABA stimulates opening which is accompanied by potassium uptake (Rodriguez *et al*, 1982; Eamus *et al*, 1984). There are some reports indicating that ABA-induced closure is dependant on the presence of CO_2 (Snaith *et al*, 1982; Eamus *et al*, 1984), however it should be noted that once ABA-induced closure has been established CO_2-free air

does not relieve it (Jones et al, 1970). Another possible requirement for ABA-induced closure is the free movement of Ca^{2+} ions across the cell membrane (DeSilva, Hetherington and Mansfield, 1985). The presence of calmodulin antagonists (e.g. Lanthanum, verapamil and nifedipine) reduced the ABA response significantly, as did the addition of ethyleneglycol-bis (- amino ethyl ether) N, N, N^1, N^1-tetra acetic acid (EGTA), a chelating agent which removes calcium from the apoplastic space (DeSilva, Cox, Hetherington and Mansfield, 1985; DeSilva et al, 1985). These findings are consistent with the hypothesis that ABA increases the permeability of the guard cell plasma membrane to Ca^{2+} which may then operate as a secondary messenger to regulate ionic fluxes (DeSilva et al, 1985).

The actual mechanism of ABA-induced closure is not fully understood. It would appear that metabolic energy is required as azide inhibits ABA-induced solute loss (Weyers, Paterson, Fitzsimons and Dudley, 1982). Some workers argue in favour of ABA inhibition of ion uptake as the cause of stomatal closure (Horton and Moran, 1972; Weyers and Hillman, 1980; Das et al, 1982; Gepstein et al, 1982/83), whilst others favour ABA stimulation of ion efflux as the mechanism (Kasamo, 1981; MacRobbie, 1981; Mansfield, 1983). MacRobbie (1981) claimed that the stimulation of ion efflux was transient and was not due to an increase in the "leakiness" of the membrane, a concept favoured by others (Dittrich and Mayer, 1983). A totally different mechanism for ABA-stimulated closure was offered by Raghavendra, Rao and Das (1976) who suggested that its effect was that of inhibiting cyclic photophosphorylation in guard cell chloroplasts and also blocking organic acid formation within the guard cell. These conclusions may however be due to the buffers used in the experiment which were phosphate and Tris buffers, the latter being known to uncouple photosynthesis (Good and Izawa, 1972). Furthermore, ABA may exert its effect via an interaction with cytokinins (Cooper, Digby and Cooper, 1972; Das et al, 1976; Blackman et al, 1984) and IAA (Pemadasa, 1982; Mansfield, 1983).

1.4.2 Auxin (IAA)

IAA is most widely studied for its role in cell wall elongation in young tissues, in which it is thought to activate a vanadate-sensitive plasma membrane-bound electrogenic ATPase proton-pump (Brummer, Potrykus and Parish, 1984; Evans, 1985). Similar proton pumps may also be associated with the IAA-stimulated uptake of potassium ions from the xylem (DeBoer, Katou, Mizuno, Kasima and Okamoto, 1985). As the stomatal system is thought to possess a proton pump which is responsible for potassium uptake, it is possible that IAA may have an effect on stomata. However, some early work on barley leaves showed that application of IAA had no effect on stomatal opening or transpiration (Livne and Vaadia, 1965). Later, Govil (1985) was able to demonstrate IAA-stimulated opening in epidermal peels of *C. communis* at 10^{-3}M IAA, a high concentration with respect to endogenous levels which are around 10^{-7}- 5 x 10^{-5}M (Goodwin and Mercer, 1983). Pemadasa (1982) also showed that IAA was able to eliminate the normal disparity between adaxial and abaxial stomata with respect to both opening and potassium accumulation. It is thought that IAA may effect the transport of potassium either directly or through its control of the proton pump. Pemadasa (1982) postulates that the inherent disparity between abaxial and adaxial stomata is a result of differences in endogenous IAA levels between the two epidermes and/or of differential sensitivity to IAA, and this is why the addition of IAA can relieve the disparity. IAA also has the ability to increase malate production in adaxial stomata (Pemadasa, 1982) and to reduce the response of all stomata to CO_2 (Snaith *et al*, 1982). IAA can also promote opening during the night phase, although this opening is not as high as that induced by light (Snaith and Mansfield, 1985).

It appears therefore that there may be a role for IAA in the control of stomatal movement, but more research is required to elucidate its action on stomata.

1.4.3 Cytokinins

Cytokinins have been shown to enhance stomatal opening in *Avena sativa* L. (Biddington and Thomas, 1978), *C. benghalensis*, *T. procumbens* (Das *et al*, 1976), *P. vulgaris* (Goring *et al*, 1984), *Anthephora pubescens* Nees (Incoll and Whitelam, 1977; Jewer and Incoll, 1980), *Kalanchoe daigremontiana* Hamet et Perr (Jewer and Incoll, 1981), *V. faba* (Wardle and Short, 1981), *Hordeum* spp. (Livne *et al*, 1965) and *Helianthus annus* L. (Kuraishi, Hashimoto and Shiraishi, 1981). In *Z. mays*, although zeatin and kinetin did not promote opening, they did alleviate ABA-induced closure of the stomata (Blackman and Davies, 1983) and also reduced CO_2 control of stomatal movement in the absence of ABA (Blackman *et al*, 1984). A significant interaction between kinetin and ABA was also reported in barley (Cooper *et al*, 1972), although there was no interaction of these two growth regulators on the stomata of *C. communis* (Blackman *et al*, 1983). Several workers have suggested that synthetic cytokinins, such as benzyladenine, have the greatest affects on stomata (Das *et al*, 1976; Biddington *et al*, 1978; Jewer *et al*, 1980; Das *et al*, 1982), however this may only reflect the fact that the naturally occurring cytokinin used, e.g. zeatin, was not endogenous to the species being studied. The mechanism of the action of cytokinins on stomata is unknown at the present time.

1.4.4 Ethylene

Ethylene is the only gaseous plant growth regulator that is known to be endogenous to most plant species. The response of stomata to ethylene seems to vary between species. Ethylene has little or no effect on the stomata of *P. vulgaris*, *Sedum pachyphyllm* L. (Madhavan, Chrominski and Smith, 1983), *Z. mays* and *P. sativum* (Pallaghy *et al*, 1972), although under conditions of dryness, chemical stimulation of ethylene production in *P. vulgaris* did lead to an inhibition of stomatal closure which could be reversed by spraying with aminoethoxyvinylglycine (AVG), an ethylene synthesis inhibitor (Elstner, Keller and Paradies, 1983). Exposure to ethylene concentrations between 60 and 70 ppm

caused increased stomatal closure within 12 hours in both tomato and carnation, however recovery was complete within 96 hours of treatment (Madhavan _et al_, 1983). It may be that ethylene has an effect on the stomata of certain plant species, and that this effect may depend on the condition of the plant and the surrounding environment.

1.4.5 Gibberellins

Little information exists on the effects of gibberellic acids on stomatal movement. However, it has been reported that gibberellin enhanced stomatal opening in leaf discs of _P. vulgaris_ (Goring _et al_, 1984) and on the leaves of _V. faba_ (Horton, 1971). Gibberellin application also stimulated transpiration in barley (Livne _et al_, 1965) once more indicating an increase in stomatal aperture. There are no reports of gibberellin activity on epidermal peels, and where this has been studied no effects have been observed (Horton, 1971).

1.5 The influence of other chemicals on stomatal movement

1.5.1 Fusicoccin

The phytotoxin fusicoccin is produced by _Fusicoccum amygdali_ Del. and chemically is a glucoside of a carbotricyclic terpene with a molecular weight of 680 (Graniti and Turner, 1970). This chemical has been shown to stimulate the electrogenic activity of xylem pumps and to stimulate uptake of potassium from the xylem (DeBoer _et al_, 1985). It also stimulates stomatal opening and transpiration in _Cornus florida_ L., _Phytolacca americana_ L., _P. vulgaris_, _Nicotiana tabaccum_ L., _Sorghum bicolar_ L., _Cucumis sativus_ L., _Medicago sativa_ L. (Graniti _et al_, 1970), _C. benghalensis_ (Reddy _et al_, 1983) and _C. communis_ (Squire and Mansfield, 1974; Travis _et al_, 1979; Snaith _et al_, 1985). In coleoptiles of _Z. mays_ fusicoccin exerts its effect by stimulating a potassium/hydrogen ion exchange pump (Nelles, 1978) of the type which is also thought to exist in the stomatal complex. In stomata, fusicoccin stimulates the uptake of potassium into the guard cell and is able to overcome environmental

conditions which would normally inhibit stomatal opening (Squire et al, 1974; Travis et al, 1979; Das et al, 1982). Fusicoccin may have effects other than those at the potassium/hydrogen ion exchange pump as it also stimulates an increase in malate and sugars, and increases starch hydrolysis within the guard cell (Travis et al, 1979; Reddy et al, 1983; Snaith et al, 1985). It is likely that fusicoccin has an effect on all these processes, however the effect at the ion pump may be the initial cause of fusicoccin-stimulated opening as this can occur within one minute of exposure to low levels of fusicoccin (Squire et al, 1974).

1.5.2 Gaseous pollutants

Stomata are the principal sites for gas exchange and are thus prime targets for the action of gaseous pollutants. One of the major gaseous pollutants is sulphur dioxide (SO_2), which is taken up into the plant through the stomata (Willmer, 1983; Treshaw, 1984). The amount of SO_2 entering the plant is regulated by stomatal aperture, since when stomata are open the most damage is observed (Mansfield and Majernik, 1970; Willmer, 1983; Treshaw, 1984). SO_2 however, may control its own uptake to some extent as it can alter the stomatal aperture. At low concentrations of SO_2, stomatal aperture and transpiration are increased, whereas at high SO_2 concentrations stomatal closure is observed (Kimmer and Kazlowski, 1981; Willmer, 1983; Treshaw, 1984). It is thought that low concentrations of SO_2 damage epidermal cells allowing hydropassive stomatal opening, whilst higher SO_2 concentrations cause severe ultracellular disorganisation and damage the guard cells causing closure (Mansfield et al, 1970; Willmer, 1983; Treshaw, 1984). Plants containing higher levels of ABA or that have the ability to detoxify, e.g. certain varieties of Populus tremuloides L., suffer less damage and can recover within 24 hours of being exposed to SO_2 (Kimmer et al, 1981; Treshaw, 1984).

The response of stomata to ozone (O_3) is usually closure, although it is not known whether this is due to a direct effect on stomata or due to inhibition of photosynthesis leading to a

build up of CO_2 levels in the leaf (Willmer, 1983). Again stomata appear the major sites of uptake and plants with closed stomata, lower stomatal frequency and higher ABA levels tend to be least damaged (Willmer, 1983; Treshaw, 1984). There are, however, exceptions to this as it is impossible to relate the sensitivity of P. vulgaris to any of the above criteria (Treshaw, 1984).

Hydrogen fluoride can cause stomatal closure, however this can be alleviated after 24 hours in clean air, again the amount of uptake can be related to stomatal aperture (Treshaw, 1984). There are some workers however who find no effect of hydrogen fluoride at concentrations which occur normally in industrial areas (Bonte, Bonte and DeCormis, 1983). NO_2 also causes a decrease in transpiration which is thought to be related to a decrease in photosynthesis, however, NO has no reported effect on stomata (Willmer, 1983). Although plants react to individual pollutants, it is well known that they react more strongly to pollutant mixtures, often called photochemical smogs (Willmer, 1983). The use of pollutant mixtures may therefore increase our understanding of how pollution as a whole affects stomata.

1.5.3 Synthetic auxins and auxin-type herbicides

The synthetic auxins 2-napthoxyacetic acid (NOXA) and 1-napthyl acetic acid (NAA) have been observed to cause stomatal closure in epidermal peels (Zelitch, 1965; Mansfield, 1967; Pemadasa, 1979). The closure caused by NAA was very similar to that caused by ABA (Snaith et al, 1984; DeSilva et al, 1985) except in that it did not appear dependant on calcium ions (DeSilva et al, 1985) and that closure caused by it could be reversed by flushing the intercellular spaces with CO_2-free air (Mansfield, 1967). The herbicide 2,4-Dichlorophenoxyacetic acid (2,4-D) also caused stomatal closure (Zelitch, 1965; Mansfield, 1967) which could not be reversed by CO_2-free air, suggesting a more direct action on the guard cell (Mansfield, 1967). 2,4,5-trichlorophenoxyacetic acid (2,4,5-T) was able to reverse normal

stomatal movements in Carrisa spinarum L., Gymnosporia emarginata Laws, Maba buxifolia Cl., Flacourtia sepiana Roxb, Dodonaea vicosa L. and Randia malabarica Lamk (Rao, Swamy and Das, 1977). In these species transpiration rates and stomatal apertures were significantly enhanced by 2,4,5-T leading to desiccation (Rao et al, 1977). Another halogenated acetic acid, monoiodoacetic acid, also altered stomatal movement by inhibiting potassium excretion and therefore stomatal closure in both light and darkness (Fujino, 1969). Synthetic and auxin-type herbicides are therefore capable of altering stomatal movement although they may not all have similar modes of action.

1.5.4 Herbicides

Apart from the auxin-type herbicides, members of several other herbicide groups have been shown to alter stomatal movement. Triazine and urea-type herbicides were shown to stimulate stomatal opening within 2 hours in plants of Amaranthus viridis L., Digera arvensis Forsk, Boerhaavia diffusa L., B. repanda Willd., C. benghalensis, Cyperus rotundus L., P. sativum, Pennisetum typhoides Stapf and Hubb (Das and Santakumari, 1975). However, atrazine was shown to produce a 50% closure of stomata in sprayed leaves of P. vulgaris (Zelitch, 1965). He considered that this reflected an action at the cell membrane as well as on photosynthesis. Rees (1986) showed that the major function of atrazine was as a potent and rapid inhibitor of leaf cell photosynthesis, although he also showed that uptake of radiolabelled uridine, leucine and acetate into cells was significantly inhibited which may show some cell membrane activity. However, it seems most likely that the stomatal closure produced by atrazine was due to a build up of intercellular CO_2 caused by inhibition of photosynthesis. Thiocarbamates showed differential responses in C_3 and C_4 plants (Das et al, 1975). The general trend with thiocarbamates was to stimulate stomatal opening in C_3 plants and to cause stomatal closure in C_4 plants (Das et al, 1975). This study also showed a definite correlation between transpiration rate and stomatal response to herbicides, and led to the suggestion that the resistance or susceptibility of the

plant may be determined by selective stomatal behaviour towards the herbicide. The closure of the stomata of P. vulgaris was inhibited by two defoliants, thidiazuron (Elstner et al, 1983) and dimethipin (Metzger and Keng, 1984). Thidiazuron is thought to cause this response by stimulating ethylene production, and its effect on stomata can be nullified by spraying the plants with AVG, an ethylene synthesis inhibitor (Elstner et al, 1983). Conversely, dimethipin inhibits protein synthesis and acts similarly to the protein synthesis inhibitor cycloheximide, which also caused loss of stomatal control (Metzger et al, 1984). Glyphosate, a translocated herbicide which inhibits aromatic amino acid synthesis, caused stomatal closure after 7 days in M. sativa and Trifoluim protense L., this effect is likely to be caused by loss of turgor within the plant (Munoz-Rueda, Gonzalez-Murua, Bercerril and Sanchez-Diaz, 1986). The powerful contact herbicide paraquat induces a reversal of normal stomatal behaviour leading to a rapid desiccation in several woody species (Rao et al, 1977). Some herbicides may actually increase crop yields when they are sprayed to control post-emergence weeds (Das et al, 1982). This is the case with alachlor and butachlor which increase the yield of Z. mays by reducing stomatal aperture and reducing water loss while still allowing photosynthesis to take place (Das et al, 1982). Bentazone also has effects on stomatal movement and these will be presented on their own as this chemical forms a major part of this study. It should be stressed that as stomata are very sensitive to internal conditions, many herbicides will alter stomatal movement as a secondary effect.

1.5.5 Metabolic inhibitors

As stomatal movement is thought to require metabolic energy it is probable that metabolic inhibitors will alter stomatal movement or prevent certain aspects of it occurring. In this section some of the known effects of inhibitors on stomata are noted. Sodium vanadate, which is widely used to inhibit membrane bound ATPases (Brummel, 1986) was able to block stomatal opening in epidermal peels of V. faba and also inhibited light-induced proton efflux and potassium uptake, therefore

appearing to act directly on the membrane pump. This inhibition was overcome once vanadate was removed from the bathing medium (Gepstein et al, 1982/83). 2,4-dinitrophenol (DNP), an inhibitor of oxidative phosphorylation and cyclic photophosphorylation, prevented stomatal opening on illumination (Willmer et al, 1970; Pemadasa and Koralege, 1977; Das et al, 1982) and closure in the dark (Pemadasa et al, 1977), and affected concurrent changes in stomatal starch and potassium concentrations in guard cells. CO_2-free air was unable to reverse these effects and the DNP effect was largely attributed to inhibition of the hydrolysis of stomatal starch and active transport of potassium ions to and from guard cells. It may also be possible for DNP to affect the energy supply of stomata by inhibition of oxidative phosphorylation (Pemadasa et al, 1977). Other inhibitors of cyclic photophosphorylation such as chloromercuridinitrophenol and salicylaldoxime also reduce transpiration (Willmer et al, 1970; Das et al, 1982). An inhibitor of non-cyclic photophosphorylation, DCMU, causes partial closure of stomata in the light, this, however can be relieved by CO_2-free air suggesting that non-cyclic photophosphorylation is not essential for stomatal opening (Willmer et al, 1970). The well known inhibitor of mitochondrial respiration and oxidative phosphorylation, sodium azide, prevents stomatal closure in the dark (Zelitch, 1965) and in the presence of ABA (Weyers et al, 1982) it also prevented stomatal opening in Pelargonium spp., Veronica beccabunga L. and Lycopersicum esculentum L. (Zelitch, 1965). Azide, therefore, appears to prevent changes in stomatal aperture irrespective of the conditions, however, variation in azide concentration can alter the stomatal response. At low azide concentrations opening is inhibited completely while closure is only partly inhibited, at higher concentrations both are inhibited completely. It is suggested that at the higher azide concentrations the functioning guard cell membranes are permanently impaired (Zelitch, 1965). Potassium cyanide also prevented stomatal closure induced by ABA and this would suggest that metabolic energy is required for ABA action involving solute loss from the guard cell (Weyers et al, 1982). Two other inhibitors,

sulphite and arsenite, rapidly inhibit the process of stomatal opening and also reduce the activity of PEPC and nicotinamide adenine dinucleotide phosphate (NADP)- dependant malate dehydrogenase, two enzymes which may have key roles in stomatal function. Rao and Aderson (1983) suggested that the inhibition of stomatal movement by these inhibitors may result from the inhibition of light modulation of key enzymes within the guard cell. It is interesting to note that arsenite stimulates stomatal opening in the dark and increases the activity of PEPC at low concentrations (Rao et al, 1983). Other inhibitors tend to act by altering membrane permeability, for example, phenylmecuric acetate is thought to interact with the sulphydryl groups on the membrane to alter permeability (Zelitch, 1965).

1.5.6 Miscellaneous

A wide range of other chemicals also affect stomatal movement. Synthetic plant growth regulators derived from fluorenecarboxylic acid (Morphactins) are characterised by a pronounced effect on plant growth and development, they are often used with other plant growth regulators and may have herbicidal activity to combat weeds in corn and grassland (Ziegler, 1970). Some of them including chloroflurenol, flurenol and EMD 7301 W produced a striking decrease in stomatal opening in the dark, but had no effect on opening in the light in C. benghalensis (Das, Rao, Swamy and Raghavendra, 1976). Chemicals which stimulated and inhibited photophosphorylation had no influence on this morphactin-induced closure (Das et al, 1976). Effects of ATP, pyruvate and potassium chloride on opening were all suppressed by morphactins, however, benzyladenine was able to stimulate stomatal opening even in the presence of a morphactin. This suggests that the influence of morphactins closely mimics that of ABA (Das et al, 1976).

The fungicide and metal-chelating compound 8-hydroxyquinoline sulphate was shown to close the stomata of chrysanthemum leaves when the end of the petiole was immersed in a dilute solution (Zelitch, 1965). At higher concentrations it also closed the stomata when sprayed onto leaf discs of tobacco (Zelitch, 1965).

Another major group of compounds which have recently been found to have a major effect on stomata are the macrocyclic polyethers (Crown ethers), which have the ability to complex with alkali metals by ion-dipole interactions. On complexation the crown gives the alkali metal a lipid-like exterior therefore facilitating transport across the membrane. Indeed, in the presence of such ionophores the movement of potassium across the guard cell membrane increased (Sharp, Simmons, Georgiou, Truter, Richardson and Wingfield, 1980). At a certain point the crown starts to inhibit stomatal opening and this point correlates well with lipophilicities measured by octanol:water partitioning and also with the binding stoichiometries for potassium as observed in organic solvents and in isolated crystalline compounds (Georgiou, Richardson, Simmons, Truter and Wingfield, 1982). One of the most commonly used crown compounds in stomatal studies is benzo-18-crown-6. In C. communis, increasing the concentration of this ionophore progressively suppressed stomatal opening on abaxial epidermal peels, while up to an optimum concentration it enhances adaxial stomatal opening, although at higher concentrations it caused closure and the stomatal concentration of potassium changed correspondingly. Increasing potassium concentration progressively alleviated ionophore-induced closure and adding ABA produced a strongly antagonistic effect. The ionophore is postulated to eliminate the inherent disparity between membranes of abaxial and adaxial guard cells. It is suggested that in normal abaxial functioning a net potassium gain leads to wide opening, the ionophore then enhances permeability leading to a potassium loss and thus closure because the intensified efflux down a concentration gradient outweighs the influx driven by the proton pump (Pemadasa, 1983). The normal low adaxial potassium accumulation may be due to restricted membrane permeability and the ionophore increases permeability leading to a net gain of potassium and wider apertures. However, further increases in the ionophore concentration leads to the membrane becoming 'leaky' and this leads to loss of potassium and stomatal closure (Pemadasa, 1983). This idea is supported by the progressive reduction of ionophore-induced closure with

increasing external potassium and the strong antagonistic interaction with ABA (Pemadasa, 1983). This ionophore is also known to cause reduced transpiration in tomato seedlings (Richardson, Truter, Wingfield, Travis, Mansfield and Jarvis, 1979).

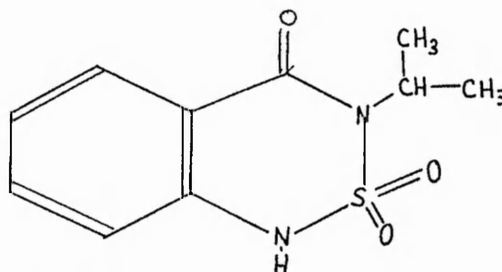
Vitamins have also been shown to alter stomatal movement. Ascorbic acid has been shown to open (Sharma and Singhvi, 1983) and close (Zelitch, 1965) stomata, and riboflavin has been shown to open stomata in peels of *Clematis gouriana* RoxB (Sharma *et al*, 1983). There was no dark effect of ascorbic acid or riboflavin in *C. gouriana* and starch was deposited heavily in the dark or where stomata were completely closed. With these two vitamins there was a two phase response curve showing opening at low concentrations and inhibition at high concentrations similar to those observed with IAA (Sharma *et al*, 1983).

The above chemicals were exogenously applied to the plant however, several plant stress products other than ABA have effects on stomata. Plant phenolic's such as, p-coumaric acid, salicylic acid and in particular sinapic acid were strong inhibitors of stomatal opening in *C. communis* (Plumbe and Willmer, 1986b). The effects on open stomata was less marked but sinapic acid brought about considerable closure while ferulic acid stimulated wider opening (Plumbe *et al*, 1986b). Wounding or fungal attack can initiate the production of phytoalexins and these too can have an effect on stomatal opening. Rishitin, pisatin, Wyerone acid and phaseollin were tested on *C. communis* and *V. faba* peels and on guard cell protoplasts of *C. communis*. Wyerone acid and phaseollin strongly inhibited stomatal opening to the same extent as ABA, while rishitin had no significant effect and pisatin gave an intermediate effect in *C. communis* (Plumbe and Willmer, 1986a). Phaseollin and ABA were able to close open stomata while pisatin and rishitin had no effect and Wyerone acid initially stimulated opening prior to returning to the control value (Plumbe *et al*, 1986a). In *V. faba* the response was different, none of the phytoalexins gave as much closure as ABA and the most inhibitory was Wyerone acid. Phytoalexins caused immediate loss in viability of guard cell protoplasts and

therefore demonstrated that their effects on stomata movement were likely to be associated with phytotoxicity (Plumbe et al, 1986a).

1.6 Current knowledge on the activity of Bentazone

1.6.1 The uses of bentazone



Bentazone

Bentazone (3-isopropyl-1,H,2,1,3 benzothia diazin-(4), 3,H one 2,2 dioxide) is a contact herbicide with some residual action (Davies and May, 1974). It is used to control a range of broad leaved weeds including Urtica urens L., Polygonum convolvulus L., Solanum nigrum L., Stellaria media L., Galium aparine L., Chenopodium album L., Senecio vulgaris L., Sinapis alba L., Matricaria spp, Capsella bursa-pastoris L., Sonchus spp., Brassica kaber L. C Wheeler, Ambrosia artenisiifolia L., Abutilon theophasti Medic., Polygonum pensylvanicum L., Helianthus annus L. and Amaranthus spp. in major crops such as P. vulgaris, Z. mays, Arachis hypogea L., P. sativum, G. max and O. sativa (Davies et al, 1974). The best control is found when bentazone is sprayed as an early post-emergent herbicide, later growth stages of weeds require more than one application and two applications at fourteen day intervals is favoured (Taylor, Baltensperger, Perry and Teem, 1985). Tank mixes are now favoured by farmers and when tank mixed with sethoxydim bentazone is able to reduce foliar toxicity and at lower concentrations it significantly enhances sethoxydim activity (Chernicky and Slife, 1986). However, if bentazone is tank mixed with mefluidide it

causes necrotic spots on the trifoliate leaves of soybeans and causes significant dwarfing of the crop (Almeida, Olivera and Filho, 1983). It has also been shown that pretreatment of plants with gibberellin caused an increased susceptibility to bentazone both in the field and in growth chamber experiments (Sterrett, 1980; Sterrett and Hodgson, 1983).

1.6.2 The effect of the environment on bentazone activity

In greenhouse experiments most damage occurred in *C. album* plants when they were given low temperature pretreatments followed by high temperatures, the greater the difference in pre- and post-treatment temperatures the greater the damage (Davies, Cobb and Taylor, 1979). Higher temperatures were also shown to increase the rate of bentazone uptake in *S. alba* plants (Retzlaff, 1983). Furthermore, increasing light intensity after bentazone application increases the toxicity of the herbicide and effects greater control (Davies *et al*, 1979; Retzlaff, 1983; Teasdale, 1984). Neither temperature nor light exert their effect by changing wax composition or deposition in *C. album* (Davies *et al*, 1979). Another environmental factor with a large effect on the efficacy of bentazone is humidity. Bentazone was found to give increased control of *C. album* (Davies *et al*, 1979) and *Amaranthus retro flexus* L. (Nalewaja, Pudelko and Adamezewski, 1975) at higher humidities and oil adjuvants were able to overcome the detrimental effects of low humidity (Nalewaja *et al*, 1975; Taylor, Davies and Cobb, 1981).

Closely connected with the environment are the effects of application time. It was found that *Xanthuim pensylvanicum* L. and *A. theophrasti* were poorly controlled when bentazone was applied late in the evening, at night or early in the morning (Doran and Anderson, 1976). However, Lunsford, Zarecer, Cole and Ellison (undated BASF report) claim that for this to occur either the rate of application must be low, or the weeds must be too large or unfavourable environmental conditions must exist.

1.6.3 The action of bentazone on photosynthesis

Bentazone inhibits electron flow at photosystem II in

isolated chloroplasts of both resistant and susceptible plant species (Mine and Matsunaka, 1975; Baltazar, Monaco and Peele, 1984). In bentazone-susceptible plants inhibition of CO_2 assimilation is fast and irreversible, however, in tolerant plants the effects on CO_2 assimilation appear more slowly and after some hours photosynthesis recovers fully (Dannigkiet, 1977). Bentazone inhibits photosynthetic electron transport at the reducing side of photosystem II between the primary electron acceptor Q and plastoquinone (Suwanketnikom, Hatzios, Penner and Bell, 1982). The degree of inhibition is strictly dependant on the amount of bentazone reaching the chloroplast (Retzlaff and Hamm, 1978). The amount of inhibition in chloroplast suspensions is also dependant on the pH of the medium (Retzlaff, Hilton and St.John, 1979; Rees, 1986). Retzlaff et al (1979) showed that the uptake of CO_2 was inhibited to a greater extent at pH 6 than at 7 or 8 and demonstrated that it was proportional to the amount of bentazone uptake. Rees (1986) however, although showing similar pH effects on photosynthesis by isolated G. max cells, found a slight lag in inhibition at pH 6 and claimed that as the lipophile concentration would be high there would be an unhindered path to the chloroplast active site unless binding of bentazone occurred elsewhere. He therefore contends that there is not a close correlation between the amount of bentazone taken into isolated cells and the rate of inhibition of photosynthesis. It should however, be noted that pH 6 is not optimal for photosynthesis and that photosynthetic competence of the G. max cells could only be maintained for a short period (Rees, 1986) and also that cell function could be altered in other ways which may have caused the lag. Furthermore, pH is unlikely to be identical throughout the cell and this may slightly impede the passage of bentazone to its site of action. In sensitive plants bentazone also reduces the efficiency of photosynthesis by inducing the longer term formation of shade-type chloroplasts (Lichtenthaler, Meier, Retzlaff and Hamm, 1982).

1.6.4 Transport and metabolism of bentazone within the plant

In soybeans and navy beans, both of which are resistant to bentazone, the movement of ^{14}C labelled bentazone was primarily acropetal (Mahoney and Penner, 1975; Retzlaff and Hamm, 1978), although some basipetal movement did occur. In these two species the resistance appeared to be due to conjugation of the absorbed bentazone and four conjugates were isolated from the mature plants (Mahoney et al, 1973). Hayes and Wax (1975) also found two methanol-extractable metabolites in soybeans, however, only one was a sugar-conjugate, the second metabolite which they observed may have been 6-hydroxybentazone which was isolated by Marana and Palmieri (1982). Wheat plants have been shown to metabolise bentazone to 6- and 8-hydroxybentazone. The 8-hydroxybentazone can only be detected for 30 minutes after application and is probably converted into the more abundant 6-hydroxybentazone (Retzlaff and Hamm, 1976). It would therefore seem that tolerant plant species have the ability to metabolise bentazone to non-toxic hydroxyderivatives and conjugates and thus are able to prevent it from causing permanent damage to the chloroplasts. The movement of bentazone within the plant is affected by leaf maturity and environmental conditions (Wills, 1984). Adding oils to bentazone formulations increases both its uptake (Retzlaff and McAvoy, 1984) and movement within the plant (Irons and Burnside, 1982), and this is thought to be because the solubility of the lipophillic undissociated molecule in the oil droplets shifts the dissociated:undissociated ratio towards undissociated, therefore increasing the concentration of the readily transportable lipophillic form (Retzlaff et al, 1984).

1.6.5 Non-photosynthetic effects of bentazone

Bentazone has been shown to inhibit RNA synthesis by 75% after 30 minutes in leaf cells of *P. vulgaris* and at higher concentrations also inhibits protein synthesis and lipid synthesis, although at much lower concentrations it can stimulate lipid synthesis after 120 minutes (Al-mendoufi and Ashton, 1984). Rees (1986) however challenged these findings as these authors did not first examine the uptake of the relevant precursors into

the cell. Rees (1986) himself found that bentazone was able to inhibit uptake of radiolabelled thymidine, uridine and acetate, whilst it enhanced the uptake of leucine.in *G. max* cells. However, incorporation of thymidine into DNA was unaffected, although decreases in incorporation of uridine into RNA (ribonucleic acid) and acetate into lipids were shown as well as an increase in inccrporation of leucine into protein. Therefore, although bentazone may have a major effect on photosynthesis it is also able to alter other metabolic processes within the cell. By far the most studied non-photosynthetic effect however, is the ability of bentazone to alter transpiration and stomatal movement. Cobb, Dunleavy and Davies (1983) were able to show that in *C. album* differential changes in transpiration, which were dependant on bentazone concentration, occurred in advance of any effect on photosynthesis. Plants treated with bentazone also showed a departure from the normal circadian rhythm of stomatal movement (Dunleavy, Cobb, Pallett and Davies, 1982). It was shown using abaxial epidermal peels from *C. album* that bentazone could close open stomata and prevent potassium-stimulated opening (Dunleavy and Cobb, 1984a) and also open closed stomata at low potassium concentrations (Dunleavy and Cobb, 1984b). Stomata are highly sensitive to the environment and it may well be that they are implicated in the poor bentazone control of *C. album* under certain environmental conditions (Anderson, Leuschen, Warnes and Nelson, 1974), and the response of weeds when sprayed at different times of the day (Doran *et al*, 1976). Indeed, it has been shown that plants treated when the stomata were open suffered more damage and took up greater amounts of ^{14}C-labelled bentazone (Dunleavy *et al*, 1982).

More recent work in this department on the effect of bentazone on other non-photosynthetic systems, such as coleoptile elongation and apical dominance, have indicated that bentazone may also be active in these growth responses (Miller, unpublished data).

1.7 Aims of the investigation

This study is essentially a continuation and expansion of the work started by Taylor (1979) and Dunleavy (1983) who characterised the effects of bentazone on C. album. They found that bentazone had an effect on transpiration and stomatal movement as well as on photosynthesis. The intention of this study is to examine in detail the stomatal action of bentazone and will lock at the effect of bentazone in several key areas.

1. Determination of the species specificity of the bentazone effect:

   It is intended to examine epidermal peels from a wide range of species to see if the effects observed in C. album are widespread. It is also hoped that this small study will yield a plant species which is more suited to epidermal peel studies.

2. The effect of bentazone on stomatal aperture at different KCl concentrations in both light and darkness.

3. The effect of bentazone at different pH's and its similarity and possible interactions with IAA: It is hoped that this study will further illuminate the possible membrane activity of bentazone.

4. The effect of bentazone on malate dehydrogenase activity:

   This study is intended to examine the possibility of bentazone altering stomatal movement by changing the activity of key enzyme systems within the guard cell.

5. The effect of bentazone on the uptake of $^{86}Rb^+$: This study will use $^{86}Rb^+$ as an analogue of K^+ to look for bentazone effects on the movement of the major ion involved in regulating guard cell turgor.

Thus, the whole study is designed to look at the effects of bentazone on stomatal aperture, guard cell metabolism (enzyme studies), guard cell membranes and ionic movement across the guard cell membrane. It is hoped that these studies will give a good indication of how bentazone exerts its effect on stomata.

CHAPTER 2 EPIDERMAL PEEL METHODOLOGY

2.1 INTRODUCTION

Although this study is a continuation of work carried out on *C. album* by Taylor (1979) and Dunleavy (1983), it was considered more prudent to use species which was better characterised in the literature with respect to stomatal movement. In this respect the most obvious choice was *C. communis* which has been extensively used by many stomatal researchers (Fujino, 1969; Willmer *et al*, 1969; Tucker and Mansfield, 1971; Squire *et al*, 1972; Willmer *et al*, 1973; Penny *et al*, 1976; Dittrich *et al*, 1977; Dittrich and Mayer, 1978; Itai and Meidner, 1978; Wilson, Ogunkanmi and Mansfield, 1978; Donkin and Martin, 1980; Jarvis *et al*, 1980; MacRobbie *et al*, 1980; Travis and Mansfield, 1981; Georgiou *et al*, 1982; Pemadasa, 1982; Snaith *et al*, 1982; Blackman *et al*, 1983; Morison *et al*, 1983; Robinson, Zeiger and Preiss, 1983; Weyers and Travis, 1983; Birkenhead *et al*, 1984; DeSilva *et al*, 1985; Govil, 1985; Grantz *et al*, 1985; Iino, Ogawa and Zeiger, 1985; Inoue *et al*, 1985; Schwartz, 1985; Plumbe *et al*, 1986; Rai, Sharma and Sharma, 1986). In 1983 Weyers *et al* examined in great detail the selection and preparation of leaf epidermis for experiments on stomatal physiology. They found that the stomatal responses of *C. communis* epidermis were more predictable than those obtained with *V. faba*. They concluded that *C. communis* was the plant species most suited to epidermal peel studies as the leaves readily yielded peels which had a low level of mesophyll contamination. They also found that peels from *C. communis* floated best on test solutions and were easier to use for stomatal aperture estimations than other species including *V. faba*.

The stomata of *C. communis* respond quickly to environmental conditions and in order for experimental results to be valid it is important to standardise the methodology allowing results to be readily reproducible. This chapter aims to show how epidermal peel methodology was standardised with respect to plant growth conditions, leaf pretreatment and peel pretreatment, in order to obtain reproducible stomatal responses to light, darkness and potassium concentrations. These findings are then compared with those found by other workers.

2.2 MATERIALS AND METHODS

2.2.1 Plant growth conditions

Plants of *C. communis* were grown in a 1:1 mixture of John Innes No. 2 and J. Arthur Bowers potting compost in a greenhouse at 25°C ± 3°C (day) to 15°C ± 2°C (night), and watered regularly from below. Supplementary lighting was provided by Phillips 40W fluorescent tubes with a 16 h photoperiod. At the 5-6 leaf stage plants were transferred to a controlled environment cabinet (Fisons 600 G3, Type TTL) under a 16 h photoperiod at a photon flux density of 200 µmol $m^{-2}s^{-1}$ (PAR) and a mean temperature of 20°C. After 4-5 days in this controlled environment the plants were considered suitable for experimental use.

2.2.2 Standardisation of leaf pretreatment time

Young fully expanded leaves were removed from the plants and floated abaxial surface down on 25 ml of 0.1 mol m^{-3} $CaCl_2$ (pH 6.0, adjusted with minimal amounts of KOH and HCl) in a Petri dish, placed in a tank with a water-jacket which maintained the temperature at 23°C ± 1°C, and illuminated from above with light of photon flux density 50 µmol $m^{-2}s^{-1}$ (PAR) for 50, 60, 70 and 80 minutes. After these times, abaxial epidermal peels were carefully prepared using watchmakers forceps, all visible mesophyll contamination cut away with iris scissors and placed in pretreatment conditions for 1 h (2.2.3 below). Following this pretreatment 3 peels were removed and 15 apertures determined microscopically from each, using a Vickers light microscope (magnification x 1000) fitted with a calibrated micrometer eyepiece. The leaf pretreatment time chosen for subsequent experiments was the one which resulted in the peels being most responsive to pretreatment conditions designed to cause opening or closure.

2.2.3 Epidermal peel pretreatment conditions

Following leaf pretreatment clean abaxial epidermal peels were removed with watchmakers forceps, cut into 5 mm x 5 mm pieces with iris scissors and transferred to conditions

which would induce either stomatal opening or stomatal closure, thus ensuring that at the commencement of any experiment all the stomata were of similar aperture.

2.2.3.1 To produce stomatal opening: Clean epidermal peels were floated cuticle upwards for 1 h in a Petri dish containing 25 ml of bathing medium, which contained 100 mol m^{-3} KCl (AnalaR) and 0.1 mol m^{-3} $CaCl_2$ at pH 6.0 (adjusted with minimal amounts of KOH and HCl). The Petri dish was placed in the temperature-controlled tank at $23^{o}C \pm 1^{o}C$ and laboratory air, which had been passed through soda lime (Sigma Chemicals) to give a CO_2 concentration of less than 50 $\mu l\ l^{-1}$, was bubbled through the medium via a hypodermic needle to ensure constant mixing. The Petri dishes were illuminated from above by Phillips 40W fluorescent tubes (cool white) at a photon flux density of 50 umol $m^{-2}s^{-1}$ (PAR).

2.2.3.2 To produce stomatal closure: Clean epidermal peels were floated cuticle upwards for 1 h in a Petri dish containing 25 ml of 0.1 mol m^{-2} $CaCl_2$ at pH 6.0 and $23^{o}C$. Untreated laboratory air of approximately 500 $\mu l\ l^{-1}$ CO_2 was bubbled through the solution via a hypodermic needle to cause continuous mixing. These Petri dishes were kept in a darkened tank.

After 1 h, 2 peels were selected at random from each pretreatment condition and 10 apertures were determined microscopically from each to ensure that stomatal opening or closure had occurred.

2.2.4 Treatment conditions

During the subsequent 120 minute observation period environmental conditions were the same as those used for pretreatment. Thus, the influence of the bathing solution was determined under conditions designed to maintain stomata either open or closed. The pH of solutions was adjusted using minimal amounts of KOH and HCl. pH was monitored throughout the treatment period and no changes were observed.

2.2.5 Design of initial experiments

2.2.5.1 Determination of the effect of potassium concentration on stomatal aperture: Ten peels were floated on 25 ml of each treatment solution, containing 0.1 mol m^{-3} $CaCl_2$ and 0, 10, 25. 50, 100 and 150 mol m^{-3} KCl, and kept under conditions designed to maintain closure or opening for 120 minutes. Two peels were removed at times 0 and 120 minutes and 10 stomatal apertures from each were directly measured as described previously. Each experiment was repeated a minimum of 3 times and results calculated as $\bar{x} \pm$ SE (n = 60).

2.2.5.2 Determination of the effect of $CaCl_2$ on stomatal movement: Fifteen epidermal peels were placed in each of 4 petri dishes; 2 dishes were pretreated to close and 2 pretreated to open. After pretreatment, 1 dish was maintained under each of the pretreatment conditions and peels from the remaining 2 dishes transferred to opposite conditions (i.e. those pretreated to close were transferred to Petri dishes containing 100 mol m^{-3} KCl + 0.1 mol m^{-3} $CaCl_2$, bubbled with low CO_2 and placed under the light to induce opening, and those pretreated to open were transferred into Petri dishes containing 0.1 mol m^{-3} $CaCl_2$, bubbled with high CO_2 and placed in darkness to induce closure). This procedure was repeated in the absence of $CaCl_2$. Apertures were determined after 0, 30, 60, 90 and 120 minutes.

As the effects of $CaCl_2$ on the stomata of C. communis were observed by other workers in the presence of 10 mol m^{-3} MES (Morpholino ethane sulphonic acid) buffer pH 6.1 (Willmer et al, 1969), the above experiment was repeated with this buffer.

2.2.5.3 Histochemical staining for potassium: In order to ascertain whether stomatal opening was due to potassium uptake, a histochemical staining procedure was employed. Peels were removed from the treatment solutions and washed thoroughly, at least twice, in distilled water to remove exogenous potassium. The peels were next floated on ice cold, freshly

prepared 20% (w/v) cobaltinitrite stain (20 g sodium cobaltinitrite (Sigma Chemicals) dissolved in 100 ml of 10% (aq.) acetic acid)and left for 30 minutes before rinsing twice in distilled water. Rinsed peels were transferred to Petri dishes containing undiluted ammonium polysulphide (BDH Chemicals) and left in the fume cupboard for 5-10 minutes to fix the stain. Peels were then mounted on microscope slides and observed under the microscope. Areas which had retained potassium were indicated by a black deposit of cobalt sulphide.

2.2.5.4 Neutral red staining for cell viability: Peels removed from treatment solutions were floated on a 1 gl^{-1} (w/v, aq.) solution of neutral red (Sigma Chemicals) for 10 minutes, rinsed once, mounted on a slide and observed microscopically at x 400 magnification. Viable cells were observed to take up the red stain.

2.2.5.5 Photography of epidermal peels: Photographs were taken using a Leitz microscope with a camera attachment. All photographs included were taken at x 400 magnification. The exposure time was set manually and determined by taking lightmeter readings for each slide. The black and white photographs were taken on an Ilford panf film and the colour photographs were taken on Kodak 400 cm film.

2.3 RESULTS

It can be seen from Fig. 2.1 that a leaf pretreatment time of 70 minutes was optimal for increasing the responsiveness to experimental conditions, as at 80 minutes the response was unchanged. At pretreatment times below 70 minutes the opening response of stomata was low. Fig. 2.2 showed that increasing the KCl concentration in the bathing medium increased the ability of the stomata to remain open and also allowed opening under conditions which would otherwise maintain stomatal closure. This potassium-stimulated stomatal opening is supported by plates 1a and 1b which show that when the stomata were closed there was no cobalt sulphide precipitation in the guard

cells, but when they were stimulated to open by 150 mol m^{-3} KCl abundant cobalt sulphide precipitation was observed.

In the absence of MES buffer, $CaCl_2$ did not have any obvious effect on stomatal movement (Fig. 2.3(a)), whereas in the presence of MES buffer $CaCl_2$ reduced normal potassium-stimulated stomatal opening (Fig. 2.3(b)(iii)). However, its presence did not alter stomatal closure (Fig. 2.3(b)(iv)) or the maintenance of opening (Fig. 2.3 (b)(i)) or closure (Fig. 2.3 (b)(ii)). Neutral red staining (Plate 2) suggested that there was little or no observed loss of guard cell viability during any of the treatments investigated in this study.

Figure 2.1 The effect of leaf pre-treatment time (min) on the stomatal aperture (um) in *C. communis* after pretreating abaxial epidermal peels to open (open circles) or close (closed circles). Each point is a mean of 60 observations from 3 replicated experiments and the error bar represents twice the standard error.

Figure 2.1

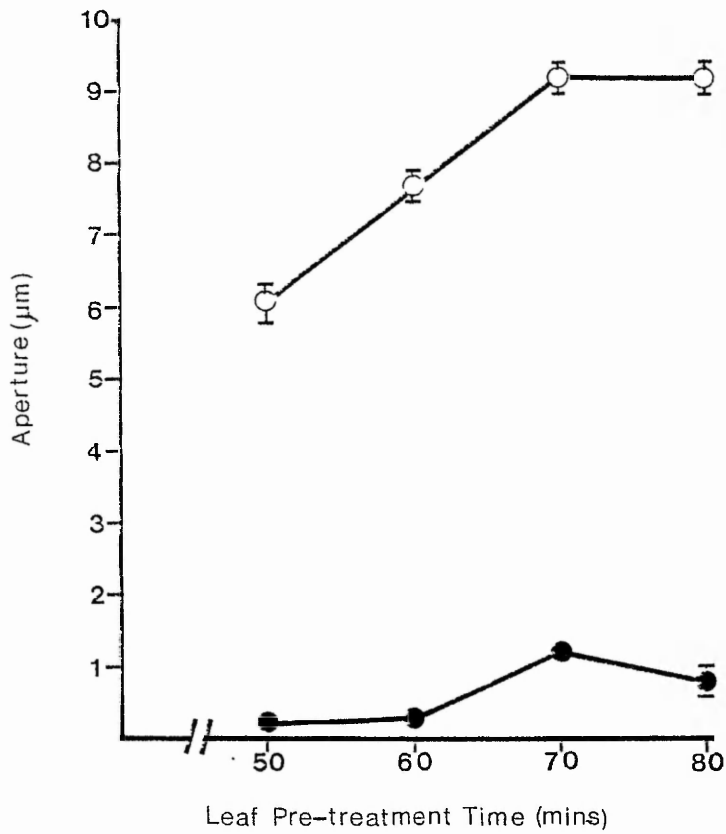


Figure 2.2 The effect of KCl concentration (mol m^{-3}) on stomatal opening (um) on abaxial epidermal peels of C. communis in light (open circles) and darkness (closed circles). Each point is a mean of 60 observations from 3 replicated experiments and the error bars represent twice the standard error.

Figure 2.2

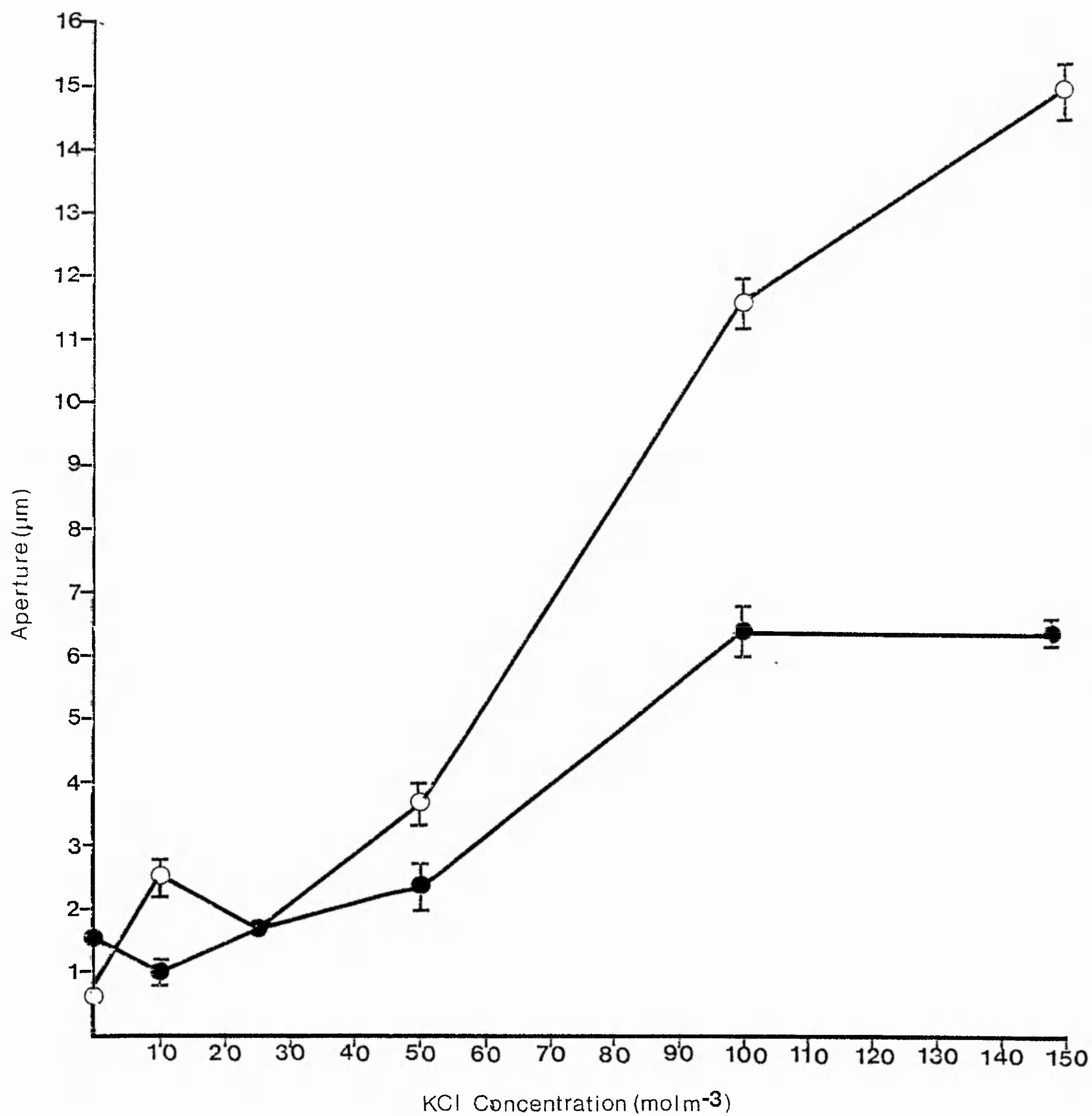


Plate 1. Cobaltinitrite staining of potassium in the guard cells of C. communis after treating stomata a) to close (darkness + $CaCl_2$ + high CO_2) and b) to open (light + $CaCl_2$ + 150 mol m^{-3} KCl + low CO_2) x 400 magnification. Taken using a Leitz Dialux microscope with a camera attachment.

Plate 1a)

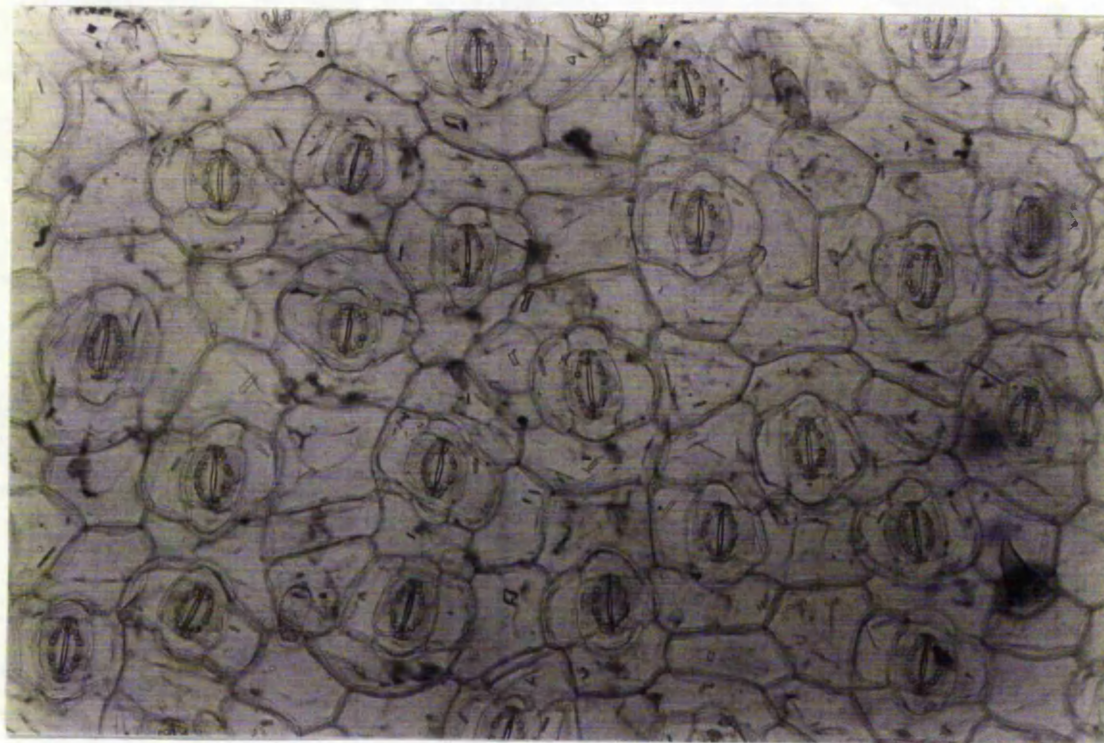


Plate 1b)

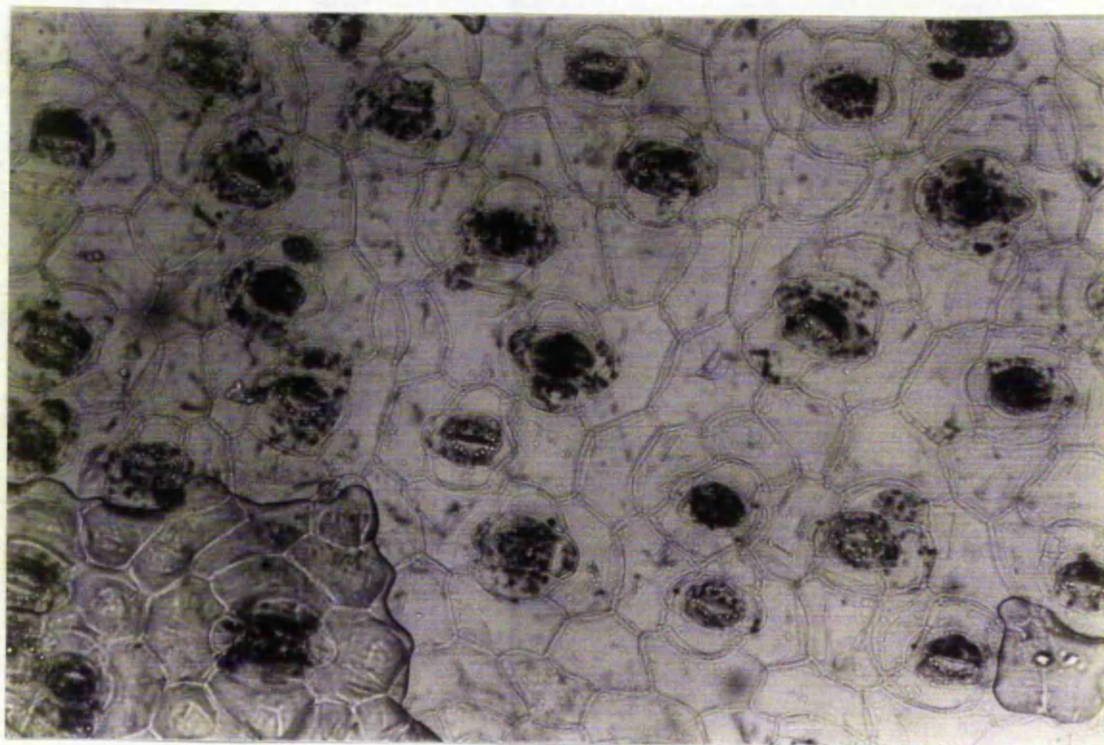


Plate 2 Neutral red staining to show the viability of *C. communis* guard cells, a) at the onset of the experiment (time = 0 minutes) and, b) at the end of the experiment (time = 120 minutes), incubation with 100 mol m^{-3} KCl x 400 magnification. Taken using a Leitz Dialux microscope with a camera attachment.

Plate 2a)

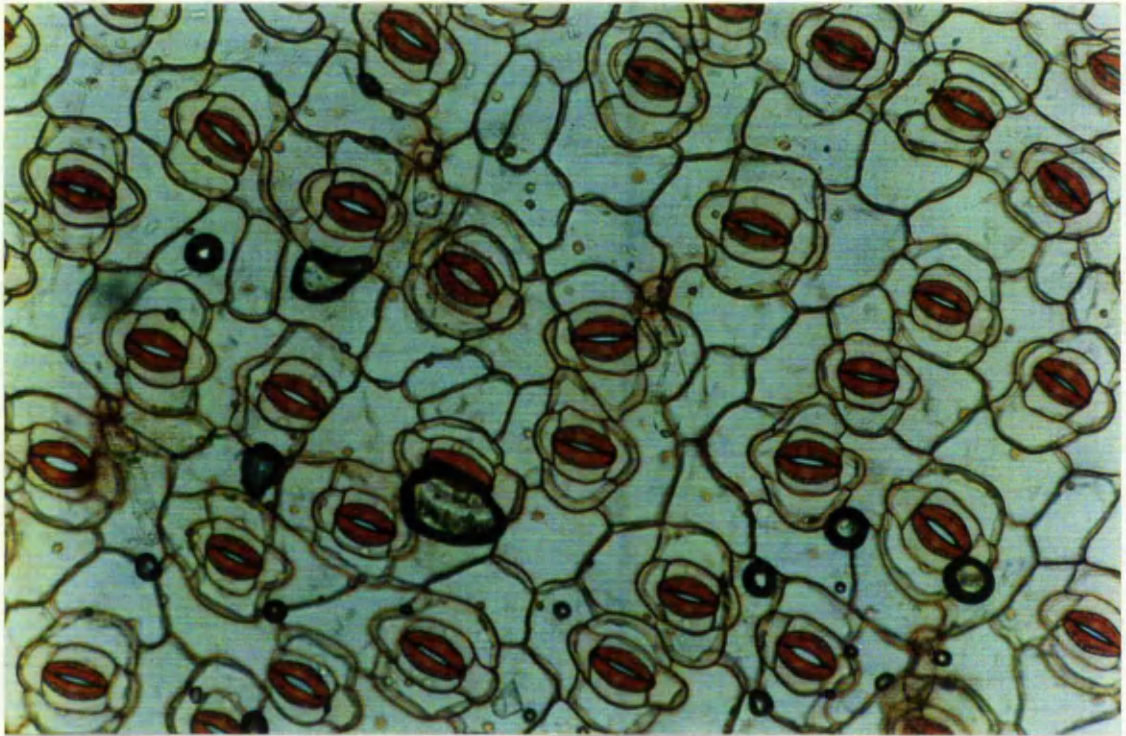


Plate 2b)

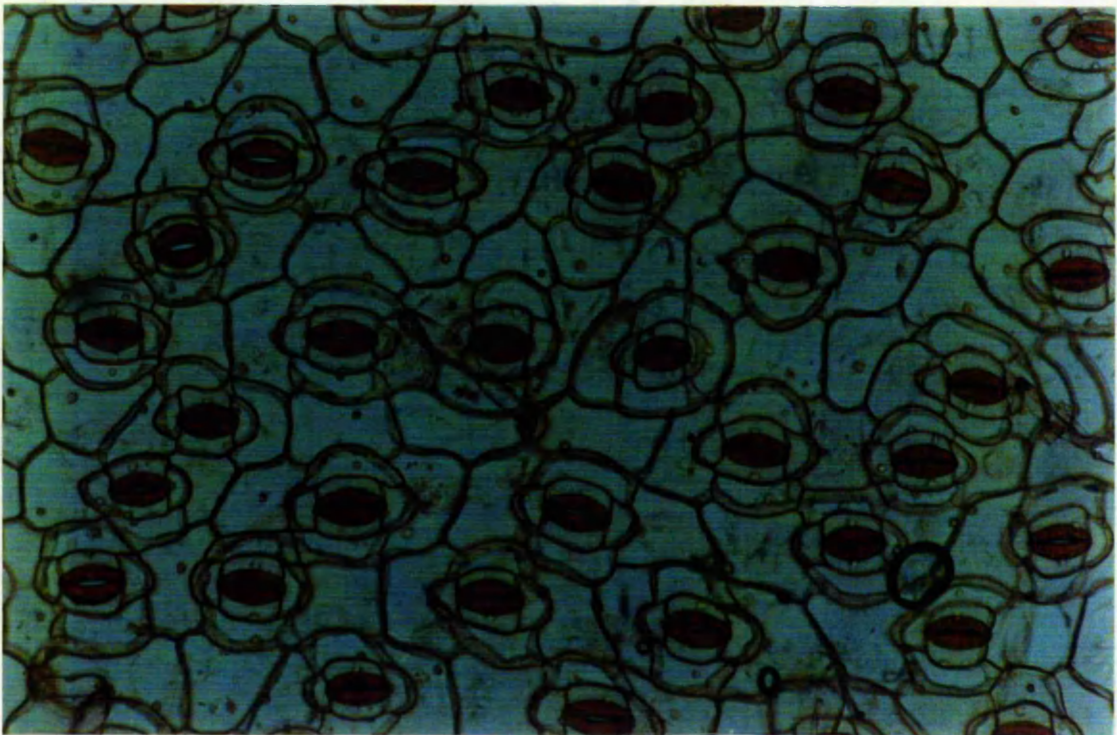


Figure 2.3 a) The effects of 0.1 mol m^{-3} $CaCl_2$ on:- (i) Maintenance of opening, (ii) maintenance of closure, (iii) stomatal opening and, (iv) stomatal closure on epidermal peels of *C. communis*. Broken lines represent the response in the absence of $CaCl_2$ and solid lines the response in its presence. Error bars represent twice the standard error about the mean (n = 60).
Open circles represent treatment in the light
Closed circles represent treatment in darkness.

Figure 2.3 b) The effects of 0.1 mol m^{-3} $CaCl_2$ in the presence of 10 mol m^{-3} MES buffer on:- (i) maintenance of opening, (ii) maintenance of closure, (iii) stomatal opening and, (iv) stomatal closure in epidermal peels of *C. communis*. Broken lines represent the response in the absence of $CaCl_2$ and solid lines the response in its presence. Error bars represent twice the standard error about the mean (n = 60).
Open circles represent treatment in the light.
Closed circles represent treatment in darkness.

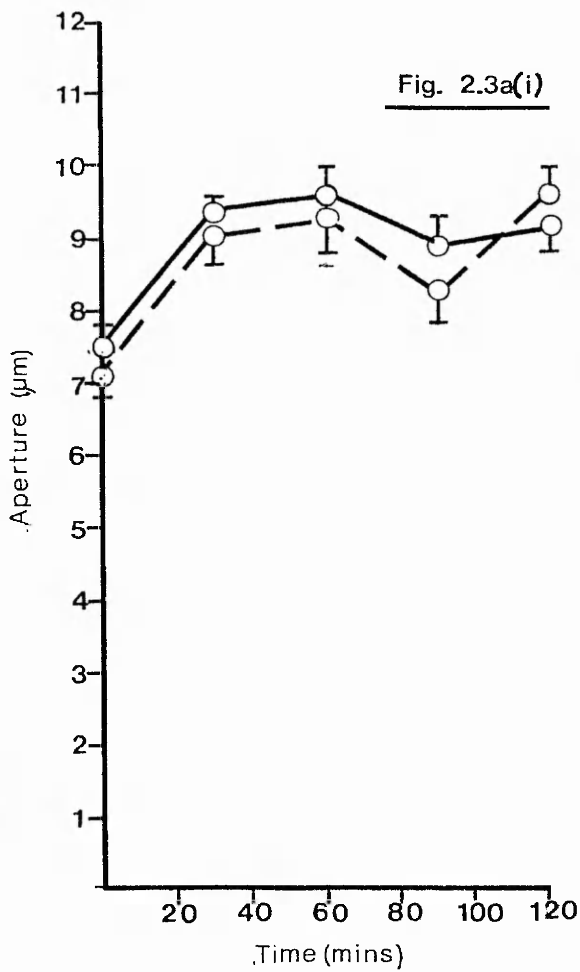


Fig. 2.3a(i)
Aperture (µm)
12
11
10
9
8
7
6
5
4
3
2
1
20
40
60
80
100
120
Time (mins)

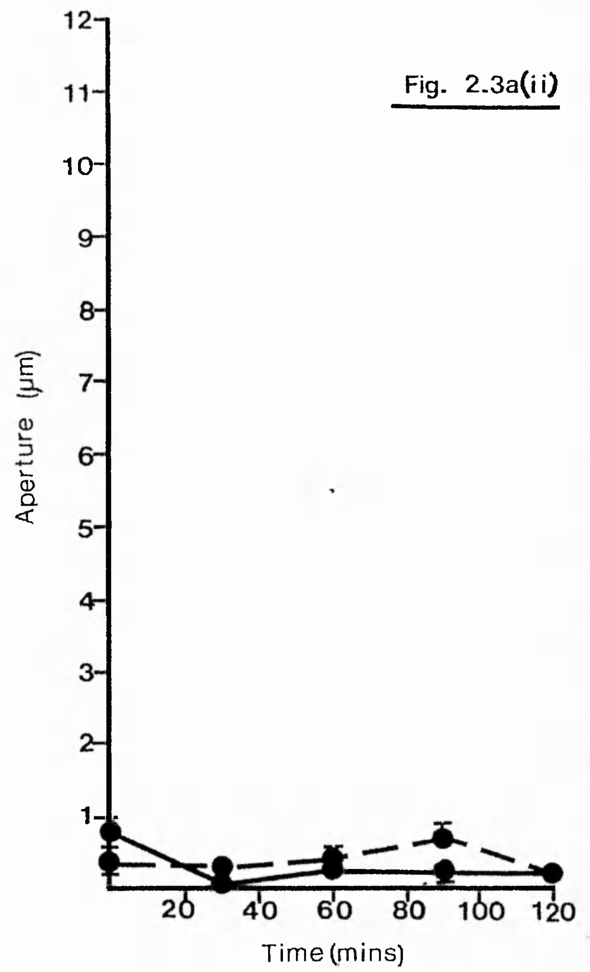


Fig. 2.3a(ii)
Aperture (µm)
12
11
10
9
8
7
6
5
4
3
2
1
20
40
60
80
100
120
Time (mins)

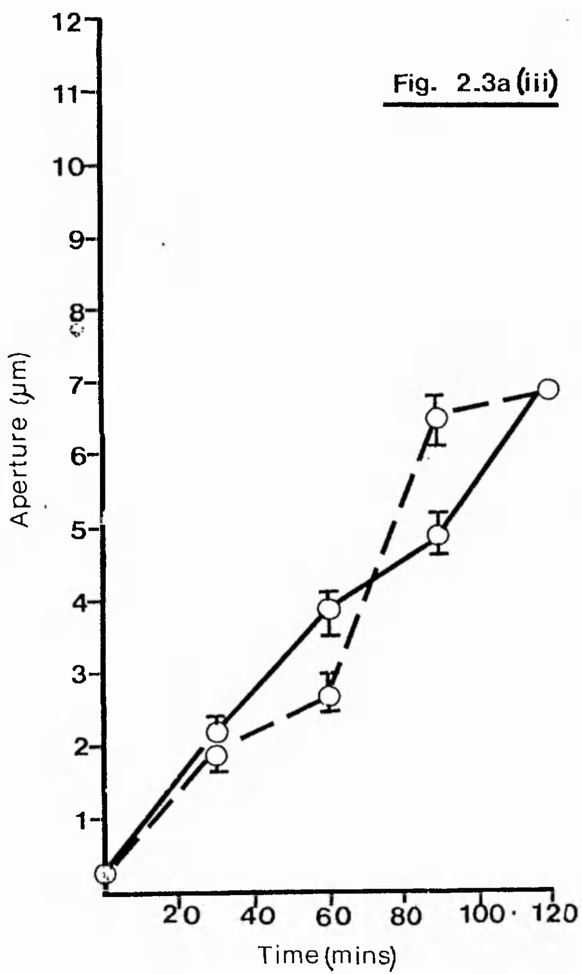


Fig. 2.3a (iii)
Aperture (µm)
12
11
10
9
8
7
6
5
4
3
2
1
20
40
60
80
100
120
Time (mins)

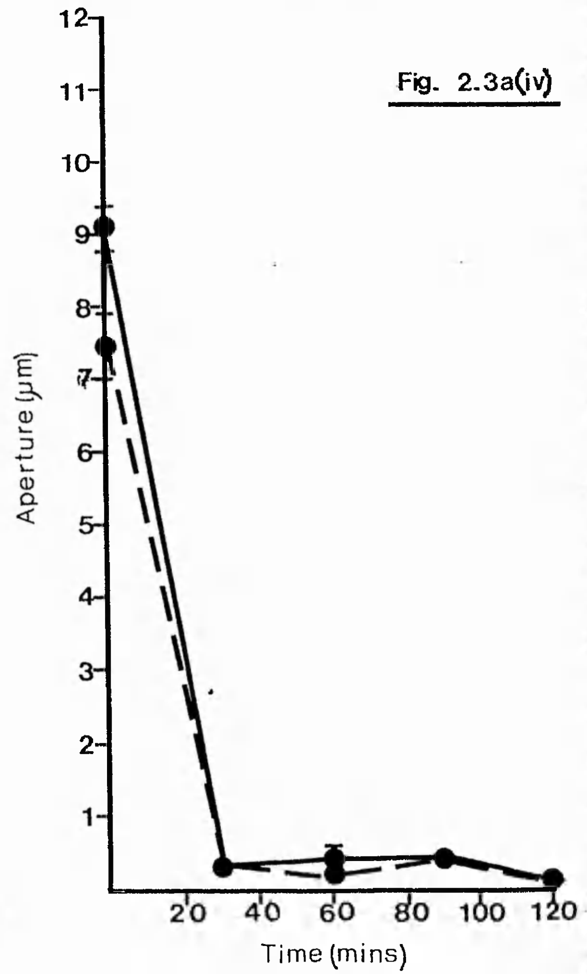


Fig. 2.3a(iv)
Aperture (µm)
12
11
10
9
8
7
6
5
4
3
2
1
20
40
60
80
100
120
Time (mins)

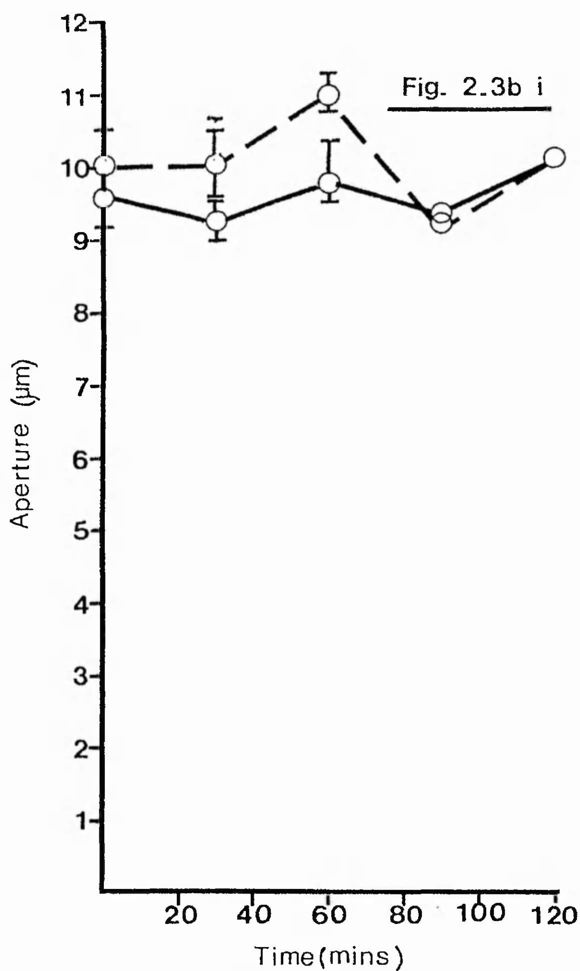


Fig. 2.3b i
Aperture (μm)
Time (mins)
12
11
10
9
8
7
6
5
4
3
2
1
20
40
60
80
100
120

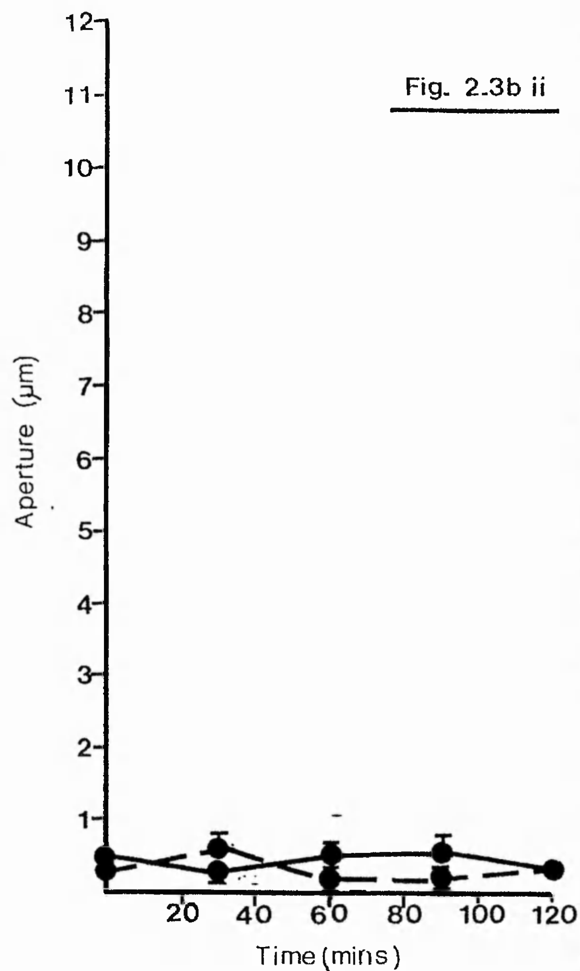


Fig. 2.3b ii
Aperture (μm)
Time (mins)
12
11
10
9
8
7
6
5
4
3
2
1
20
40
60
80
100
120

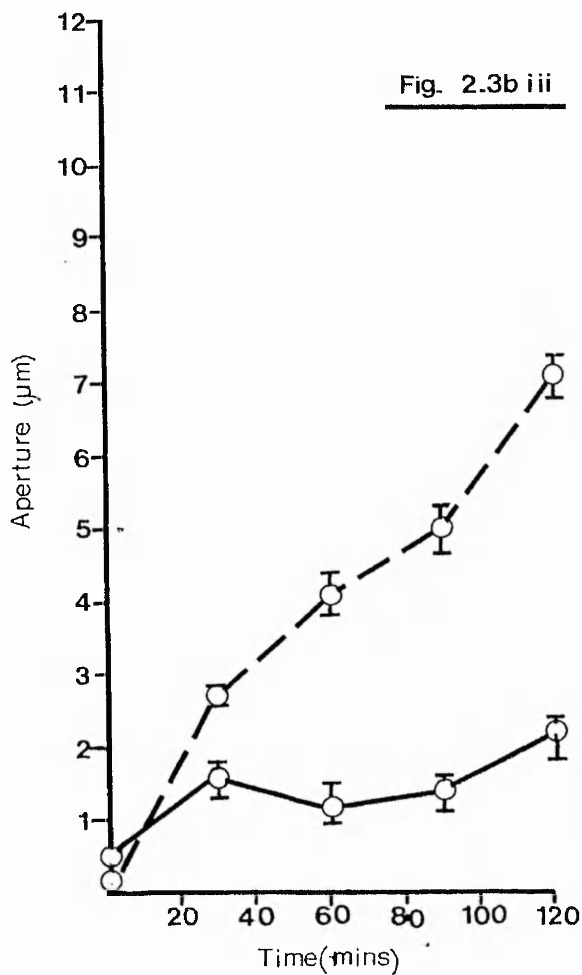


Fig. 2.3b iii
Aperture (μm)
Time (mins)
12
11
10
9
8
7
6
5
4
3
2
1
20
40
60
80
100
120

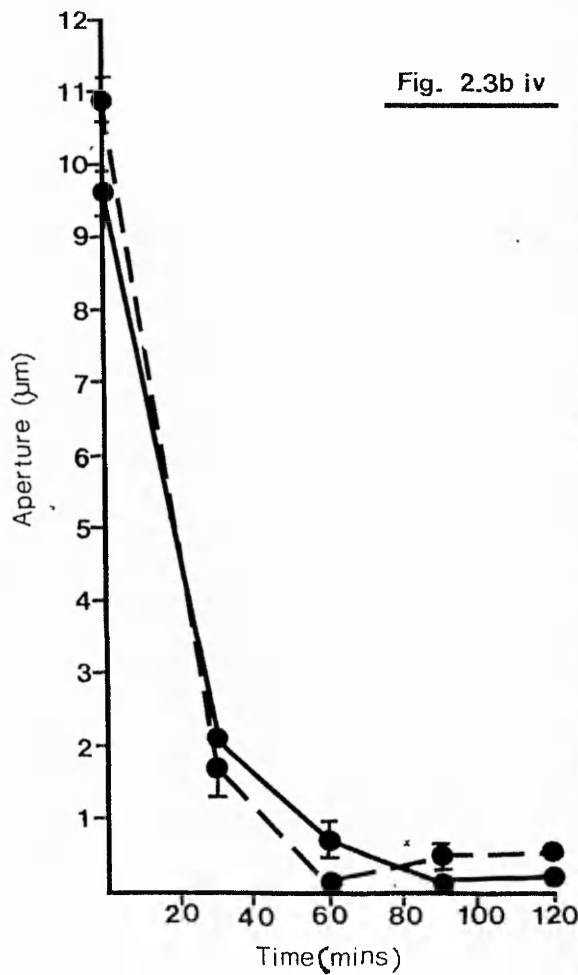


Fig. 2.3b iv
Aperture (μm)
Time (mins)
12
11
10
9
8
7
6
5
4
3
2
1
20
40
60
80
100
120

2.4 DISCUSSION

These observations demonstrate that C. communis is a valuable test species for research in stomatal physiology since it provides large-scale, reliable and reproducible responses as well as sensitivity to environmental change. This sensitivity, however, may also be a disadvantage, as changes in the growing conditions can greatly alter the response of the stomata on epidermal peels in later experiments. Thus, stomatal responses were checked after each pretreatment, and if abnormal responses to known pretreatment conditions were observed, the experiment was abandoned. The leaves of C. communis were found to peel easily and yield clean viable epidermal peels which floated readily on the test solutions as observed by other workers (Weyers et al, 1983). The response of stomata to conditions was easily measurable as the stomata of C. communis were large and changes in aperture were of a measurable size.

Pretreatment of leaves for 70 minutes proved the best way to ensure that the stomata on epidermal peels responded maximally to pretreatment conditions. Indeed, other workers have shown that pre-illumination of the epidermal peels themselves can increase the rate and extent of ion-stimulated stomatal opening (Inoue, Noguchi and Kubo, 1985). It is also suggested that this light energy can be stored, in some form, for some hours and then used for delayed stomatal opening (Inoue et al, 1985). It may, therefore, be that pre-illumination of the leaves causes a similar sensitisation of the stomata in epidermal peels.

The responses to potassium chloride concentration are similar to those found by Travis et al (1979), even though these experiments were conducted in the absence of MES buffer. The responses in both light and darkness showed that increasing the potassium ion concentration in the bathing medium increased the mediums ability to open stomata and to maintain stomatal opening, which would suggest that the amount of stomatal opening is related to the amount of available potassium. Indeed, the cobaltinitrite stains show that on stomatal opening (Plate 1b) the potassium concentration in the guard cells

increases markedly when compared to that observed in closed stomata (Plate 1a). This, therefore, demonstrates that under the experimental conditions described, stomatal movement in C. communis is mediated by potassium uptake and is thus in agreement with other workers (Fischer, 1968; Dayanandan et al, 1975; Outlaw, 1977; MacRobbie et al, 1980; Mansfield, 1983).

The effect of calcium chloride has been much debated in the literature in relation to stomatal movement. It's use is favoured when dealing with epidermal peels from V. faba, where it is claimed to increase the specificity of the guard cell membrane for potassium uptake (Pallaghy, 1970). There have been no reports of any calcium-induced inhibition of stomatal opening in V. faba, however such claims have been made in connection with C. communis (Willmer and Mansfield, 1969). It is interesting to note that another difference between the epidermal peel incubation conditions for these two species is that buffer is rarely used with V. faba (Pallaghy, 1970; Horton et al, 1972; Fischer, 1973; Hsaio et al, 1973; Lurie, 1978; Gepstein et al 1982/83), whereas buffer is routinely used with C. communis (Willmer et al, 1969; MacRobbie et al, 1980; Blackman et al, 1983; Pemadasa, 1983; Snaith et al, 1985). The results obtained in the present study (Fig. 2.3a and b) demonstrate that in the absence of 10 mol m^{-3} MES buffer, calcium has no inhibitory effects on stomatal opening in epidermal peels of C. communis, and that inhibitory effects were only observed when MES buffer was introduced into the bathing medium. It may therefore be that the apparent sensitivity of the stomata of C. communis to low levels of calcium chloride is the result of an interaction with the MES buffer. Calcium has recently been reported to play a major role in the normal responses of stomata to ABA (DeSilva, Cox, Hetherington, and Mansfield, 1985; DeSilva, Hetherington and Mansfield, 1985), and it may be that low levels of calcium are required to facilitate other normal responses of stomata. As stomatal viability did not decline over the experimental period (Plate 2a and b) and pH remained constant, it was considered unnecessary to use buffer in the incubation media, and thus no

problems were observed due to the inclusion of calcium chloride at the concentration of 0.1 mol m^{-3}.

2.5 CONCLUSIONS

The following scheme outlines the basic methods which were employed throughout this investigation and which proved to give reliable and reproducible results. The stomatal responses obtained appeared to result primarily from potassium movement across the guard cell membranes and were not due to any observable loss in viability or interaction with calcium.

LEAF AND EPIDERMAL PEEL INCUBATION

(*Commelina communis*)

Leaf pretreatment

Abaxial surface downwards on
25 ml of 0.1 mol m^{-3} $CaCl_2$ at
50 μ mol $m^{-2}s^{-1}$ (PAR) and 23°C
± 1°C 70 minutes

Preparation of abaxial epidermal peels

Epidermal peel pretreatment

To open stomata

Light (50 μ mol $m^{-2}s^{-1}$ (PAR))
Temp 23 ± 1°C
Air passed over sodalime
(< 50 μl l^{-1} CO_2)
100 mol m^{-3} KCl
0.1 mol m^{-3} $CaCl_2$
60 minutes

To close stomata

Darkness
Temp 23 ± 1°C
Air (≥ 500 μl l^{-1} CO_2)

KCl omitted
0.1 mol m^{-3} $CaCl_2$
60 minutes

Epidermal peel treatments

On open stomata

Light (50 μ mol $m^{-2}s^{-1}$ (PAR))
Temp 23 ± 1°C
Air passed over sodalime
(< 50 μl l^{-1} CO_2)
+ required levels of KCl and
other test chemicals
0.1 mol m^{-3} $CaCl_2$
120 minutes

On closed stomata

Darkness
Temp 23 ± 1°C
Air (≥ 500 μl l^{-1} CO_2)

+ required levels of KCl and
other test chemicals
0.1 mol m^{-3} $CaCl_2$
120 minutes

CHAPTER 3. CHARACTERISATION OF THE STOMATAL RESPONSE TO BENTAZONE IN EPIDERMAL PEELS

3.1 INTRODUCTION

Recent studies have shown that the herbicide bentazone can alter stomatal movement in C. album in epidermal peels (Taylor, Cobb and Davies, 1980; Cobb et al, 1983; Dunleavy et al, 1984a and b) and affect the transpiration of intact leaves (Dunleavy et al, 1982; Cobb et al, 1983). Cobb et al, 1983, were able to show that the differential changes in transpiration which were dependant on bentazone concentration, occurred in advance of any effect on photosynthesis. The studies on abaxial epidermal peels of C. album showed that bentazone could close open stomata and prevent potassium-stimulated opening (Dunleavy et al, 1984a), as well as being able to open closed stomata at low potassium concentrations (Dunleavy et al, 1984b).

The initial aim of this investigation was to examine the responses of the stomata of other species to this herbicide and so establish the species specificity of the response observed with C. album. Following these investigations it was intended to further elucidate the response of stomata to bentazone using C. communis as the experimental species since it grows quickly, can produce high yields of clean, viable epidermes and it's stomatal physiology is well characterised in the literature (e.g. Willmer et al, 1969; Mansfield, 1983; Weyers et al, 1983).

3.2 MATERIALS AND METHODS

The basic epidermal peel methodology used was as outlined in Chapter 2, and, unless otherwise stated, the plant species used was C. communis.

3.2.1 Design of experiments

3.2.1.1 The effect of bentazone concentration on stomatal aperture:

Treatment solutions contained 0.1 mol m^{-3} $CaCl_2$, 50 mol m^{-3} KCl and bentazone at the following concentrations, 0, 5 x 10^{-5}M, 1 x 10^{-4}M and 5 x 10^{-4}M. All solutions were adjusted to pH 6.0 by adding minimal amounts of KOH and HCl, and pH was seen to remain constant throughout the experimental period. Peels were exposed to

treatment solutions for the usual 120 minutes time period under conditions designed to maintain opening or closure, after which 10 apertures were measured on each of 2 peels using a microscope. The whole experiment was repeated 3 times.

3.2.1.2 The effect of 10^{-4}M bentazone on the response of stomata to potassium chloride concentrations: (a) In the absence of 10 mol m^{-3} MES buffer. Treatment solutions contained 0.1 mol m^{-3} $CaCl_2$ and 0, 10, 25, 50, 100 or 150 mol m^{-3} KCl in the presence or absence of 10^{-4}M bentazone and were adjusted to pH 6.0 using minimal amounts of KOH and HCl. pH was monitored throughout the experimental period and found to remain constant. Peels were left in treatment solutions for 120 minutes under conditions designed to maintain opening or closure, after which 2 peels were removed and 10 apertures measured from each. The experiment was repeated 2 times in the presence of 10^{-4}M bentazone, and also performed 3 times in the presence of 10^{-4}M 6-hydroxybentazone.
(b) In the presence of 10 mol m^{-3} MES buffer. Details as in (a) above, buffer was adjusted to pH 6.0 using minimal amounts of KOH.

3.2.1.3 The effect of 10^{-4}M bentazone on the stomatal responses of several plant species: The plant species used in this investigation were:- V. faba L., C. communis L., Beta vulgaris L., Brassica napus L. (cotyledons), Pisum sativum L., Galium aparine L. and Nicotiana tabaccum L. All of the plants were grown under conditions similar to those used for C. communis, however, in experiments using species other than C. communis, the leaf pretreatment stage was found to be unnecessary. Peel pretreatment and treatment conditions were as outlined for C. communis. The treatment solutions used contained 0.1 mol m^{-3} $CaCl_2$ and 5 or 150 mol m^{-3} KCl in the presence or absence of 10^{-4}M bentazone. The peels were floated, cuticle upwards, on the treatment solutions for

120 minutes under conditions designed to maintain opening or closure, 2 peels were removed at the end of the treatment period and 10 apertures measured from each. The experiment was repeated 3 times.

3.2.1.4 The effect of pH on the stomatal response to bentazone: Treatment solutions contained 0.1 mol m^{-3} $CaCl_2$ and 40 mol m^{-3} KCl in the presence or absence of 10^{-4}M bentazone. These solutions were prepared at pH 4, pH 5, pH 6 and pH 7 by adding minimal amounts of KOH and HCl. pH was monitored and was seen to remain constant throughout the experimental period. Treatment time was 120 minutes after which apertures were determined in the usual manner.

3.3 RESULTS

Figure 3.1 illustrates the dose-response obtained from incubating epidermes in the presence of bentazone. An optimal bentazone concentration of 10^{-4}M bentazone was observed and chosen for use in further studies.

Table 3.1 indicates that bentazone can alter stomatal movement in both susceptible and tolerant plant species. In those species known to be tolerant to this herbicide, bentazone caused no change or a decrease in stomatal aperture in the light with both high and low potassium concentrations. The trend with susceptible species, G. aparine and young B. napus, was to increase the aperture in the light under both low and high potassium regimes. The response of stomata to bentazone in darkness, however, did not appear to have any correlation with the susceptibility of the plant to the herbicide. In V. faba, C. communis, B. vulgaris and G. aparine, bentazone stimulated stomatal opening at low potassium concentrations, whilst in V. arvensis and B. napus it suppressed opening. In darkness, the results at high potassium concentrations showed that bentazone had no effect on V. faba, V. arvensis, P. sativum and G. aparine and that of the remaining four species, three once more showed bentazone stimulation of stomatal opening, whilst B. napus showed a decrease in stomatal opening.

Figure 3.2 further illustrates the effect of bentazone on the stomata of C. communis over a wide range of potassium concentrations, and includes the effect of the non-toxic bentazone derivative, 6-hydroxybentazone. In the light at low potassium concentrations (50 mol m^{-3}, Fig. 3.2(i)), 10^{-4}M bentazone stimulated stomatal opening, whilst at higher potassium concentrations (100 mol m^{-3}) apertures were lower than controls. However, 10^{-4}M 6-hydroxybentazone showed no ability to stimulate stomatal opening but markedly reduced opening above 100 mol m^{-3} KCl. In conditions designed to maintain stomatal closure (Fig. 3.2 (ii)) 10^{-4}M bentazone increased apertures throughout the potassium range, whilst 10^{-4}M 6-hydroxybentazone suppressed stomatal opening between 50 and 100 mol m^{-3} KCl. Figure 3.3 examines the effect of 10^{-4}M bentazone in the presence of MES buffer. In the light, the presence of buffer did not greatly alter the overall effect of bentazone (cf. Fig. 3.2 (i) with Fig. 3.3 (i)), however, in darkness MES totally negated any response to bentazone (cf. Fig. 3.2 (ii) with Fig. 3.3 (ii)).

Figure 3.4 demonstrates that bentazone is more able to open stomata at low pH in both light and darkness, and that the magnitude of the response to bentazone decreases with increasing pH. Neutral red staining indicated that none of these observed effects were due to guard cell damage, since guard cells were 95% viable at the end of incubation periods.

Figure 3.1

The effect of bentazone concentration on stomatal aperture in C. communis at 50 mol m^{-3} KCl under conditions designed to maintain opening (open circles) and closure (closed circles). Each point is a mean of sixty observations ± twice the standard error from three replicate experiments.

Figure 3.1

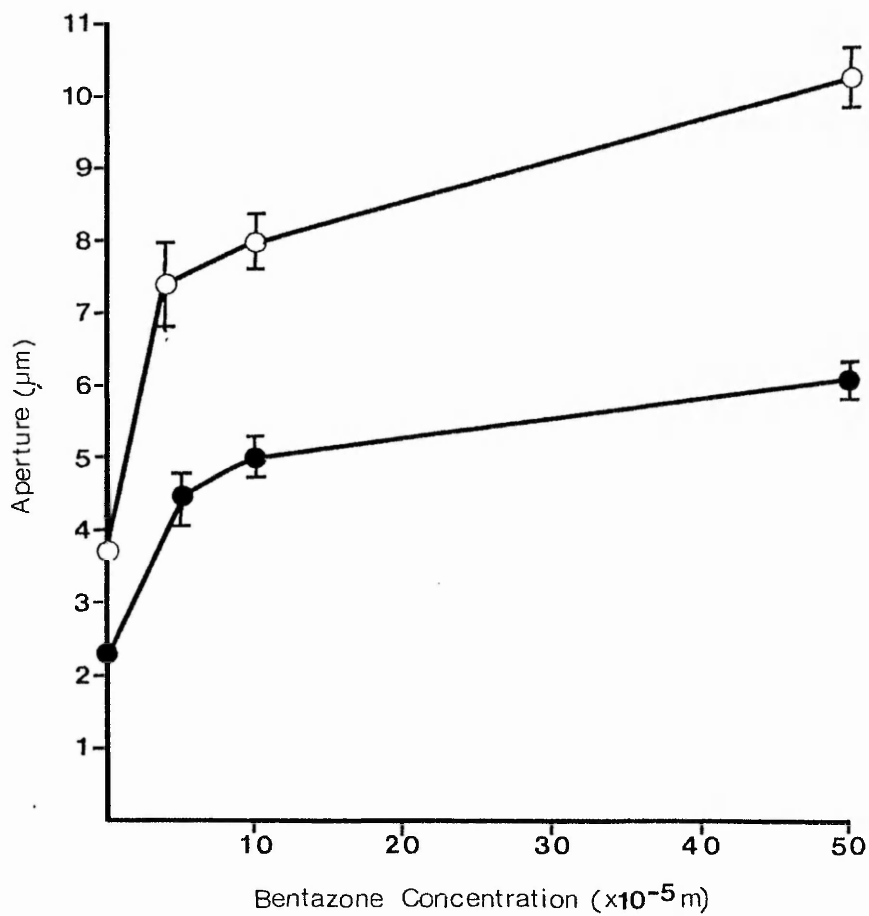


Table 3.1 Percentage change in stomatal aperture due to 10^{-4}M bentazone compared with the control

SPECIES	Light: % change in aperture caused by bentazone at 5 mol m^{-3} KCl	Light: % change in aperture caused by bentazone at 150 mol m^{-3} KCl	Dark: % change in aperture caused by bentazone at 5 mol m^{-3} KCl	Dark: % change in aperture caused by bentazone at 150 mol m^{-3} KCl	TOLERANT OR SUSCEPTIBLE
Vicia faba	23% decrease	33% decrease	18% increase	No change	tolerant
Beta vulgaris	No change	16% decrease	69% increase	44% increase	tolerant
Viola arvensis	29% decrease	28% decrease	19% decrease	No change	tolerant
Pisum sativum	33% decrease	25% decrease	No change	No change	tolerant
Brassica napus	5% increase	No change	49% decrease	17% decrease	susceptible at growth stage used
Galium aparine	20% increase	11% increase	93% increase	No change	susceptible
Commelina communis	221% increase	20% decrease	20% increase	30% increase	Not known
Nicotiana tabaccum	51% increase	7% increase	No change	23% increase	Not known

Figure 3.2

(i) The effect of 10^{-4}M bentazone and 10^{-4}M 6-hydroxybentazone on the response of C. communis stomata to potassium concentrations under conditions designed to maintain opening. Control (open circles), 10^{-4}M bentazone (open triangles), 6-hydroxybentazone (open inverted triangles).

(ii) The effect of 10^{-4}M bentazone and 10^{-4}M 6-hydroxybentazone on the response of C. communis stomata to potassium concentration under conditions designed to maintain closure. Control (closed circles), 10^{-4}M bentazone (closed triangles), 10^{-4}M 6-hydroxybentazone (closed inverted triangles).

Each point is a mean of 60 observations $\pm$ twice the standard error from 3 replicate experiments.

Figure 3.2(i)

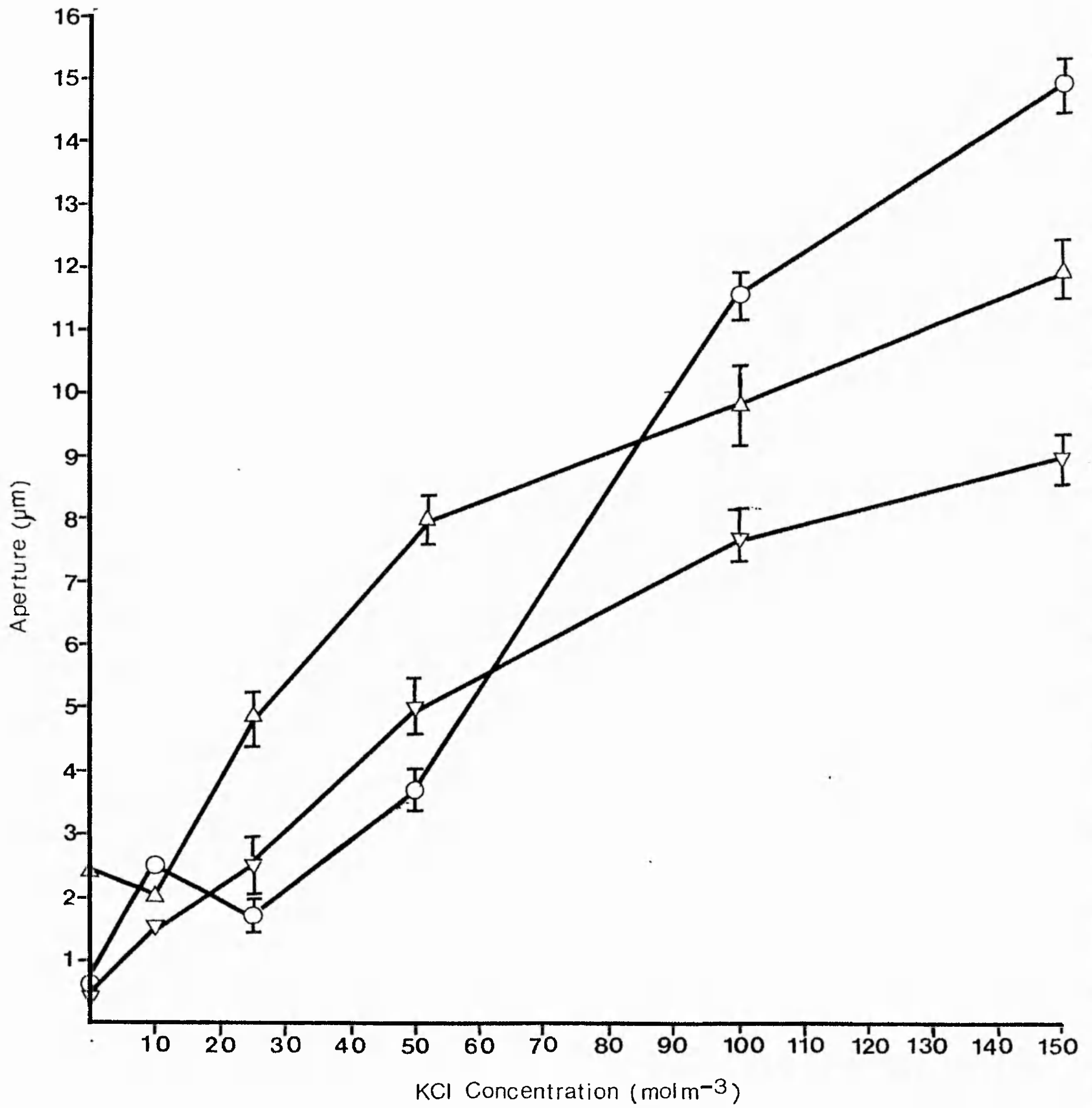


Figure 3.2(ii)

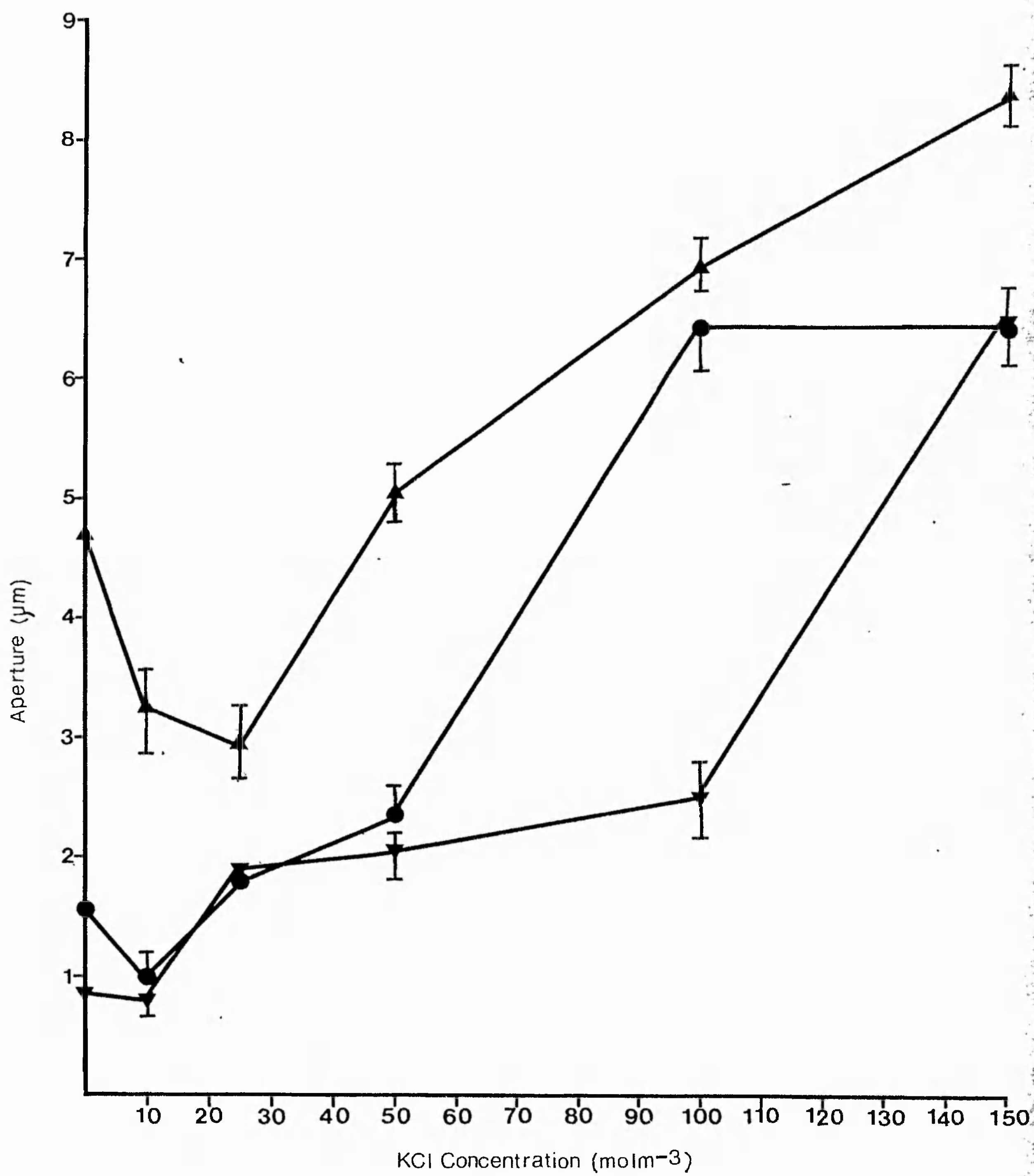


Figure 3.3

(i) The effect of 10^{-4}M bentazone on the response of C. communis stomata to potassium concentration in the presence of 10 mol m^{-3} MES buffer (pH 6.0) under conditions designed to maintain opening. Control (open circles), 10^{-4}M bentazone (open triangles).

(ii) The effect of 10^{-4}M bentazone on the response of C. communis stomata to potassium concentration in the presence of 10 mol m^{-3} MES buffer (pH 6.0) under conditions designed to maintain closure. Control (closed circles), 10^{-4}M bentazone (closed triangles).

Each point is a mean of 60 observations ± twice the standard error taken from 3 replicate experiments.

Figure 3.3(i)

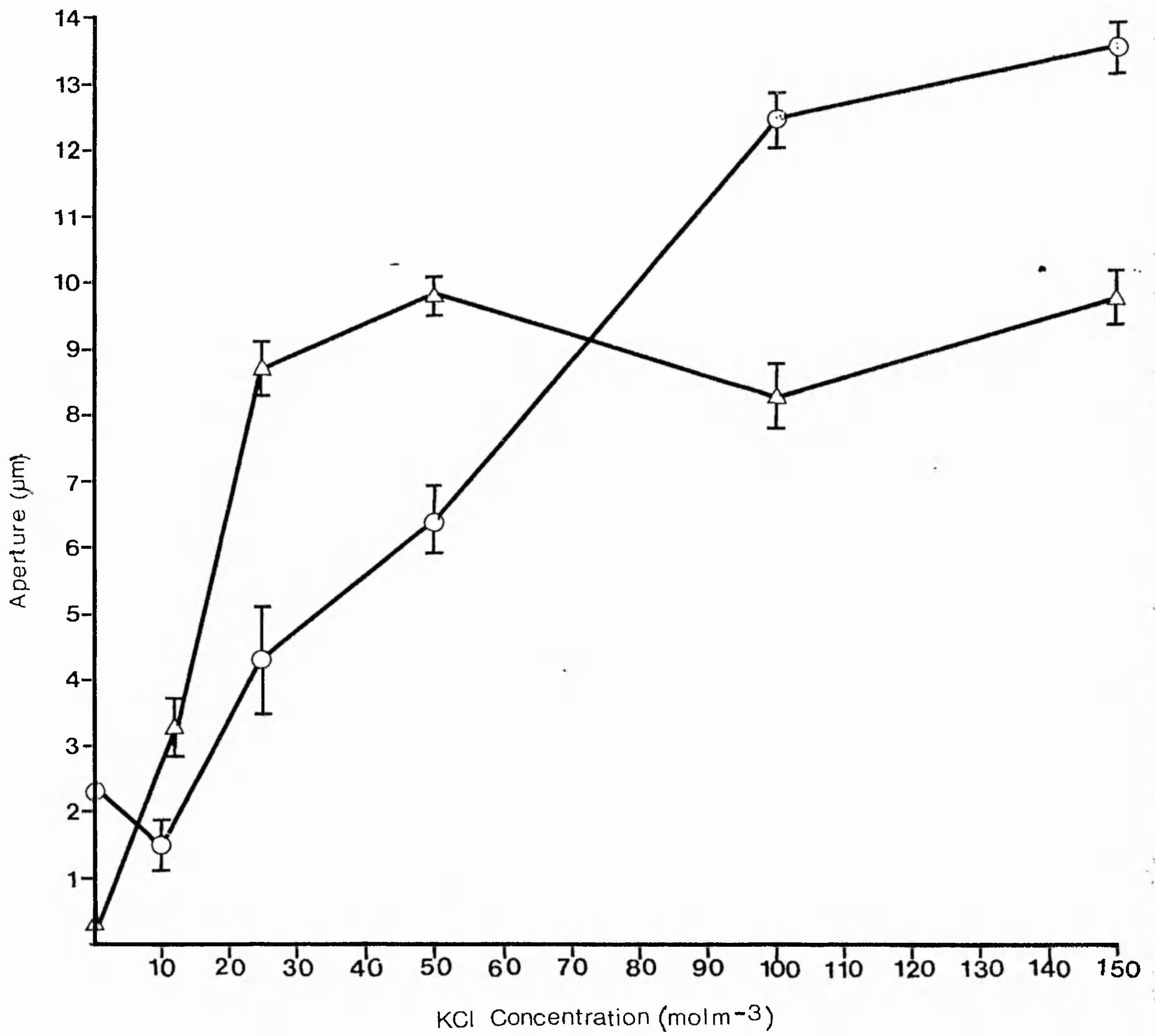


Figure 3.3(ii)

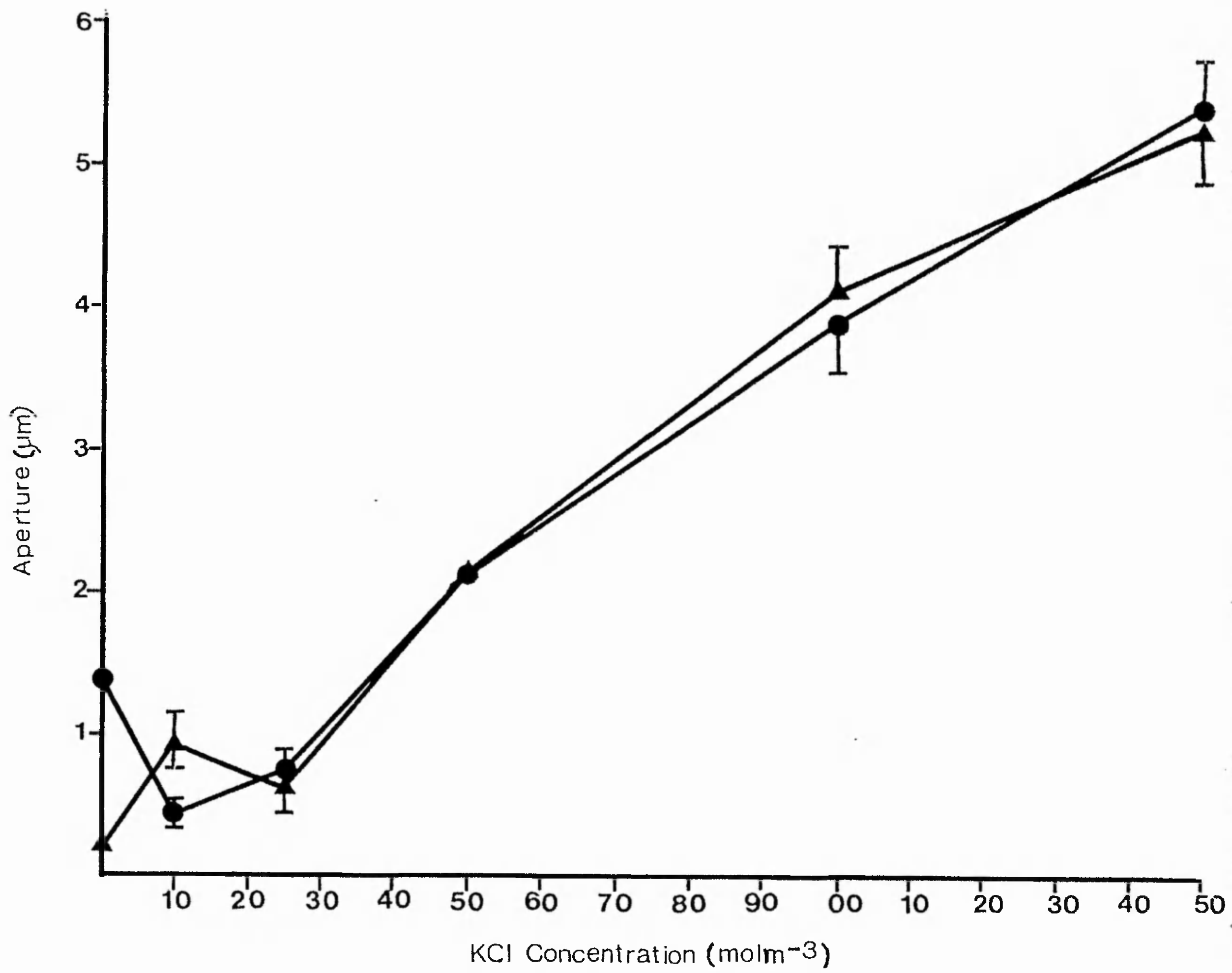


Figure 3.4

(i) The effect of pH on the stomatal response of C. communis to 10^{-4}M bentazone at 50 mol m^{-3} KCl under conditions designed to maintain opening. Control (open circles), bentazone treatment (open triangles).

(ii) The effect of pH on the stomatal response of C. communis to 10^{-4}M bentazone at 50 mol m^{-3} KCl under conditions designed to maintain closure. Control (closed circles), bentazone treatment (closed triangles).

Each point is a mean of 60 observations ± twice the standard error taken from 3 replicate experiments.

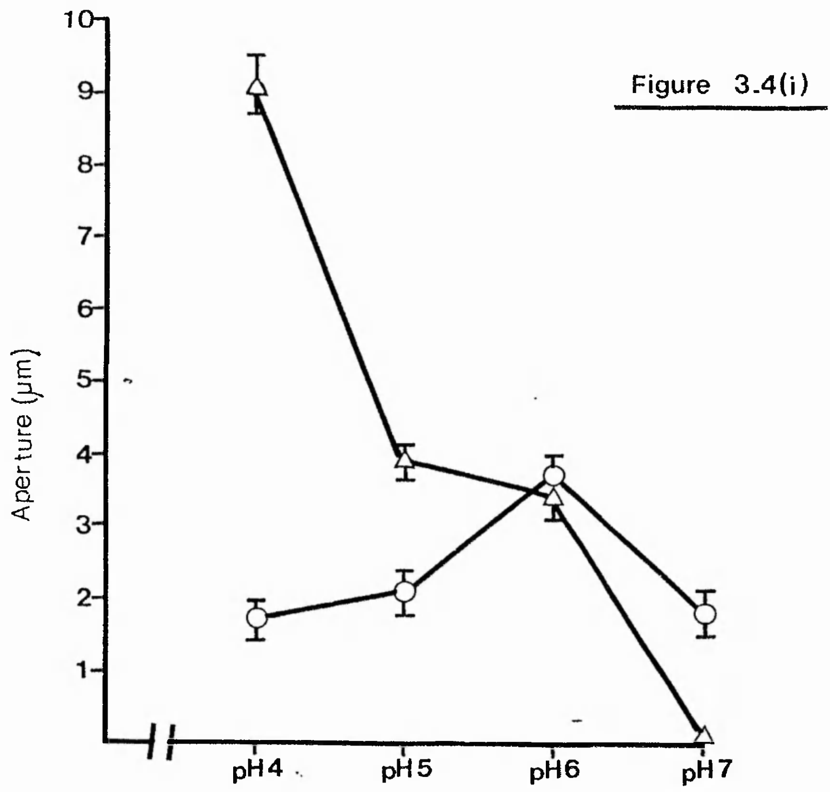


Figure 3.4(i)
Aperture (μm)
10
9
8
7
6
5
4
3
2
1
pH4
pH5
pH6
pH7

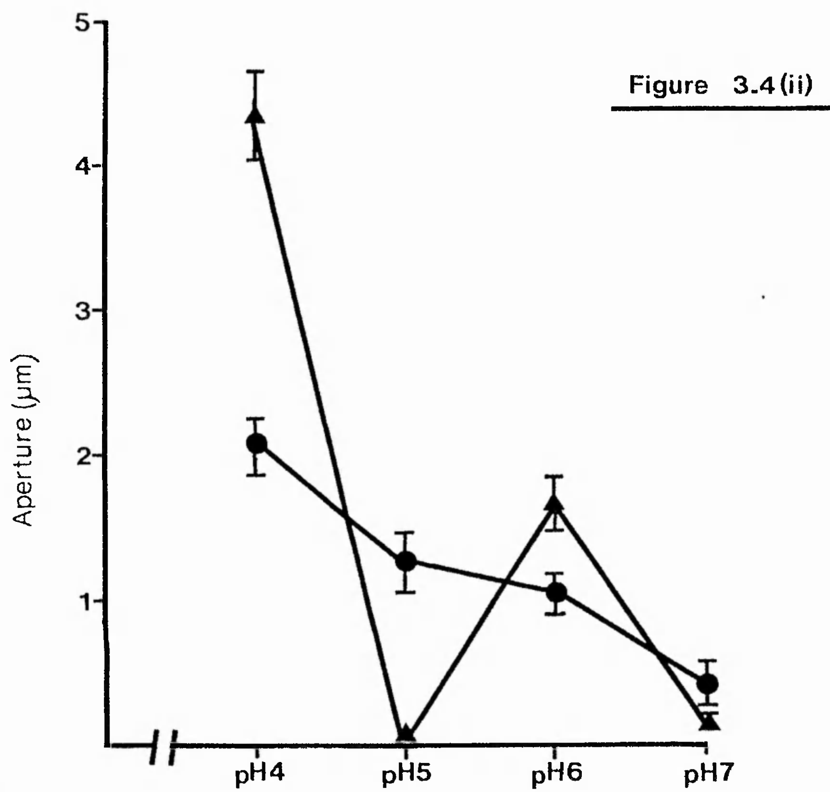


Figure 3.4(ii)
Aperture (μm)
5
4
3
2
1
pH4
pH5
pH6
pH7

3.4 DISCUSSION

The concentration of bentazone chosen for use with *C. communis* epidermal peels in this investigation was the same as that considered best for use with *C. album* i.e. 10^{-4}M (Dunleavy, 1983). Thus, it was thought that this concentration would be suitable when examining the effect of bentazone on the stomata of a range of plant species. From these studies it has been possible to show that bentazone can affect the movement of stomata in several species and that the effect is dependant on potassium concentration, light and darkness. These results therefore support and extend the findings of Taylor (1979) and Dunleavy (1983) on *C. album*. The possible correlation of susceptibility to the effect of bentazone on stomata in the light would tend to suggest that of those species whose sensitivity to the herbicide is unknown, both *N. tabaccum* and *C. communis* would be susceptible to bentazone. Indeed, the ability of bentazone to open stomata in the light would be likely to increase phytotoxicity as it has been demonstrated by Dunleavy *et al*, 1982, that more bentazone enters the plant when stomata are open. Also, when the stomata are open the rate of transport of chemicals within the plant is likely to be increased, as both photosynthesis and transpiration are increased, thus allowing more chemicals to reach their active sites. In the light, bentazone tends to decrease stomatal aperture in tolerant species, therefore possibly reducing uptake and transport of the herbicide. The reduction in rate of transport may allow for detoxification to take place before the herbicide reaches the target site. In darkness, there is no correlation between susceptibility and stomatal aperture, and this may suggest that the effect of bentazone in darkness is mediated by processes different to those employed in the light. When the response of the stomata of *C. communis* to bentazone was observed in greater detail (Figure 3.2) it was apparent that in the light the stimulation of stomatal opening occurred only at the lower potassium concentrations (50 mol m^{-3}) and that at higher potassium concentrations (100 mol m^{-3}) the ability of potassium to maintain stomatal opening was reduced. Earlier studies by Dunleavy (1983) using *C. album* had found the reduction of maintenance of stomatal opening at high potassium, but did not show the bentazone-stimulated opening in the light. These differences

may result from differences in the responsiveness of the stomata in each species. It should also be noted that due to the structure of the epicuticular lips on *C. album* small changes in aperture are difficult to measure, and this itself could yield the observed differences.

In darkness however, bentazone appeared to stimulate stomatal opening in *C. communis* over the complete potassium concentration range. This is in agreement with the trend observed by Dunleavy (1983) although the observed bentazone-stimulated opening of the stomata of *C. album* was over a much smaller range of low potassium concentrations (0-25 mol m^{-3} KCl). The non-toxic bentazone derivative 6-hydroxybentazone, the major metabolite produced in tolerant plants (Retzlaff et al, 1976), did not noticably stimulate stomatal opening in either light or darkness. In the light it gave a greater reduction in aperture at higher potassium concentrations (100 mol m^{-3}) than bentazone itself and in darkness it greatly reduced the ability of stomata to open in response to 100 mol m^{-3} potassium before returning to a value similar to that of the control at 150 mol m^{-3} potassium. Therefore, by altering the bentazone molecule slightly by the addition of a hydroxyl group in the 6-position both the toxicity of the molecule and the ability to stimulate stomatal opening are removed. MES buffer was added to see if it caused any alteration to the bentazone response. It did not greatly alter the response of stomata to bentazone in the light, however, in darkness it negated the stimulatory effect exerted by bentazone on stomatal opening. This once more demonstrates the ability of MES buffer to affect stomata that are in the process of opening (see Chapter 2) and strengthens the arguments against its further use in this investigation. The study on the effect of pH on bentazone activity showed conclusively that bentazone was more active at the lower pH (pH 4.0) where the stimulatory effect was strongly amplified. This effect was not due to guard cell damage as neutral red staining still showed 95% guard cell viability. This pH effect most likely reflects the proportion of bentazone which remains in the readily transportable lipophillic form (Retzlaff *et al*, 1984) and is also complemented by observations that at low pH bentazone also inhibits photosynthesis faster (Retzlaff *et al*, 1979).

The responses of stomata to other herbicides are poorly documented. However, of those cited 2,4-D (Zelitch, 1965; Mansfield, 1969), 2,4,5-T (Rao et al, 1977), atrazine (Zelitch, 1965), thiocarbamates (in C_4 plants, Das et al, 1975), alachlor and butachlor (Das et al, 1982) show a tendency to cause total or partial closure of the stomata in a wide range of plant species. Conversely, 2,4,5-T (Rao et al, 1977), triazine and urea type herbicides (Das et al, 1975), thiocarbamates (in C_3 plants: Das et al, 1975), glyphosate (Munoz-Rueda et al, 1986) and paraquat (Rao et al, 1977) are cited as being able to open stomata, however, of these only paraquat and 2,4,5-T seemed to show the type of rapid response observed with bentazone. The literature also claims that two defoliants thidiazuron (Elstner et al, 1983) and dimethipin (Metzger et al, 1984) have the ability to prevent stomatal closure.

The responses of bentazone, as demonstrated in this chapter, are rapid and marked, and as extensive laboratory tests have not been carried out on any of these other herbicides, no actual measurements of pore size quoted and a wide range of plant species used it is impossible to directly compare the findings of other workers with the bentazone response of C. communis stomata.

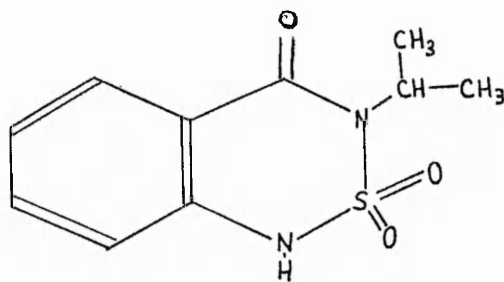
3.5 CONCLUSIONS

Bentazone can alter stomatal movement in several species by both stimulating and reducing apertures in a potassium concentration-dependant fashion, and is most active at low pH. Its non-toxic derivative 6-hydroxybentazone, is unable to stimulate stomatal opening but can reduce apertures relative to the controls. MES buffer can negate the ability of bentazone to open closed stomata on epidermal peels of C. communis maintained in closing conditions.

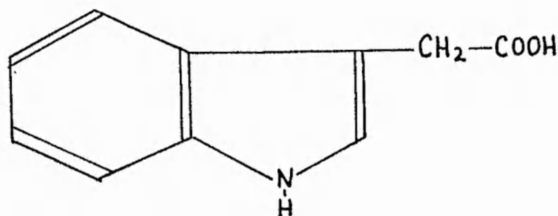
CHAPTER 4 STUDIES ON THE INTERACTION OF BENTAZONE WITH IAA. I. EFFECTS ON STOMATAL APERTURE

4.1 INTRODUCTION

The complete mode of action of the herbicide bentazone remains obscure. Bentazone has been shown to affect photosynthesis (Mine et al, 1975; Dunleavy, 1983), stomatal movement (Taylor, 1979; Dunleavy, 1983) and *Avena* coleoptile elongation (Farr, 1982; Miller and Cobb, 1986). In the coleoptile bioassay bentazone was able to stimulate coleoptile elongation in a manner similar to auxin (Miller et al, 1986). Both bentazone and auxin are complex molecules in which total depolarisation is unlikely, therefore charge separation across both molecules is possible.



Bentazone



IAA

Indeed, one of the earliest theories of auxin binding was based on the idea of a fixed charge separation across the molecules of auxin-active substances (Porter and Thimann, 1965). They postulated that the binding of auxin to a receptor was due to the separation of a fractional positive charge on the indole nitrogen and a negative charge on the carboxylic acid group. This distance they found to be 0.55 nm (Porter et al, 1965).

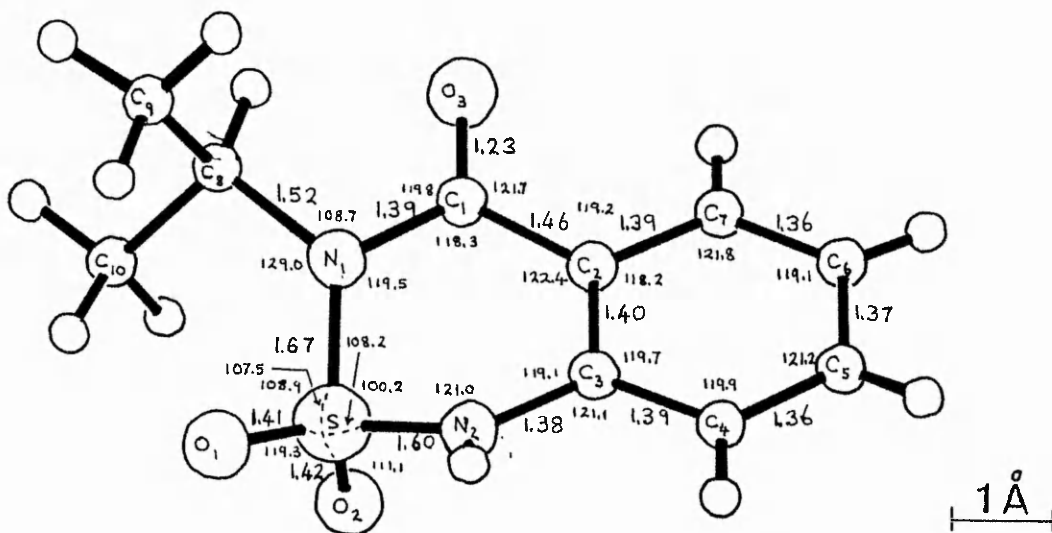


Diagram of the bentazone molecule giving the bond lengths (large print, Å) and the bond angles (small print, grads). Diagram supplied by BASF

Using detailed information on the bentazone molecule (shown above) it was calculated that a similar charge separation could occur between the C6 (δ+) and the sulphydryl oxygen. However, in 1978 Farrimond, Elliot and Clack found that the indole nitrogen of IAA was actually negative, whilst in some of the synthetic auxins, such as NAA, this nitrogen was positive. They also found that the charge separations were not exact and could be as low as 0.5 nm in some synthetic auxins. Following

further studies Farrimond, Elliot and Clack (1981) concluded that charge separation could not be cited as the sole basis of auxin activity.

Since these observations, attention has turned to models which suggest that auxin activity depends on a conformational change in the receptor induced by the binding of the auxin molecule. Two such models were proposed by Kaethner (1977) and Katekar (1979). Kaethners model required the bound molecule to undergo simultaneous conformational change at the receptor from a 'recognition' configuration to a non-planar 'modulation' configuration. This model holds for many auxin-active substances, but not all since some compounds which possess auxin activity such as arylcarboxylic acids cannot undergo the required transformational change (Venis, 1985). Katekars model is the more favoured at present (Venis, 1985) and envisages IAA binding in an extended planar conformation. The receptor is considered as an electrophilic area that accepts the indole ring and extends beyond it. It is thought that a conformational change would occur to the receptor on binding in this model, but as yet there is no proof (Venis, 1985).

The bentazone molecule is complex and may not ideally fit into any of the accepted models for auxin binding. However, Cross (1985) points out that there is no concrete proof that there is a connection between identified auxin binding sites and IAA action. Such proof, he states, would include a requirement that the site could be reconstructed, together with the appropriate macromolecular machinery to construct a model of the auxin response. Such statements merely reflect the relative paucity of information available on auxin binding sites in plants. Furthermore, although several proteins have been identified within the plant cell which will bind IAA (Venis, 1985) there have not yet been any related studies with herbicide binding.

This investigation compared the effects of bentazone and IAA on stomatal movement in C. communis, and presents observations on interactions in the presence of both molecules. The responses were observed from pH 4-7 since the activity of both chemicals is known to be altered by pH (Retzlaff et al, 1979; Kaldeway, 1984). Under conditions found to give maximal responses 6-hydroxybentazone was also included, to examine whether alterations at the carbon-6 position could affect any interaction with IAA.

4.2 MATERIALS AND METHODS

The basic treatment of *C. communis* leaves and epidermal peels was as described in Chapter 2.

4.2.1 Design of experiments

4.2.1.1 Determination of the effect of IAA concentration at different pH values: Treatment solutions contained 50 $molm^{-3}$ KCl, 0.1 $molm^{-3}$ $CaCl_2$ and 10^{-3} M, 10^{-4}M, 10^{-5}M or 10^{-6}M IAA (Sigma Chemicals, U.K.) and were adjusted to pH 4, 5, 6 or 7 using minimal amounts of KOH and HCl. Peels were floated on the treatment solutions for 120 minutes under conditions designed to maintain opening or closure after which two peels were removed and 10 apertures determined microscopically from each. The final pH value was determined and no alteration from the initial value was observed. The experiment was repeated three times.

4.2.1.2 Determination of interactions between bentazone and IAA: The above experiment was repeated in the presence of 10^{-4} M bentazone.

4.2.1.3 Time-course experiment to investigate the interaction between bentazone and IAA at pH 4: Treatment solutions contained 50 $molm^{-3}$ KCl and 0.1 $molm^{-3}$ $CaCl_2$ in the presence or absence of 10^{-4}M bentazone and/or 10^{-4}M IAA at pH 4. Treatment times were 0, 30, 60, 90 and 120 minutes. After each time interval two peels were removed from each treatment and 10 apertures determined from each. Separate Petri dishes were used at each time interval to prevent any alteration of aperture being caused by constant disturbance. Treatments were performed under conditions designed to maintain both opening and closure. Three replicate experiments were carried out.

4.2.1.4 Time-course experiment to investigate the interaction between 6-hydroxybentazone and IAA at pH 4: Experiment 4.2.1.3 was repeated, substituting 10^{-4}M 6-hydroxybentazone for bentazone.

4.2.1.5 Experiment to investigate the dependance of the bentazone/IAA interaction on potassium concentration: Treatment solutions contained 0.1 $molm^{-3}$ $CaCl_2$, 10, 50, or 150 $molm^{-3}$

KCl in the presence or absence of 10^{-4}M bentazone and 10^{-4}M IAA. All solutions were adjusted to pH 4 by adding minimal amounts of KOH and HCl. Three replicate experiments were performed.

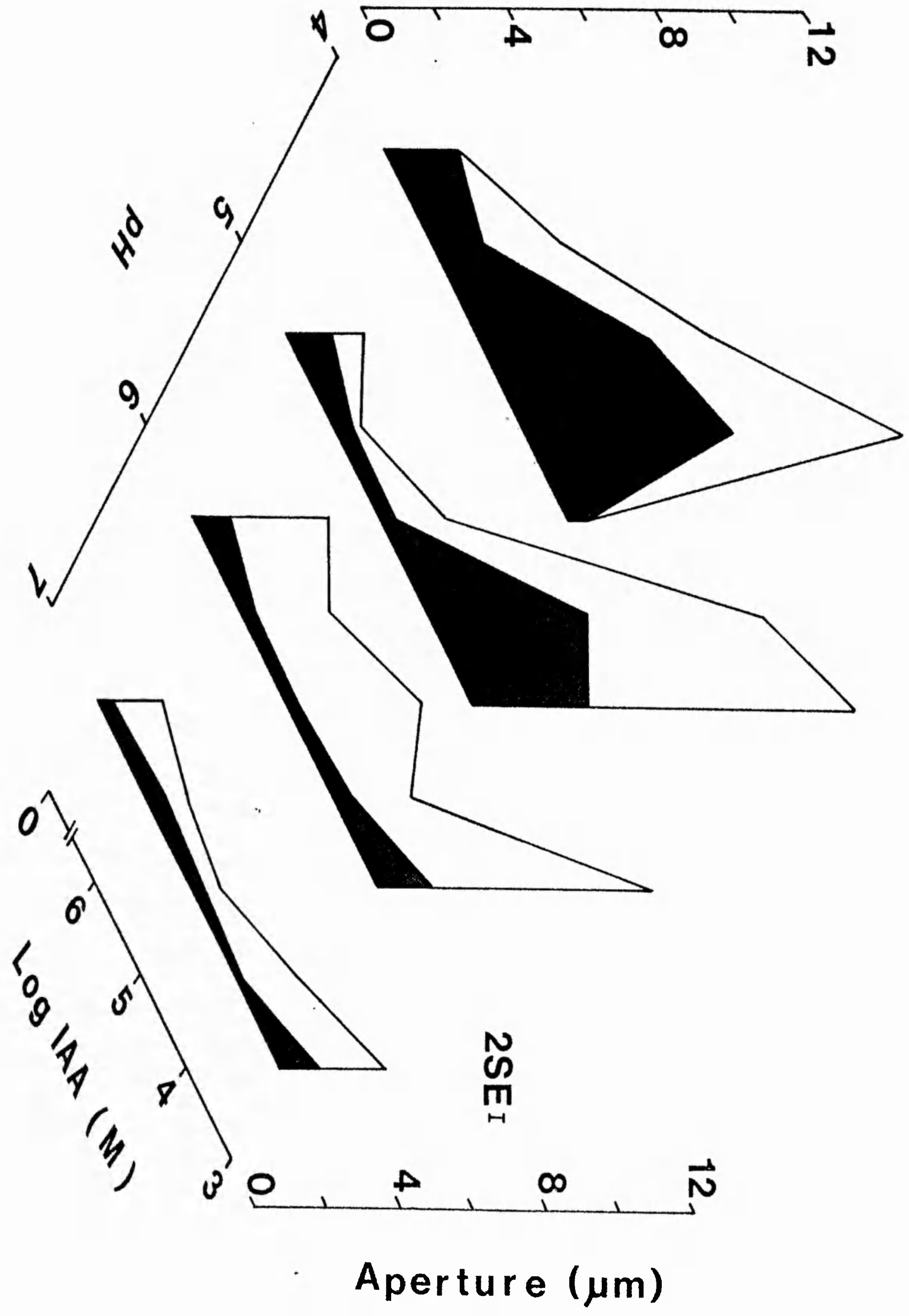
4.3 RESULTS

IAA was able to maintain and promote stomatal opening, although the concentration at which this occurred was greatly governed by the pH of the treatment solution (Figure 4.1). As pH was lowered the effect of IAA increased in both light and darkness until at pH 4, 10^{-3}M IAA became inhibitory, indicating a supra-optimal response. At lower pH values it is therefore possible to observe IAA stimulation of stomatal opening at physiologically active concentrations. At pH 6 and 7 there was little or no interaction between IAA and bentazone under either set of conditions (Figures 4.2 and 4.3), and under conditions designed to maintain closure (Figure 4.3) no interaction was observed at pH 5 either. However, in the light at pH 5 (Figure 4.2) and in darkness at pH 4 (Figure 4.3) the presence of both bentazone (10^{-4}M) and IAA (10^{-6}M) caused an increase in stomatal aperture. In the light, this effect was more than that obtained by adding the separate effects of the two chemicals together, suggesting a synergistic action. In the dark, this effect was close to that expected if the two chemicals were added together, suggesting an additive response. In the light at pH 4 (Figure 4.2) the most marked interaction was observed with 10^{-4}M bentazone and 10^{-4}M IAA. In this case, the response was well below that expected by combining the responses of the two chemicals and almost closed the stomata suggesting an antagonistic interaction. It was unlikely that these responses were attributable to guard cell damage as neutral red staining indicated guard cell viability to be around 95% at the end of the experimental period, even at pH 4. This was supported by the time-course experiments (Figures 4.4 and 4.5) which showed that the stomatal aperture continued to alter throughout the whole 120 minutes at pH 4. Figure 4.4 also shows that the interaction was established after only 60 minutes and after this time aperture fell below the control value. In darkness (Figure 4.5) the interaction was less dramatic and was not fully established until 90 minutes had elapsed, after which time the

plot of the interaction followed the control plot. In the light the final aperture produced by the interaction was almost unchanged by potassium concentration (Figure 4.6). However, in darkness the final aperture increased with potassium concentration up to 50 $molm^{-3}$ before levelling out (Figure 4.7). At pH 4 in the light (Figure 4.8), $10^{-4}M$ 6-hydroxybentazone increased aperture similarly to IAA, but when the two compounds were added simultaneously no interaction resulted. In darkness (Figure 4.9), 6-hydroxybentazone did not increase the stomatal aperture as much as $10^{-4}M$ IAA. However, when the two chemicals were applied together, the response was similar to that of IAA after 120 minutes, suggesting that in darkness the IAA response may occur regardless of the presence of 6-hydroxybentazone.

Figure 4.1 The relationship between IAA concentration (M), stomatal aperture (μm) and pH under conditions designed to maintain opening (unshaded area) and closure (shaded area).

Each point is a mean of 60 observations ± twice the standard error, from three replicate experiments.

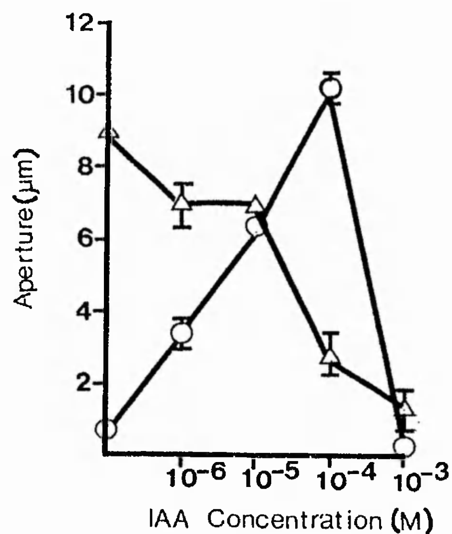


Aperture (µm)
12
8
4
0
pH
4
5
6
7
Log IAA (M)
0
6
5
4
3
2SE

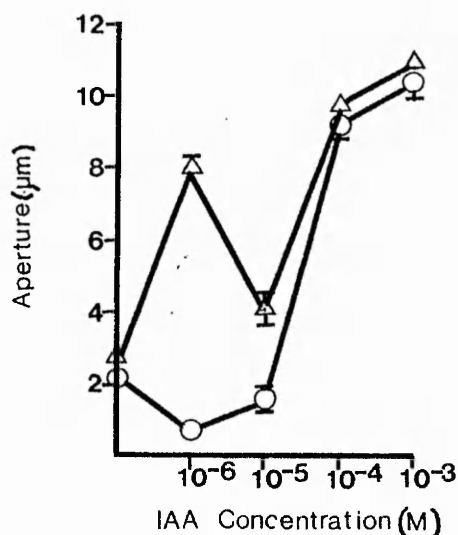
Figure 4.2 Comparison of the affect of IAA concentration (M) at different pH in the presence (open triangles) and absence (open circles) of 10^{-4}M bentazone under conditions designed to maintain stomatal opening.

Each point is a mean of 60 observations $\pm$ twice the standard error, taken from 3 replicate experiments.

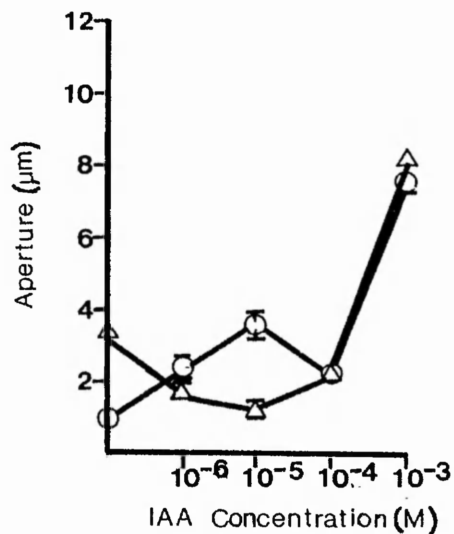
Figure 4.2



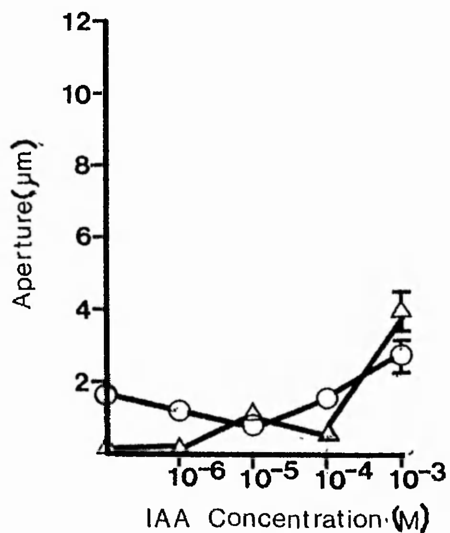
pH4



pH5



pH6

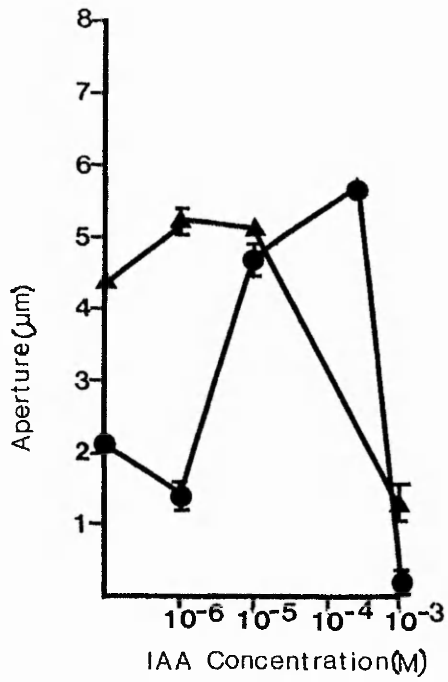


pH7

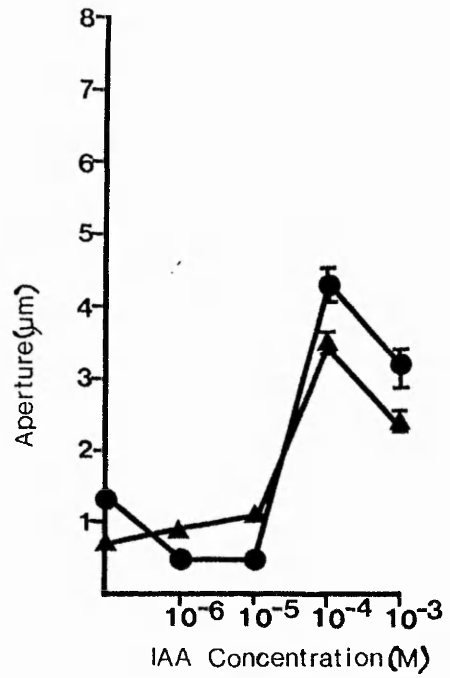
Figure 4.3 Comparison of the affect of IAA concentration (M) at different pH in the presence (closed triangles) and absence (closed circles) of 10^{-4}M bentazone under conditions designed to maintain stomatal closure.

Each point is a mean of 60 observations ± twice the standard error taken from 3 replicate experiments.

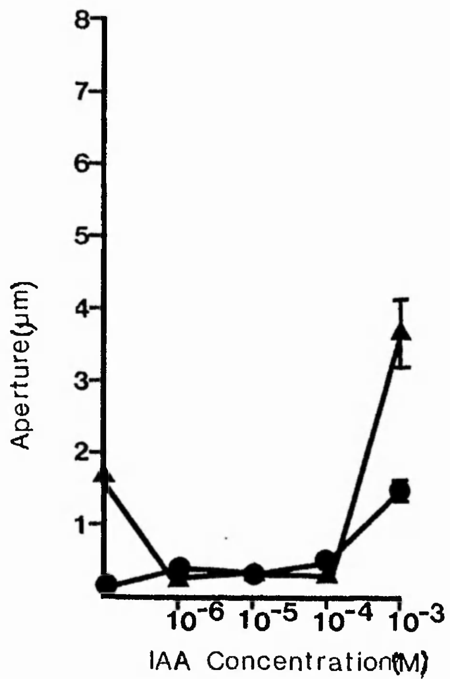
Figure 4.3



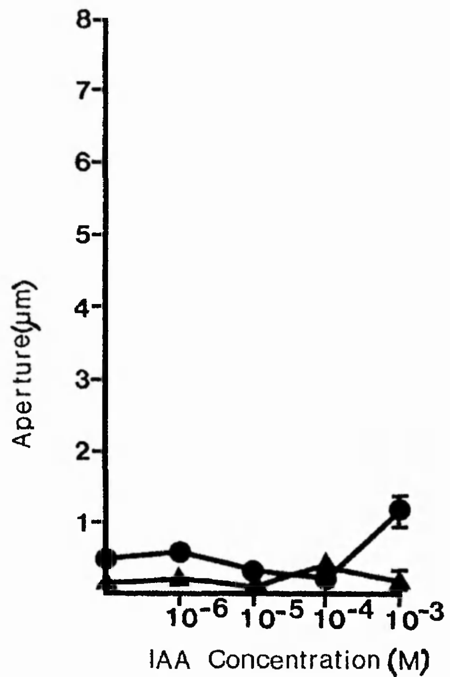
pH4



pH5



pH6



pH7

Figure 4.4 Time-course of the effect of 10^{-4}M bentazone (open triangles), 10^{-4}M IAA (open squares) and their interaction (open diamonds), in the presence of 50 molm^{-3} KCl, at pH 4 under conditions designed to maintain opening. Control value (open circles joined by a dotted line).

Each point is a mean of 60 observations $\pm$ twice the standard error taken from 3 replicate experiments.

Figure 4.4

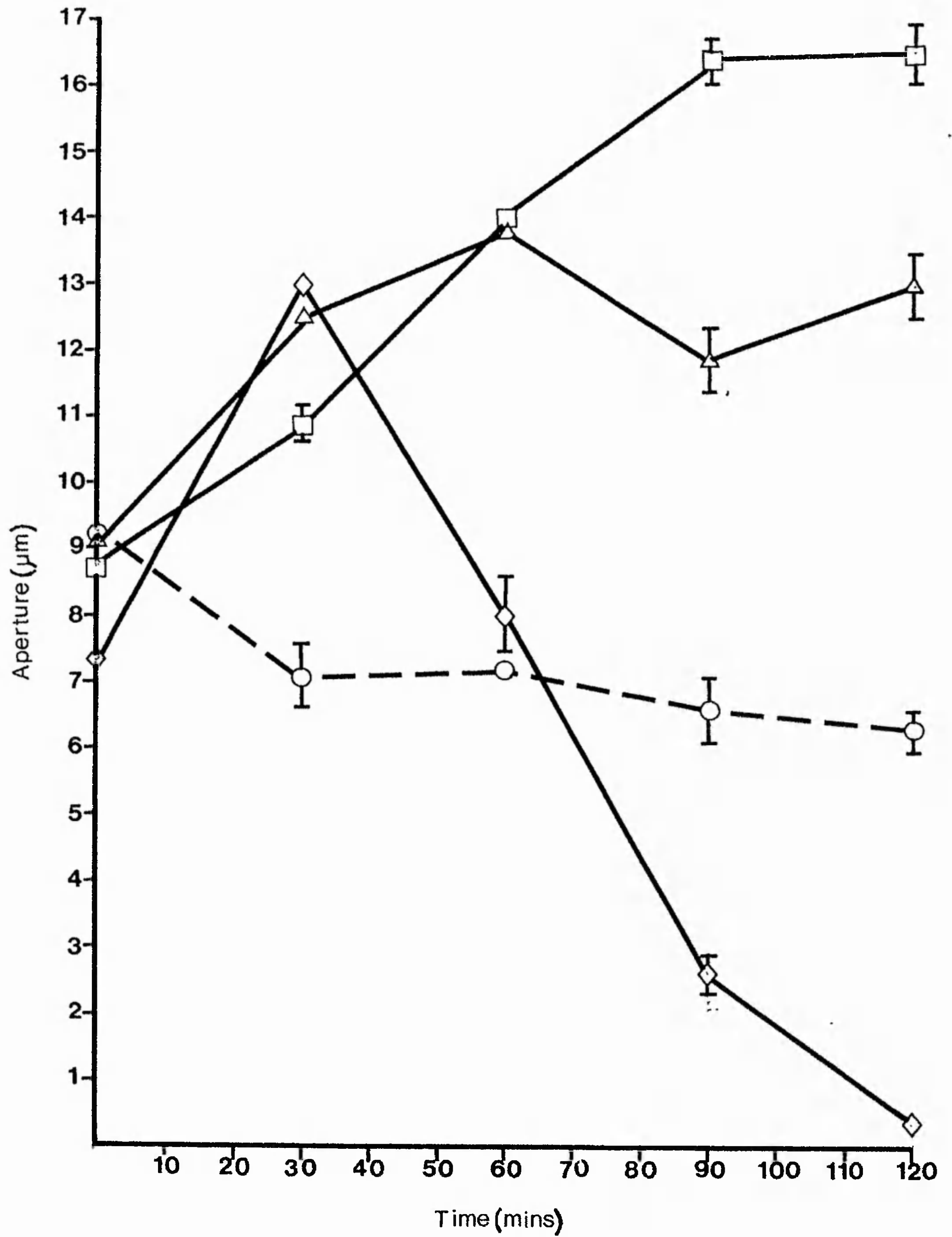


Figure 4.5 Time-course of the effect of 10^{-4}M bentazone (closed triangles), 10^{-4}M IAA (closed squares) and their interaction (closed diamonds), in the presence of 50 $mol m^{-3}$ KCl, at pH 4 under conditions designed to maintain stomatal closure. Control value (closed circles joined by dotted lines).

Each point is a mean of 60 observations ± twice the standard error taken from 3 replicate experiments.

Figure 4.5

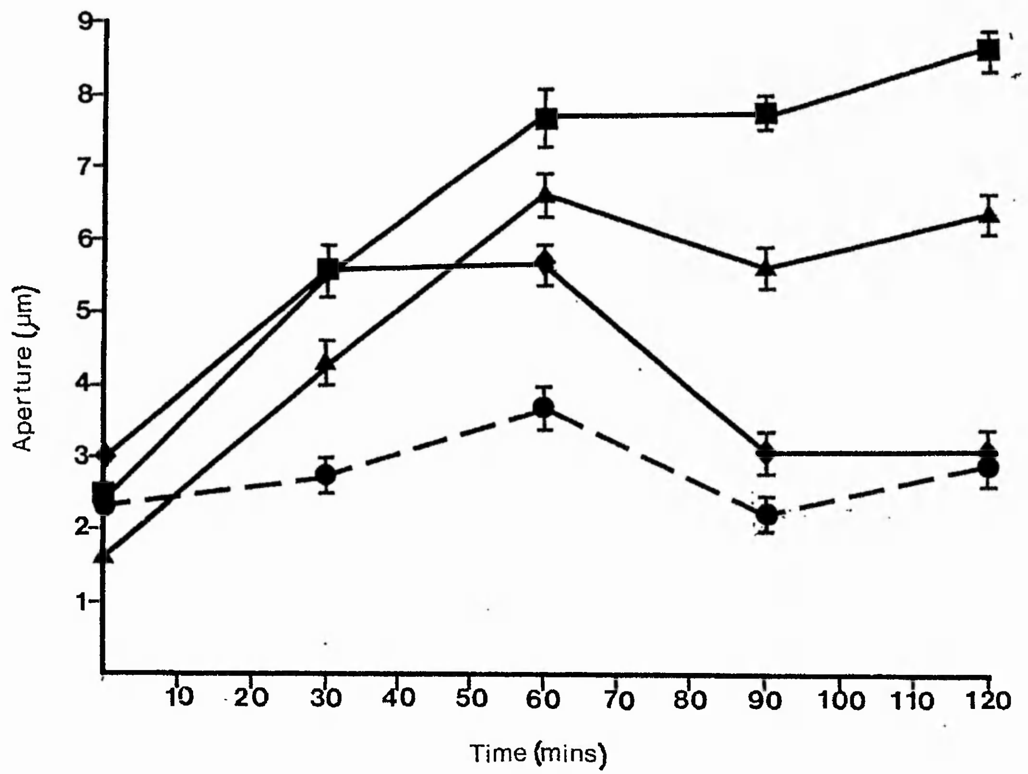


Figure 4.6 The effect of potassium concentration on the action of 10^{-4}M bentazone (open triangles), 10^{-4}M IAA (open squares) and their interaction (open diamonds) at pH 4 under conditions designed to maintain opening. Control (open circles joined by a dotted line).

Each point is a mean of 60 observations ± twice the standard error taken from 3 replicate experiments.

Figure 4.6

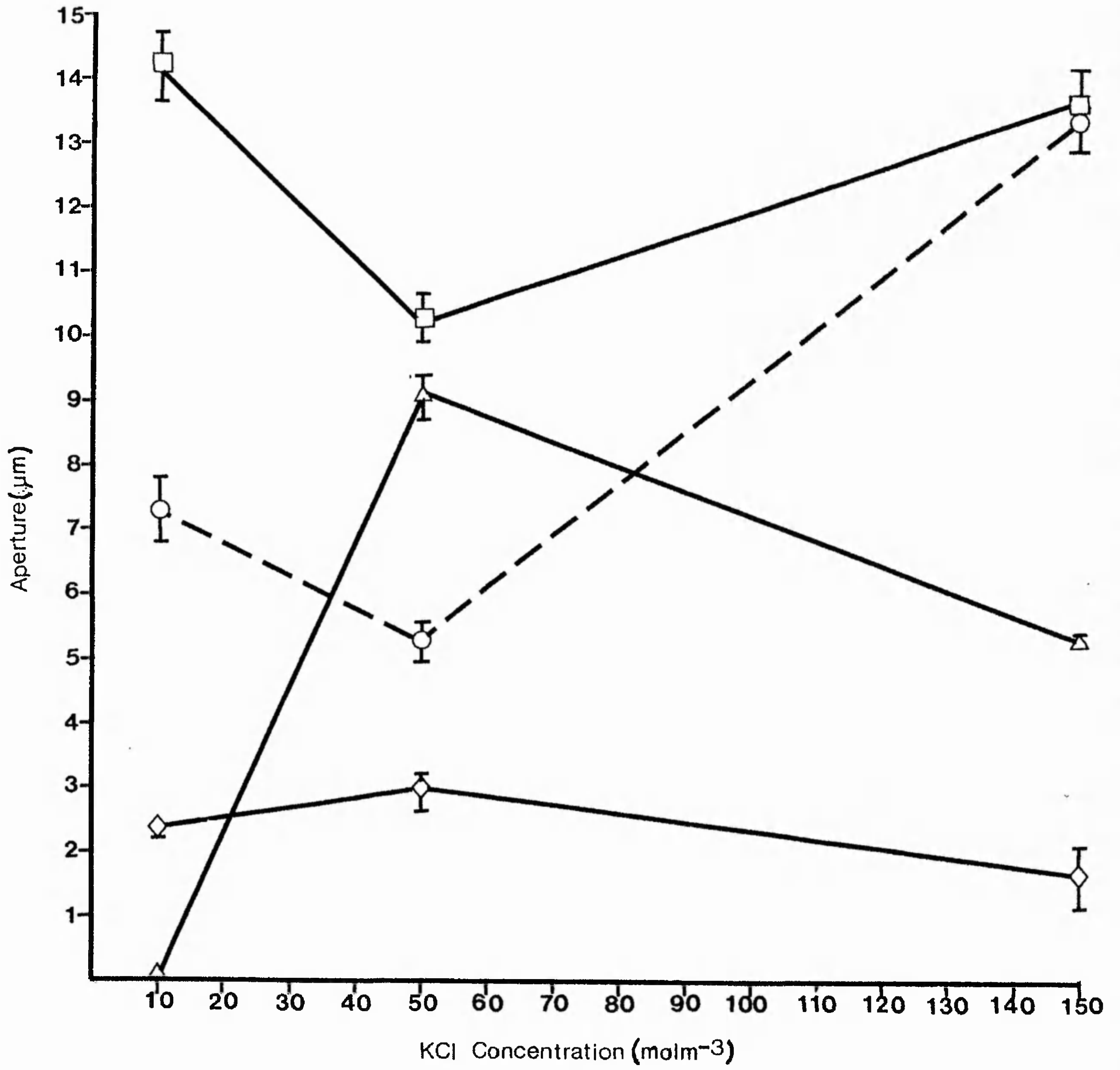


Figure 4.7 The effect of potassium concentration on the action of 10^{-4}M bentazone (closed triangles), 10^{-4}M IAA (closed squares) and their interaction (closed diamonds) at pH 4 under conditions designed to maintain stomatal closure. Control (closed circles joined by a dotted line).

Each point is a mean of 60 observations ± twice the standard error taken from 3 replicate experiments.

Figure 4.7

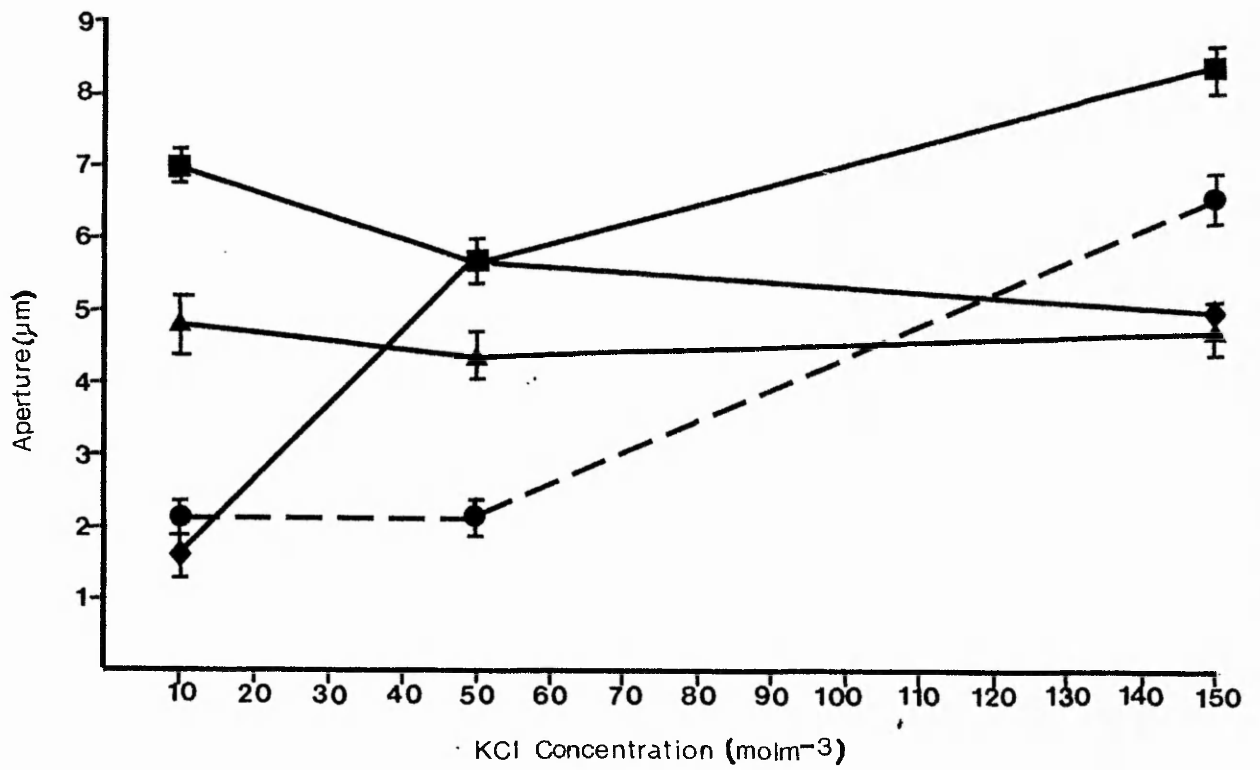


Figure 4.8 Time-course of the effect of 10^{-4}M 6-hydroxybentazone (open inverted triangles), 10^{-4}M IAA (open squares) and their interaction (open diamonds) in the presence of 50 molm^{-3} KCl at pH 4 under conditions designed to maintain stomatal opening. Control (open circles joined by a dotted line).

Each point is a mean of 60 observations ± twice the standard error taken from 3 replicate experiments.

Figure 4.8

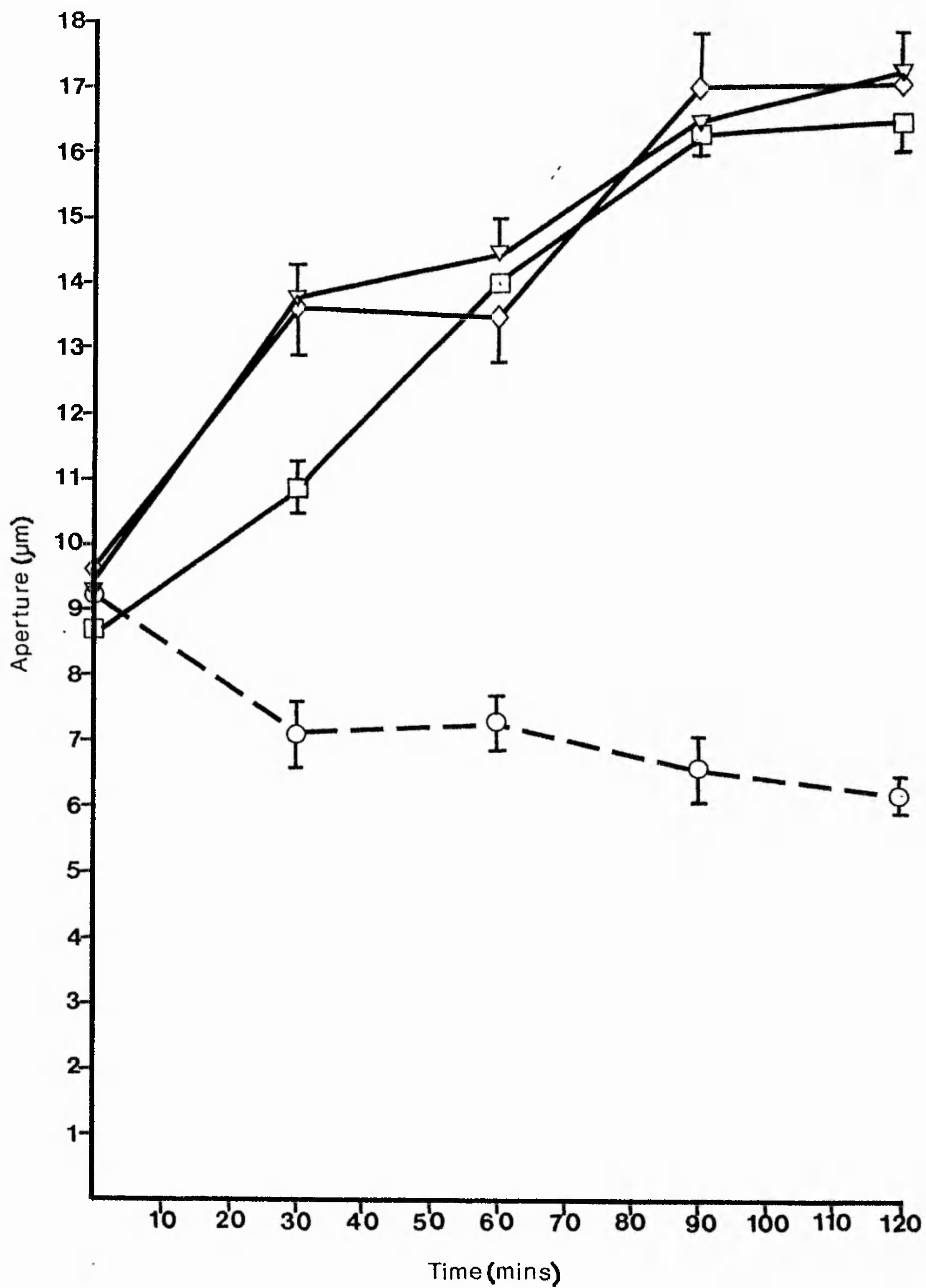
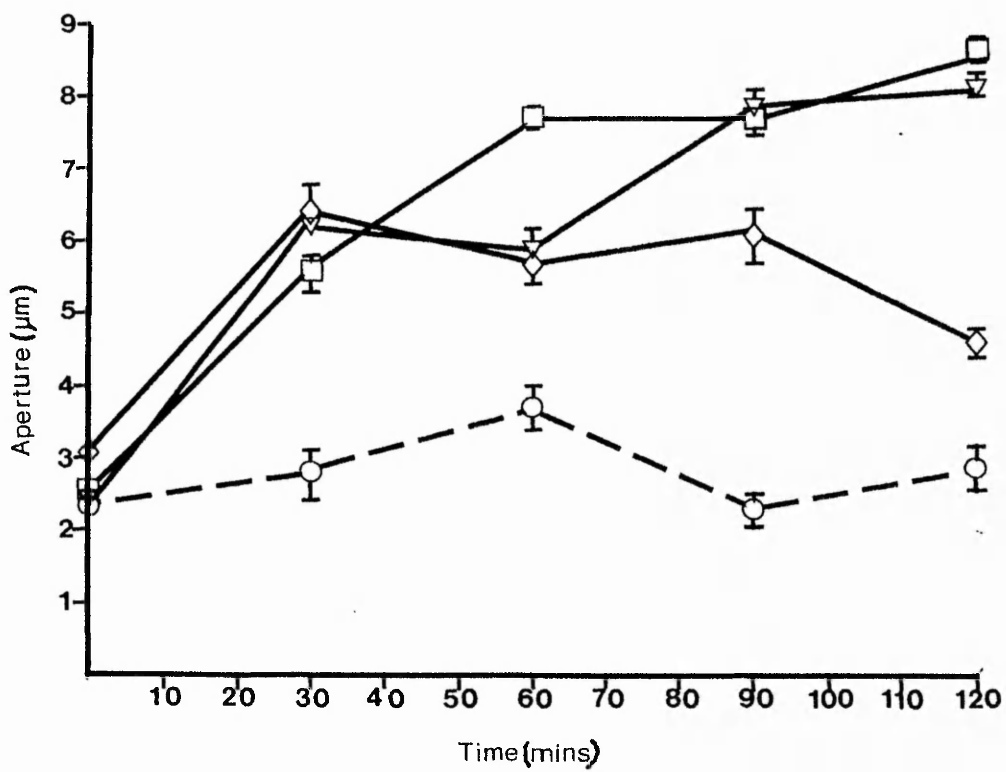


Figure 4.9 Time-course of the effect of 10^{-4}M 6-hydroxybentazone (closed inverted triangles), 10^{-4}M IAA (closed squares) and their interaction (closed diamonds) in the presence of 50 $molm^{-3}$ KCl at pH 4 under conditions designed to maintain stomatal closure. Control (closed circles joined by a dotted line).

Each point is a mean of 60 observations ± twice the standard error taken from 3 replicate experiments.

Figure 4.9



4.4 DISCUSSION

Findings presented in this chapter reveal the sensitivity of C. communis stomata to exogenous IAA. They indicate a novel action of auxin on abaxial stomata in darkness, since only responses in darkness with adaxial stomata have been previously reported (Pemadasa, 1982; Mansfield, Pemadasa and Snaith, 1983). Figures 4.4 and 4.5 show time-courses of the response to 10^{-4}M IAA at pH 4 and indicate a rapid action on the guard cell at physiologically active concentrations in both light (Figure 4.4) and darkness (Figure 4.5). The responses obtained at higher pH values are in agreement with other workers who found maximal responses around 10^{-2}M IAA (Eamus et al, 1984). However, such high concentrations are rarely, if ever, found in plants under normal circumstances. Some workers have shown an opening response to lower IAA concentrations with abaxial stomata under conditions designed to cause closure (e.g. high CO_2 and ABA; Mansfield et al, 1983), although other studies using IAA concentrations more representative of endogenous levels, under conditions designed to open stomata, did not find any IAA-initiated response on transpiration or stomatal movement (Livne et al, 1965).

The observed responses to IAA were likely to reflect the relative amounts of dissociated ($IAA^- + H^+$) and protonated (IAA - H) molecule at each experimental pH (pKa = 4.7). At pH values around neutral the equilibrium favours dissociation whilst at pH 4 the equilibrium favours the protonated form. The chemiosmotic theory predicts that the lipophilic weak acid (IAA - H) crosses the cell membrane more easily than the anion (IAA^-) (Kaldeway, 1984). Thus, although the same concentration of IAA is used over the experimental pH range, variation still exists in the concentration of readily transportable IAA-H. Consequently, at pH 4, where the majority of IAA is in the protonated form, the most marked and rapid responses were observed (Figures 4.1, 4.4 and 4.5).

Bentazone, like IAA, is a molecule which effects a stomatal response that varies with pH. It has also been shown to be rapid in the inhibition of photosynthesis at low pH in isolated cells of G. max (Retzlaff et al, 1979; Rees, 1986), although in these studies the lowest pH media used were around pH 6 since greater acidity impaired

structural and physiological integrity. Indeed, Figures 4.2 and 4.3 show that in the absence of IAA, bentazone induces the greatest variation in stomatal aperture from that of the control at the lowest pH value, in this case pH 4, under conditions designed to maintain opening (Figure 4.2) and closure (Figure 4.3). The pH-dependence may once more be a result of the degree of dissociation of the molecule. As the pH of the solution decreases the amount of undissociated bentazone increases therefore increasing the concentration of the lipophilic form which can easily traverse the membrane. It therefore follows that interactions between IAA and bentazone are most likely to occur at low pH where both molecules are able to reach their active sites more readily and are presumably taken up in greater quantities. This was indeed the case, the most pronounced interactions being found at pH 4 under both sets of experimental conditions (Figures 4.2 and 4.3).

In this Chapter, three types of auxin/bentazone interaction have been observed, synergistic (pH 5, 10^{-4}M bentazone and 10^{-6}M IAA in the light), additive (pH 4, 10^{-4}M bentazone and 10^{-6}M IAA in darkness) and antagonistic (pH 4, 10^{-4}M bentazone and 10^{-4}M IAA in the light). This range of results may be explained assuming that IAA-stimulated stomatal opening occurs over a specific IAA concentration range, that bentazone acts similarly to IAA and that there is an optimal concentration for the response after which IAA becomes inhibitory to stomatal opening. Such concentration responses to auxin are well known with respect to the growth of roots, buds, leaves and stems (e.g. Goodwin and Mercer, 1983). It must also be assumed that the important concentration of both IAA and bentazone is the concentration of the undissociated lipophilic forms of both molecules. At pH 5 in the light (Figure 4.2), the guard cells were sensitive to both IAA and bentazone, and as the pH was below neutral a substantial amount of each molecule would be present in the undissociated lipophilic form. In this form both molecules were readily available to the guard cells. Assuming that bentazone and IAA were acting similarly it was considered that the combined concentration of these chemicals reaching the active site was close to the optimum concentration for stimulation of stomatal opening and thus gave the increased stimulation of stomatal opening. This was

not seen in darkness at pH 5, possibly because the response of stomata in darkness tends to be slower. However, at pH 4 similar concentrations of IAA and bentazone were shown to interact in darkness in an additive manner. At pH 4 the guard cell would be even more sensitive than at pH 5 and the relative amounts of undissociated IAA and bentazone would also be greater. As a consequence of these factors it was possible to observe an effect in darkness. There are at least two possible explanations as to why the effect was additive and not synergistic. The first is simply that the response is slower in darkness and may not be maximal at 120 minutes, and the second is that the increased concentrations of undissociated compounds may have become supra-optimal and be starting to inhibit stomatal opening. The antagonistic interaction in the light at pH 4 with 10^{-4}M bentazone and 10^{-4}M IAA (Figure 4.2 and Figure 4.4) may be explained by the combined concentration of undissociated IAA and bentazone being well above the optimum and therefore causing a suppression of stomatal opening.

The interaction between 10^{-4}M bentazone and 10^{-4}M IAA in the light was not dependant on potassium concentration (Figure 4.7), whereas in the dark it was potassium dependant. This may be due to slightly different systems being involved in stomatal movement under each set of environmental conditions. Light itself can induce stomatal opening (see Chapter 1.3.1) and can open stomata at low potassium levels. However, in darkness the opening is almost totally due to the external potassium supply as the high CO_2 concentrations and other environmental conditions have been specifically chosen to maintain stomatal closure, and therefore the interaction is likely to be potassium dependant.

The speed of the response of C. communis stomata to IAA and bentazone would tend to suggest a "membrane effect" rather than a "metabolic change". Such an effect may be due to the action of these compounds on a plasmalemma located electrogenic pump, (possibly K^+/H^+ exchange), which has been postulated as a possible mechanism involved directly in stomatal movement (Outlaw, 1983; Edwards and Bowling, 1985). IAA is thought to exert its effect on cell wall elongation by acting on a proton pump (Goodwin et al, 1983). It is of course possible that both chemicals may exert similar effects on stomatal movement by different mechanisms as the stomatal system is complex and governed by many factors.

At pH 4, 6-hydroxybentazone was able to stimulate stomatal opening in the light to a similar extent as IAA (Figure 4.8), in darkness it was also able to stimulate stomatal opening but by less than IAA (Figure 4.9). In both light and darkness when the compounds were mixed, the apertures were similar to those observed with IAA alone. It would therefore seem that substitution by a hydroxyl group at the 6-position prevents an interaction with IAA. This may be the result of an alteration of the charge distribution which makes the molecule unable to act at a similar site to IAA.

Although these studies have shown that bentazone and IAA exert similar effects on stomatal aperture they have not shown whether the mechanism of action was the same. There are many possibilities for a mechanism and it is hoped that the studies presented in Chapters 5 and 6 will further clarify the situation.

4.5 CONCLUSIONS

This study has shown that both IAA and bentazone have the ability to open stomata at low pH, and that at pH 4 an interaction occurs between the two compounds which may be dependant on the maintenance of a specific charge separation on the bentazone molecule.

CHAPTER 5. STUDIES ON THE INTERACTIONS OF BENTAZONE WITH IAA. II. EFFECTS ON NAD-DEPENDENT MALATE DEHYDROGENASE

5.1 INTRODUCTION

Malate is considered to have an important role in stomatal movement and is thought to donate the protons which are extruded by the proton pump to balance potassium uptake (Pearson, 1973; Pearson et al, 1974; Outlaw et al, 1977; Schnabl, 1980a; Reddy et al, 1983). Many of these workers have been able to show a direct correlation between malate concentration and stomatal aperture.

Studies on the enzymic content of guard cells have shown quite high levels of phosphoenolpyruvate (PEP) carboxylase activity (Willmer, Kanai, Pallas and Black, 1973; Willmer et al, 1973; Raghavendra, 1980; Schnabl, 1980a; Schnabl, Elbert, and Kramer, 1982; Birkenhead and Willmer, 1986), whereas few studies have demonstrated ribulose bisphosphate carboxylase activity in the guard cells. Thus it would seem prudent to assume that carbon fixation in the guard cell is due almost entirely to the enzyme PEP carboxylase.

The action of PEP carboxylase is most often associated with the fixation of CO_2 in C_4 and CAM plants. PEP carboxylase catalyses the reaction PEP $\xrightarrow{H_2O\ CO_2}$ Oxaloacetic acid (OAA), the OAA can then be reduced to malate in a reaction catalysed by malate dehydrogenase. In CAM plants the malate produced is actively transported across the tonoplast membrane into the vacuole, thus keeping the cytoplasmic malate concentration low. This prevents allosteric inhibition of PEP carboxylase by malate (Kluge, 1976). Malate dehydrogenase activity has been demonstrated in guard cells (Willmer et al, 1973; Birkenhead et al, 1986) and most of this activity is attributed to the NAD-dependant malate dehydrogenase (Birkenhead et al, 1986).

A system which may operate in guard cells has been proposed by Goodwin et al, 1983. The guard cell has a proton pump located on the plasmalemma which pumps protons out of the cell. The removal

of these protons causes the cytoplasmic pH to rise to pH 8-9, which is around the optimum for PEP carboxylase activity, and also allows passive movement of potassium ions across the membrane into the cell. PEP carboxylase catalyses the conversion of PEP to OAA and then malate dehydrogenase converts the OAA to malate which is stored in the vacuole and draws water into the cell causing swelling. The malate is also capable of donating the protons which are extruded by the proton pump.

On stomatal closure the guard cell appears to dispose of malate in several ways (Dittrich et al, 1977). Some of the malate becomes involved in gluconeogenic pathways and is stored as starch (Dittrich et al, 1977; Schnabl, 1980; Birkenhead et al, 1984), whilst some is utilized in the tricarboxylic acid cycle by the numerous mitochondria (Pearson et al, 1974; Dittrich et al, 1977; Robinson and Preiss, 1985). Dittrich et al (1977) also found that epidermal peels of *C. communis* released some of their malate into the bathing medium on stomatal closure.

This study therefore, intends to examine the effect of bentazone and IAA on the NAD-dependent malate dehydrogenase (E.C. 1.1.1.37), to establish whether these chemicals may alter the metabolic processes occurring in guard cells during stomatal movement.

5.2 MATERIALS AND METHODS

The treatment of *C. communis* leaves and peels was as stated in Chapter 2.

5.2.1 Design of experiments

5.2.1.1 Determination of the effect of bentazone on malate dehydrogenase activity at varying potassium concentrations:

Treatment solutions contained 0.1 mol m^{-3} $CaCl_2$ and 0, 10, 50, 100 mol m^{-3} KCl in the presence or absence of 10^{-4}M bentazone. The number of peels treated was 85 and the incubation time was 120 minutes under both sets of environmental conditions, after which time 2 peels were removed and their apertures determined microscopically. The remaining peels were then placed in an ice-cold hand homogeniser and

ground in 1 ml of ice cold 0.25 M phosphate buffer (pH 7.4). This homogenate was transferred into 1.5 ml microfuge tubes and centrifuged at 13,000 rpm (11,600 g) for 4 minutes in a Micro Centaur microfuge. As the enzyme is cytoplasmic the activity was almost entirely restricted to the supernatant and this was retained and assayed for malate dehydrogenase activity and total soluble protein content.

5.2.1.2 Determination of the effect of bentazone, IAA and a mixture of both chemicals on malate dehydrogenase activity at pH 6 and pH 4: Treatment solutions contained 0.1 mol m^{-3} $CaCl_2$ and 50 mol m^{-3} KCl in the presence and absence of both 10^{-4}M bentazone and 10^{-4} IAA at pH 6 and pH 4. Following 120 minutes of treatment, under conditions designed to maintain both opening and closure, 2 peels were removed and their apertures determined. The remaining 80 peels were treated as described in 5.2.1.1.

5.2.2 Assay procedures

5.2.2.1 Malate drhydrogenase assay: Two glass 3 ml cuvettes were prepared as follows:-

Stock solutions	Blank	Test
0.25 M phosphate buffer, pH 7.4	0.30 ml	0.30 ml
0.0076 M OAA (Sigma Chemicals) in buffer, pH 7.4	0.10 ml	0.10 ml
H_2O	2.55 ml	2.45 ml
0.0015 M NADH in buffer, pH 7.4	-	0.10 ml
Supernatant	0.05 ml	0.05 ml

The UV/VIS Spectrophotometer (Perkin-Elmer 550S) was zeroed on a dual blank prior to the addition of NADH to the test solution. The oxidation of NADH was followed with respect to time at 340 nm. The protein content of the supernatant was measured by the Lowry protein assay and the specific activity of the enzyme was calculated for each treatment condition.

5.2.2.2 <u>Protein determination:</u> The method followed for the Lowry protein assay was that outlined by Schleif and Wensink (1981). Stock solutions of 2% aq. (w/v) copper sulphate, 4% aq. (w/v) potassium tartrate, 3% aq. (w/v) sodium carbonate in 0.1 M sodium hydroxide, and Folin-Ciocalteau phenol reagent were prepared prior to the experiment. Immediately before the assay the solutions were mixed as follows:-

2 ml copper sulphate solution plus 2 ml potassium tartrate solution plus 98 ml of the sodium carbonate solution and the Folin-Ciocalteau reagent was diluted 1:1 with water.

A standard curve was made using bovine serum albumin (BSA; Sigma fraction V). Aliquots of BSA containing 0-150 μg protein/ml H_2O were placed in test tubes and adjusted to a volume of 0.5 ml. Five ml of the copper sulphate-tartrate-sodium carbonate solution was added and the tubes left to stand at room temperature ($21^{\circ}C \pm 4^{\circ}C$) for 10 minutes. After this, 0.5 ml of the diluted Folin-Ciocalteau reagent was added and the tubes shaken vigorously. The absorbance at 650 nm was determined after a further 30 minutes at room temperature. A 0.5 ml aliquot of the supernatant was treated similarly and the resultant absorbance converted to ug protein/ml by means of the calibration curve.

5.3 <u>RESULTS</u>

In the absence of bentazone under conditions designed to

maintain opening (Figure 5.1) the specific activity of malate dehydrogenase remained fairly constant. In the presence of bentazone malate dehydrogenase specific activity appeared to decrease with increasing potassium concentration. In the absence of bentazone stomatal opening was not fully maintained at any concentration, except 100 mol m^{-3} potassium. However, even in the presence of 100 mol m^{-3} potassium, bentazone produced relative closure (Figure 5.1).

Under conditions designed to maintain stomatal closure (Figure 5.2) malate dehydrogenase specific activity rose with increasing potassium concentration. In the presence of bentazone the malate dehydrogenase specific activity was greatly enhanced in the absence of potassium and then followed a similar pattern to the control in the presence of potassium. Apertures followed a similar pattern to that observed with malate dehydrogenase.

At 50 mol m^{-3} potassium and pH 6 (Figure 5.3) malate dehydrogenase specific activity was similar in both light and darkness. Addition of bentazone in both cases caused a slight decrease in specific activity, and addition of IAA alone caused a more marked reduction in activity which was similar under both sets of environmental conditions. When the two compounds were applied together the resultant malate dehydrogenase activity in the light was similar to that obtained with bentazone alone and in darkness was similar to that obtained with IAA alone.

At pH 4 (Figure 5.4) the specific activity of malate dehydrogenase was again similar in the controls, however, bentazone enhanced the activity under conditions designed to maintain opening but had no effect under conditions designed to maintain closure. IAA did not alter control values under either set of conditions and mixing of the two chemicals caused a reduction in enzyme activity in both cases. With the exception of the light treatment at pH 4, the pattern of enzyme activity did not follow the pattern observed with change in aperture.

Figure 5.1 The effect of 10^{-4} M bentazone (open triangles) on the specific activity of malate dehydrogenase (solid lines) over a range of KCl concentrations and pH 6 under conditions designed to maintain stomatal opening. Control values are signified by open circles and the dotted lines represent the corresponding change in aperture. Each point is a mean of 6 specific activity determinations or 60 apertures taken from 3 replicate experiments and twice the maximum standard error is represented by the bars at the side of the figure.

Figure 5.1

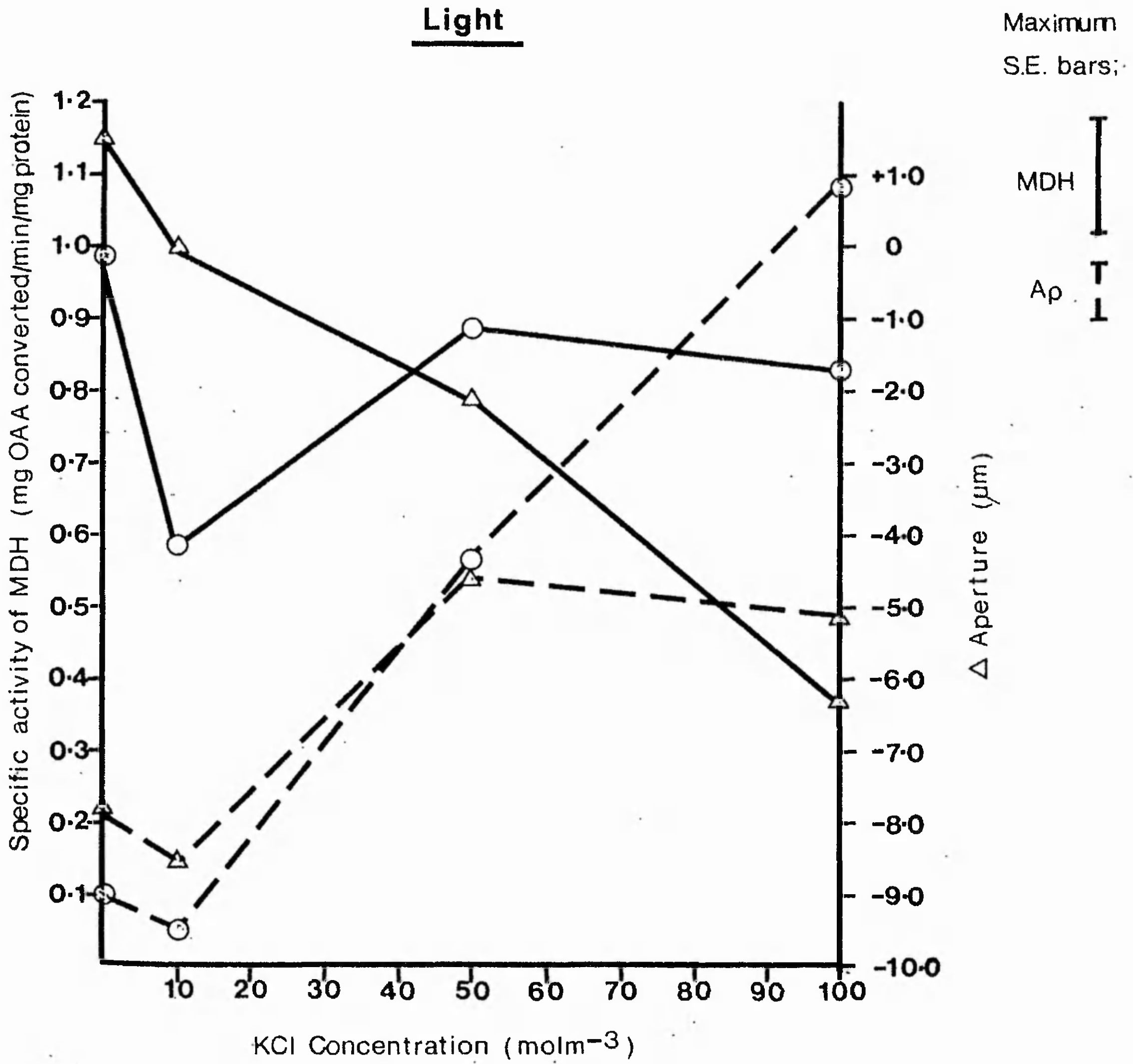


FIGURE 5.2 The effect of 10^{-4} M bentazone (closed triangles) on the specific activity of malate dehydrogenase (solid lines) over a range of KCl concentration and pH 6 under conditions designed to maintain stomatal closure. Control values are signified by closed circles and the dotted lines represent the corresponding change in aperture. Each point is a mean of 6 specific activity determinations or 60 apertures taken from 3 replicate experiments and twice the standard error is represented by the bars at the side of the figure.

Figure 5.2

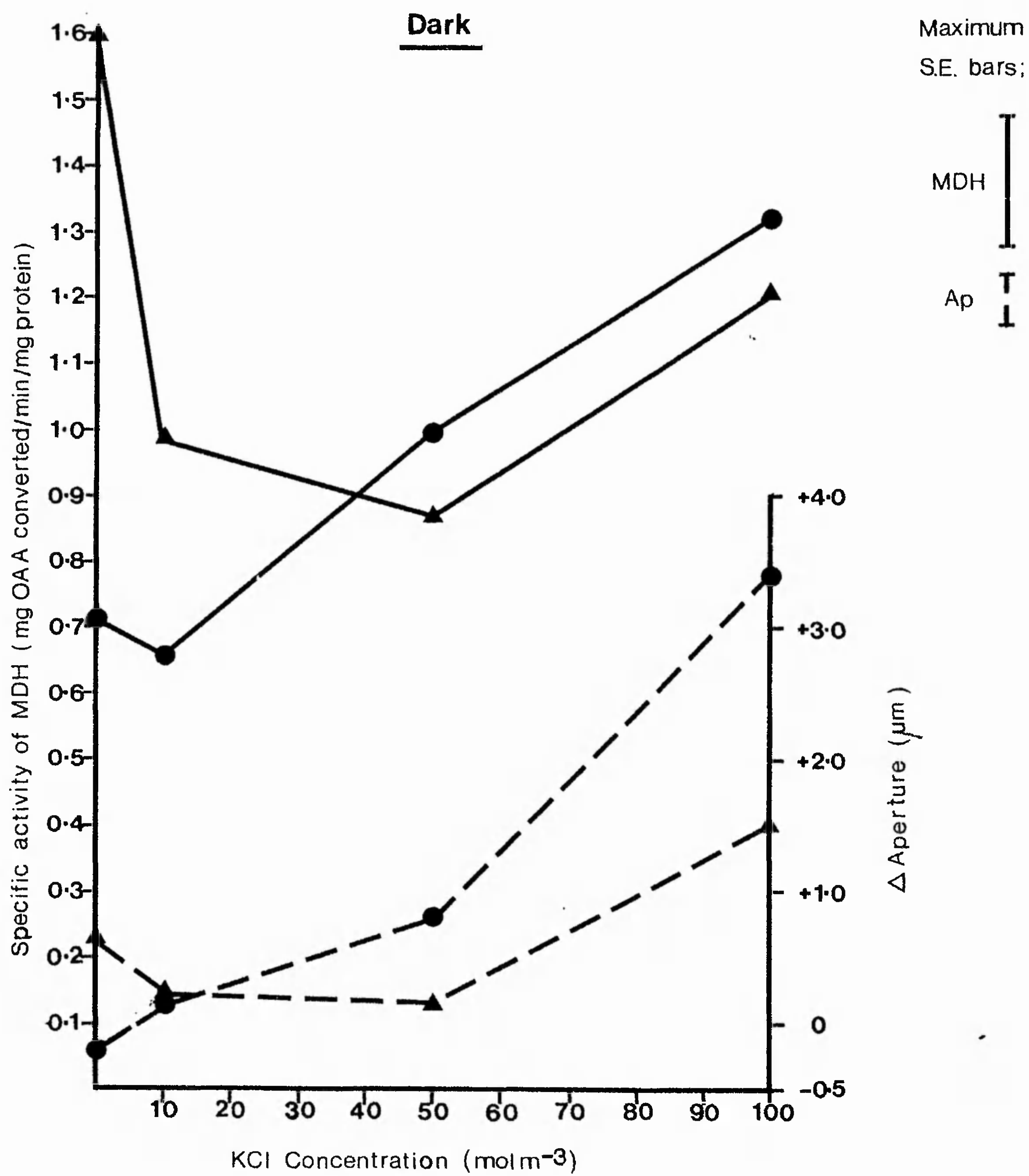


FIGURE 5.3 The effect of 10^{-4} M bentazone and 10^{-4}M IAA on the specific activity of malate dehydrogenase at 50 mol m^{-3} KCl and pH 6 (i) under conditions designed to maintain opening, (ii) under conditions designed to maintain closure. Shaded areas represent change in aperture. The results are taken from 6 determinations of specific activity taken on 4 separate occasions and the error bar represents twice the standard error.

Figure 5.3

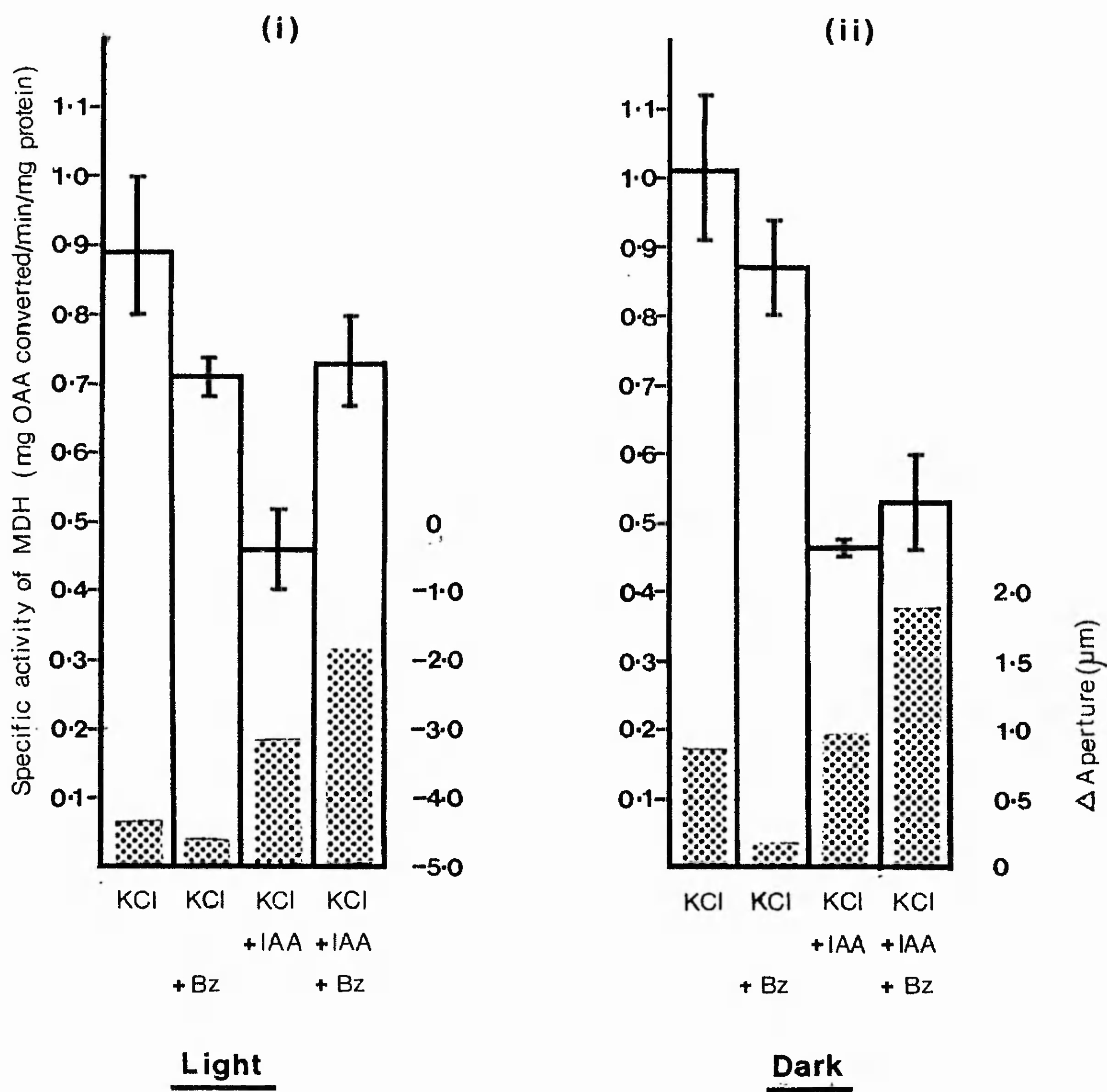
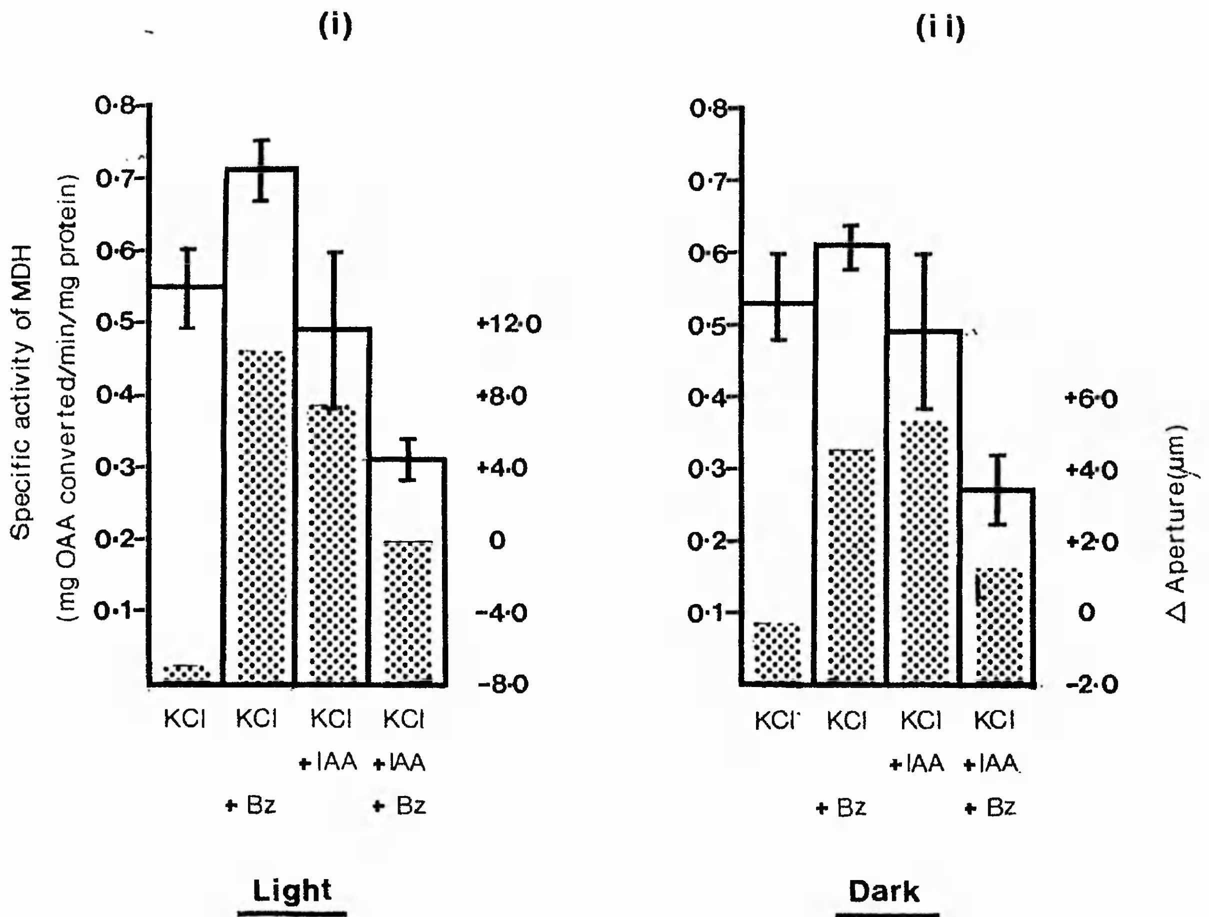


FIGURE 5.4 The effect of 10^{-4} M bentazone and 10^{-4} M IAA on the specific activity of malate dehydrogenase at 50 mol m^{-3} KCl and pH 4 (i) under conditions designed to maintain opening, (ii) under conditions designed to maintain closure. The results are taken from 6 determinations of specific activity taken on 3 separate occasions and the error bar represents twice the standard error.

Figure 5.4



5.4 DISCUSSION

The differences in the effects of bathing medium potassium concentration on the specific activity of malate dehydrogenase under conditions designed to maintain opening (Figure 5.1) and closure (Figure 5.2) may reflect differences in the pre-treatment potassium status of the guard cells. The guard cells of stomata that were pretreated to open would have contained much higher amounts of potassium than those of stomata pretreated to close, as guard cells take up potassium during opening (e.g. Fujino, 1967; Penny et al, 1974; Jarvis et al, 1980). Under conditions designed to maintain opening, only 100 mol m^{-3} potassium was actually able to do so (Figure 5.1), thus at all other concentrations the guard cells were likely to be loosing potassium. For this reason it would be unnecessary to increase malate production to balance incoming potassium, and it is also possible that some of the malate may have been extruded from the guard cells with the potassium as was found by Dittrich et al (1977). Therefore in the absence of bentazone it would be expected that malate would be produced at a constant rate to supply the cells requirements and not at an increased rate to balance ionic changes within the cell. This was in fact the finding of this study (Figure 5.1).

Under conditions designed to maintain stomatal closure (Figure 5.2), the initial potassium status of the guard cells was much lower and therefore the increasing apertures were due to uptake of potassium, not prevention of potassium extrusion.

The effect of bentazone does not appear to be directly on the enzyme itself as the specific activity of malate dehydrogenase is not reduced under conditions designed to maintain closure in the presence of the herbicide (Figure 5.2). At low potassium concentrations under these conditions, the presence of bentazone stimulated the specific activity of malate dehydrogenase. Under closing conditions bentazone stimulates stomatal opening at potassium levels which are normally too low to open stomata. It is probable that bentazone stimulates either potassium uptake or proton efflux and both of these actions could increase the activity of malate

dehydrogenase. Potassium uptake increases the need for a balancing ion and proton efflux increases the pH of the cytoplasm resulting in increased PEP carboxylase activity. In this case the specific activity of malate dehydrogenase can be seen to increase with increasing external potassium concentration as the need to produce malate as a balancing ion presumably increased.

The addition of bentazone to the treatment solution under conditions designed to maintain stomatal opening caused a decrease in malate dehydrogenase specific activity at potassium concentrations above 50 mol m^{-3} (Figure 5.1). From this point bentazone also decreased the ability of potassium ions to maintain stomatal opening. It may be that bentazone increases the rate of potassium leakage by some action at the plasma membrane and causes the cell to have a surfeit of malate. In CAM plants it has been shown that when the malate concentration reaches a certain level transport into the vacuole ceases and malate is released into the cytoplasm allosterically inhibiting PEP carboxylase, thus reducing production of OAA (Kluge, 1976). Also, if cytoplasmic pH decreases to levels considerably below pH 8, PEP carboxylase becomes inactivated by pH (Goodwin et al, 1983). It is feasible that as potassium leakage increases it is accompanied by increased malate loss of the type observed by Dittrich et al (1977) and in the process the malate concentration in the cytoplasm increases, this in turn inhibits PEP carboxylase. The subsequent loss of OAA production reduces the substrate level for malate dehydrogenase and a reduction of the specific activity is observed for this enzyme.

The effect of bentazone does not appear to be directly on the enzyme itself as the specific activity of malate dehydrogenase is not reduced under conditions designed to maintain closure in the presence of the herbicide (Figure 5.2). At low potassium concentrations under these conditions, the presence of bentazone stimulated the specific activity of malate dehydrogenase. Under closing conditions bentazone stimulates stomatal opening at potassium levels which are normally too low to open stomata. It is probable that bentazone stimulates either potassium uptake or proton efflux, and both of these actions could increase the activity of malate

dehydrogenase. Potassium uptake increases the need for a balancing ion and proton efflux increases the pH of the cytoplasm resulting in increased PEP carboxylase activity. The rise in OAA concentrations therefore increase the available substrate for malate dehydrogenase (Goodwin et al, 1983). At external potassium levels equal to 50 mol m^{-3} and above bentazone does not stimulate the specific activity of malate dehydrogenase above that of the control (Figure 5.2). Over this concentration range the specific activity of malate dehydrogenase increases with increasing potassium concentration in a similar manner in both the presence and absence of bentazone and this may be accounted for by the explanation discussed earlier.

In both Figures 5.3 and 5.4 it is apparent that the control values for the specific activity of malate dehydrogenase at 50 mol m^{-3} potassium were similar under both sets of environmental conditions, suggesting that guard cells have a similar ability to produce malate in light and darkness. This finding would be expected as the study is dealing specifically with NAD-dependent malate dehydrogenase which is not known to be photosensitive. However, if this study had included NADP-dependent malate dehydrogenase differences may have occurred as this enzyme is known to be stimulated by light (Ogawa, Ishikawa, Shimada and Shibata, 1978). This enzyme, however, was not investigated in this study as several workers, including Birkenhead et al (1986), have found it to be either absent from or in very low concentrations in the guard cell. At 50 mol m^{-3} potassium, bentazone had little effect on the specific activity of malate dehydrogenase when applied alone (Figure 5.3). However, IAA greatly reduced the specific activity of this enzyme in both light and darkness. It is possible that IAA itself is able to donate some protons to the pool in the cytoplasm decreasing pH and reducing PEP carboxylase activity and thus the activity of malate dehydrogenase. Kaldewey (1984), in his discussion on auxin uptake, suggests that any undissociated IAA taken up in the cell dissociates once it enters the cytoplasm and the IAA diffuses out. However the concentration of IAA is only 10^{-4} M and the contribution to the proton pool would only be small. Also at pH 4 (Figure 5.4) IAA has no effect on enzyme activity and it is at this pH that IAA is most

likely to be taken up in large quantities (see Chapter 4), and thus the above argument fails to satisfy the reason for IAA suppression of malate dehydrogenase activity. It is of interest that bentazone is able to overcome the IAA suppression of enzyme activity in the light but not in darkness (Figure 5.3). At pH 4, the presence of both chemicals caused a decrease in enzyme activity and aperture. Such an observation may again be a result of malate release through the cytoplasm, as the guard cell shrinks, decreasing the pH or allosterically inhibiting PEP carboxylase activity.

Therefore, bentazone and IAA can effect the specific activity of malate dehydrogenase but the effects are altered by environmental conditions.

5.5 CONCLUSIONS

Both bentazone and IAA can cause changes in the specific activity of malate dehydrogenase. However, from the evidence presented it would appear that these effects are more likely due to changes in the ionic balance of the guard cell rather than a direct action on this enzyme.

CHAPTER 6. STUDIES ON THE INTERACTIONS OF BENTAZONE WITH IAA. III. EFFECTS ON RUBIDIUM UPTAKE

6.1 INTRODUCTION

The movement of ions is crucial for plant growth and development and is thought to be responsible for many processes particularly those involving movement. Ionic fluxes have been cited as probable mechanisms for movements of leaves, leaflets and small branches (Bidwell, 1979). Here the fluxes are thought to be of potassium and occur in a specialised organ called the pulvinus. Fluxes of potassium and protons are also cited as being responsible for the movement of the trap lobes of the venus fly trap (Dionea muscipula. Ellis; Rea, 1983). Similarly, movement of potassium is thought to be the major cause of osmotic changes leading to guard cell swelling and therefore to stomatal opening. However, ions usually have a very low permeability through biological membranes because of the large size of their hydration shell and their low lipid solubility. Also the forces acting on ions include electric potential gradients or charge potential gradients, so that their movement is influenced by charge distribution as well as concentration. Thus, the movement of one ion automatically influences the charge pattern of the system, and this affects the movement of other ions regardless of their charge (Bidwell, 1979).

In stomata potassium enters the guard cell against a concentration gradient (e.g. Raschke, 1975). Such movement of ions often involves active transport and Bidwell (1979) highlights the criteria for active transport. He states that when active transport occurs the rate of transport will exceed that predicted from the permeability and the electrochemical gradients and that the final steady-state electrochemical potential will not be in equilibrium across the region of transport. Also, a quantitative relationship will exist between the amount of transport and the amount of metabolic energy expended, and finally, such transport will only occur in a metabolically active cell.

As early as 1967 Fujino showed that ATP was utilized when stomata opened suggesting some form of active transport, he also suggested that the transport of potassium ions across the guard cell membrane was important. Since then it has been shown that upon opening guard cells are able to increase their potassium content by 4 times the amount that would be found in the guard cells of closed stomata (Outlaw et al, 1977). The most popular model proposed for active transport of potassium into the guard cells is an electrogenic ATPase pump on the plasmalemma (Moody and Zeiger, 1978; Kasamo, 1979b; Outlaw, 1983; Edwards et al, 1985; Shimazaki et al, 1986). The exact type of ATPase pump which exists is as yet unknown. Some workers view it as a K^+/H^+ exchange pump (Outlaw, 1983) of the type which has been postulated as a mechanism of potassium transport in the xylem of *Vigna unguiculata*. L (DeBoer et al, 1985), maize coleoptiles (Nelles, 1978) and the trap lobes of *D. muscipula* (Rea, 1983). Most workers are in agreement that the ATPase is likely to pump protons, however, some like Bowling and Edwards (1984) claim that if a K^+/H^+ exchange pump existed a higher acidity would be expected in the apoplasm. Goodwin et al (1983) suggested that the ATPase pumped protons out of the cell setting up a gradient along which the potassium could flow into the cell. The specific ATPase involved has not as yet been identified, however, some work has been done on epidermal ATPases and this has shown that the ATPase is activated by divalent cations (Fujino, 1967; Kasamo, 1979a), has a pH optimum around pH 6.0 and can be stimulated up to a further 20% by potassium chloride (Kasamo, 1979a). Kasamo (1979b) also noted that there was proton influx into peels which was greatest in darkness but also existed to a lesser extent in the light. An ATPase pump may not provide all the answers. Indeed, Schroeder, Hedrich and Fernandez (1984) suggested that a significant contribution to the uptake and release of potassium by guard cells during stomatal movement was made by a cation-selective channel which had a high selectivity for potassium. As can be seen much work still needs to be done on the mechanism of potassium uptake by guard cells.

While it may prove difficult to study the mechanism of potassium movement it is quite possible to examine the movement of potassium using radioisotopic techniques. Very few studies have been carried out using potassium isotopes due to their short half-lives, whereas rubidium (Rb) has similar properties to potassium, and the isotope ^{86}Rb (half-life 18.6 days) has been successfully used to monitor potassium movement in guard cells (Fischer and Hsiao, 1968; Mansfield, 1970; Weyers and Hillman, 1979; MacRobbie et al, 1980; MacRobbie, 1981 and 1982). In this study ^{86}Rb was used to ascertain whether bentazone, IAA or a mixture of the two compounds affected the potassium content of guard cells. Inhibitor studies were also included to further elucidate bentazone and IAA action. The inhibitors used were sodium orthovanadate (VO_4) an inhibitor of plasmalemma-bound ATPase activity (Brummel, 1986) that was shown not to affect tonoplast ATPase activity in oats (Rea, Manolson and Poole, 1986 unpublished data); diethylstilbestrol (DES) which can inhibit potassium absorption by decreasing mitochondrial ATP production in addition to inhibiting membrane bound ATPase activity (Balke and Hodges, 1979); potassium nitrate which may inhibit the tonoplast ATPase (Rea et al, 1986), although this is questioned by Leigh (1986, unpublished data); and carbonyl cyanide-m-chlorophenyl hydrazine (CCCP) which increases the membrane permeability to protons (Leigh, 1986, unpublished data) and thus interferes with controlled proton transport by ATPase pumps.

6.2 MATERIALS AND METHODS

The basic C. communis peel and leaf pretreatment was as outlined in Chapter 2.

6.2.1 Design of experiments

6.2.1.1 Determination of the effect of rubidium chloride concentration on stomatal aperture:

Treatment solutions contained 0.1 mol m^{-3} $CaCl_2$ and 0, 6.25, 12.5, 25, 50 or 100 mol m^{-3} RbCl at pH 6.0. Treatment time was 120 minutes under conditions designed to maintain both opening and closure. Following

treatment 3 peels were removed and measured microscopically, the experiment was repeated twice.

6.2.1.2 Determination of the effect of 10^{-4}M bentazone and 10^{-4}M IAA on the ability of guard cells to take up and maintain ^{86}Rb: Treatment solutions contained 0.1 mol m^{-3} $CaCl_2$ and 50 mol m^{-3} potassium in the presence and absence of 10^{-4}M bentazone and 10^{-4}M IAA plus 1 x 10^8 DPM of ^{86}Rb. The volume of the treatment solutions was 10 ml not 25 ml as used in previous experiments and small size Petri dishes (50 mm diameter) were used. Ten peels were used for each treatment. The treatment time was 120 minutes and following this peels were washed three times in unlabelled 50 mol m^{-3} KCl plus or minus IAA and bentazone and then placed in plastic 20 ml volume scintillation vials containing 10 ml of unlabelled test solution and transferred to a liquid scintillation counter to be counted. Experiments were carried out under conditions designed to maintain both opening and closure at pH 6 and pH 4. Parallel experiments were run in unlabelled solutions and stomatal aperture was determined from these peels. Each treatment was repeated three times.

6.2.1.3 Determination of the effects of inhibitors on stomatal aperture and the ability of guard cells to take up and maintain ^{86}Rb at pH 4: Treatment solutions contained 0.1 mol m^{-3} $CaCl_2$ and 50 mol m^{-3} KCl at pH 4 plus 1 x 10^8 dpm of ^{86}Rb in a 10 ml volume. The inhibitors used were CCCP (10 μm made up in 5% (v/v) ethanol), DES (20 μm initially dissolved in a minimal amount of 1 M HCl and then made up to the required concentration with distilled water), vanadate (VO_4, 50 μm, made up in 5% (v/v) ethanol) and nitrate (NO_3, 50 mol m^{-3} aqueous). All chemicals were purchased from Sigma and the concentrations in brackets represent the final concentrations in the treatment solutions.

All four inhibitors were used in the absence of 10^{-4}M bentazone and 10^{-4}M IAA and in the presence of 10^{-4}M IAA, however due to a lack of ^{86}Rb only CCCP and DES were used in the presence of bentazone alone. The treatment time was 120 minutes and the labelled peels were treated the same as in the previous experiment. The study was repeated in the absence of ^{86}Rb and 3 peels were removed after 120 minutes and 15 apertures determined microscopically from each peel. This experiment was carried out under conditions designed to maintain both opening and closure and performed only once.

6.2.2 <u>Operation of the liquid scintillation counter to count ^{86}Rb by the Cerenkov method</u>

<u>Theory of Cerenkov counting</u>. Cerenkov counting can only be used when the energy of the emitted B-particle is greater than about 300 k eV. When these particles travel through a medium of high dielectric constant (e.g. water) they produce local molecular polarisation and the polarised molecules then return to their ground state by the emission of electromagnetic radiation. When the velocity of the particle through the medium is greater than that of light through the medium the radiation is visible as U.V. light. This phenomenon is the Cerenkov effect and the light can be detected by liquid scintillation counters designed to cope with very low intensity light flashes (Dyer, 1974).

6.2.2.1 <u>Determination of the energy distribution of the emission</u>: The counter (Packard 3375) was set to non-coincidental counting and the gain set to 50%. The window settings were set at 50-100. 100-150, 100-200, 200-300, 300-400, 400-500, 500-600 and 700-800 MeV. A solution containing 2.8 x 10^{-5} dpm ^{86}Rb in 10 ml of water was counted at each setting. The results were plotted (Figure 6.1) and showed that even at the extreme window settings emissions were still being detected at a high enough level to warrant their inclusion. Thus,

it was decided to use a wide window setting of 50-1000 MeV throughout the experiments. The efficiency of counting was found to be 34%. This figure was calculated by dividing the total number of counts per minute (cpm) by the known number of disintegrations per minute (dpm) and multiplying by 100.

6.2.2.2 <u>Determination of the gain setting for counting</u>: The counting window was set to 50-1000 MeV, the gain was then set to 10, 20, 30, 40, 50, 60, 70, 80 and 90%. A solution containing 2.8×10^5 dpm in 10 ml was once more counted and the counting efficiency was found to be optimal at 50% gain (Figure 6.2) and thus this was used for the rest of the experiments.

6.2.2.3 <u>Determination of the quenching effect of epidermal peels</u>: Two vials containing 10 ml of water and 3.1×10^4 dpm ^{86}Rb 0, 3, 5, 7 and 10 epidermal peels were added. These were counted and no loss in efficiency was detected (Figure 6.3) and it was assumed that there was a negligible quenching effect by the peels.

6.2.2.4 <u>Determination of the percentage recovery of ^{86}Rb from the experimental system</u>: One $\times 10^5$ dpm of ^{86}Rb was added to 10 ml of 100 mol m^{-3} KCl in small Petri dishes (50 mm diameter) and 10 peels were floated on each dish. These were then incubated under opening conditions for 120 minutes, rinsed 3 times in 10 ml of unlabelled solution and transferred to a plastic scintillation vial containing 10 ml of unlabelled solution. All the rinses and the remaining labelled solution were then transferred in to scintillation vials and were all counted. Total recovery was never less than 94%, however the amount entering the peels was minimal. In order to increase the uptake of the peels to a level above 1000 cpm, the amount of label added to the test solution was increased to 1×10^8 dpm, this did not

adversely effect counting efficiency or percentage recovery.

6.3 RESULTS

Figures 6.1, 6.2 and 6.3 justify the liquid scintillation counter settings, showing the wide energy band over which disintegrations from ^{86}Rb can be detected by Cerenkov counting (Figure 6.1), the effect of gain on counting efficiency (Figure 6.2) and the lack of quenching by epidermal peels (Figure 6.3). Figure 6.4 showed that rubidium, like potassium, was able to increase stomatal aperture with increasing concentration in both light and darkness and thus may be used as a tracer for potassium.

At pH 6, ^{86}Rb uptake did not appear to alter between treatments under either set of environmental conditions, nor reflect the aperture changes under conditions designed to maintain opening (Figure 6.5). At pH 4, however, the pattern of ^{86}Rb uptake was similar to the pattern of aperture changes with bentazone reducing both factors and IAA increasing then (Figure 6.6). Mixing the two compounds appeared to reduce ^{86}Rb uptake under both sets of environmental conditions to a level below that of the control, however under conditions designed to maintain closure the aperture remained similar to the control (Figure 6.6). Thus, at pH 4 ^{86}Rb uptake and retention appeared to follow the pattern of aperture in almost all cases (Figure 6.6).

Under conditions designed to maintain both opening and closure, 10 um CCCP appeared to increase both stomatal aperture and ^{86}Rb uptake in the absence of both IAA and bentazone (Figure 6.7 and 6.8). In the presence of IAA, however, CCCP slightly reduced stomatal aperture under both sets of conditions. This, however, did not appear to reflect the amounts of ^{86}Rb uptake in the presence of IAA in the light (Figure 6.7). When bentazone was added with CCCP there was little effect on either aperture or ^{86}Rb uptake under either set of conditions (Figures 6.7 and 6.8). DES at 20 um had the most consistent effect on stomatal aperture reducing it under both sets of conditions regardless of the presence of IAA and bentazone, giving values below that of the zero time reading at all times in the light (Figure 6.7) and in the presence of bentazone in

darkness (Figure 6.8). However, under conditions designed to maintain stomatal opening more ^{86}Rb was detected in the presence of DES than in the control (Figure 6.7), this was also observed in the absence of IAA and bentazone under conditions designed to maintain closure (Figure 6.8). Vanadate at 50 um reduced aperture in the light in both the presence and absence of IAA (Figure 6.7) but not to the same extent as with DES and in darkness it reduced the aperture in the presence of IAA (Figure 6.8). However, this inhibitor only reduced the amount of ^{86}Rb detected in the absence of IAA under conditions designed to maintain opening (Figure 6.7) under all other treatments it had little effect on ^{86}Rb uptake and retention. Nitrate at 50 mol m^{-3} reduced aperture in the presence and absence of IAA under conditions designed to maintain opening (Figure 6.7). Under conditions designed to maintain closure nitrate reduced aperture in the presence of IAA and slightly increased aperture in its absence (Figure 6.8). The effect of nitrate on ^{86}Rb uptake and retention is somewhat different, in the absence of IAA in the light detectable ^{86}Rb is reduced (Figure 6.7) however under all other treatments studied the trend was that nitrate increased the amount of ^{86}Rb in the peels (Figures 6.7 and 6.8).

FIGURE 6.1 <u>Distribution of the energy of disintegrations from ^{86}Rb</u>

Figure 6.1

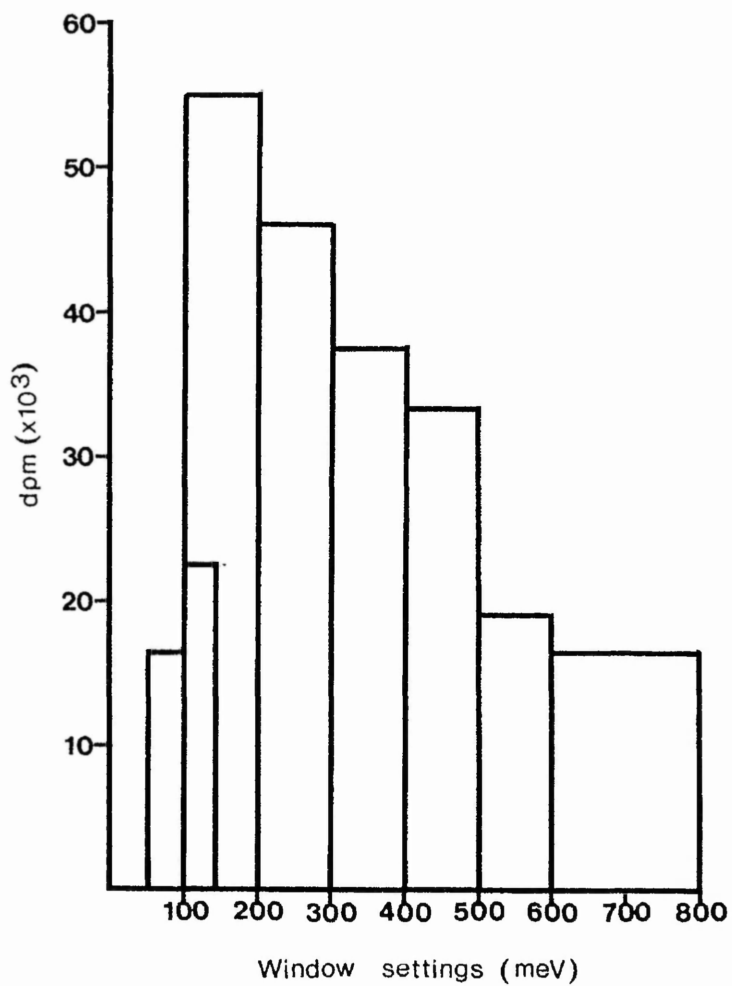


FIGURE 6.2 The effect of altering gain settings on the counting efficiency of ^{86}Rb

FIGURE 6.3 The effect of epidermal peel number on the counting efficiency of ^{86}Rb

Figure 6.2

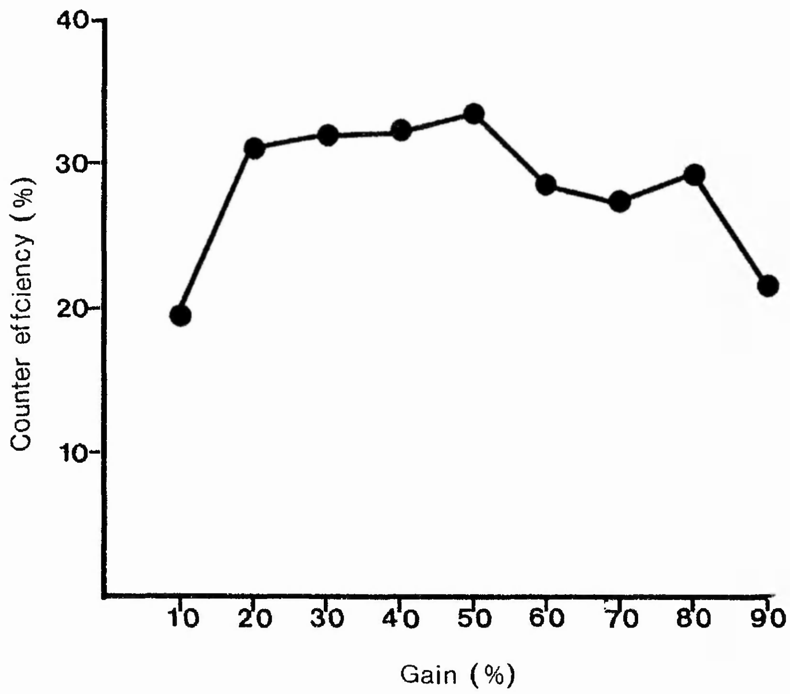


Figure 6.3

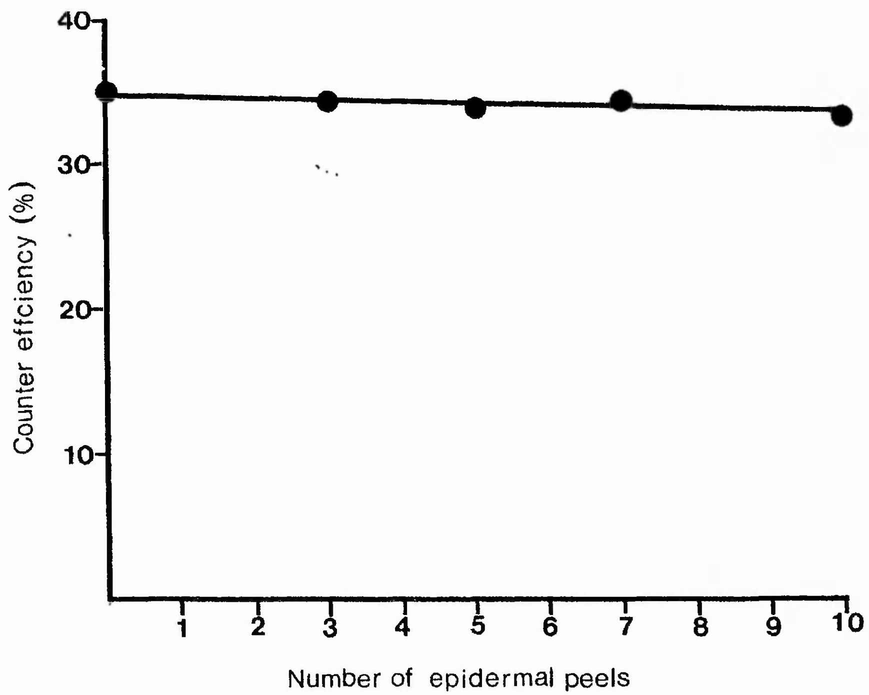


FIGURE 6.4 The effect of rubidium chloride concentration on aperture under conditions designed to maintain stomatal opening (open circles) and stomatal closure (closed circles). Each point is a mean of 90 observations from two replicate experiments and the error bar represents twice the standard error.

Figure 6.4

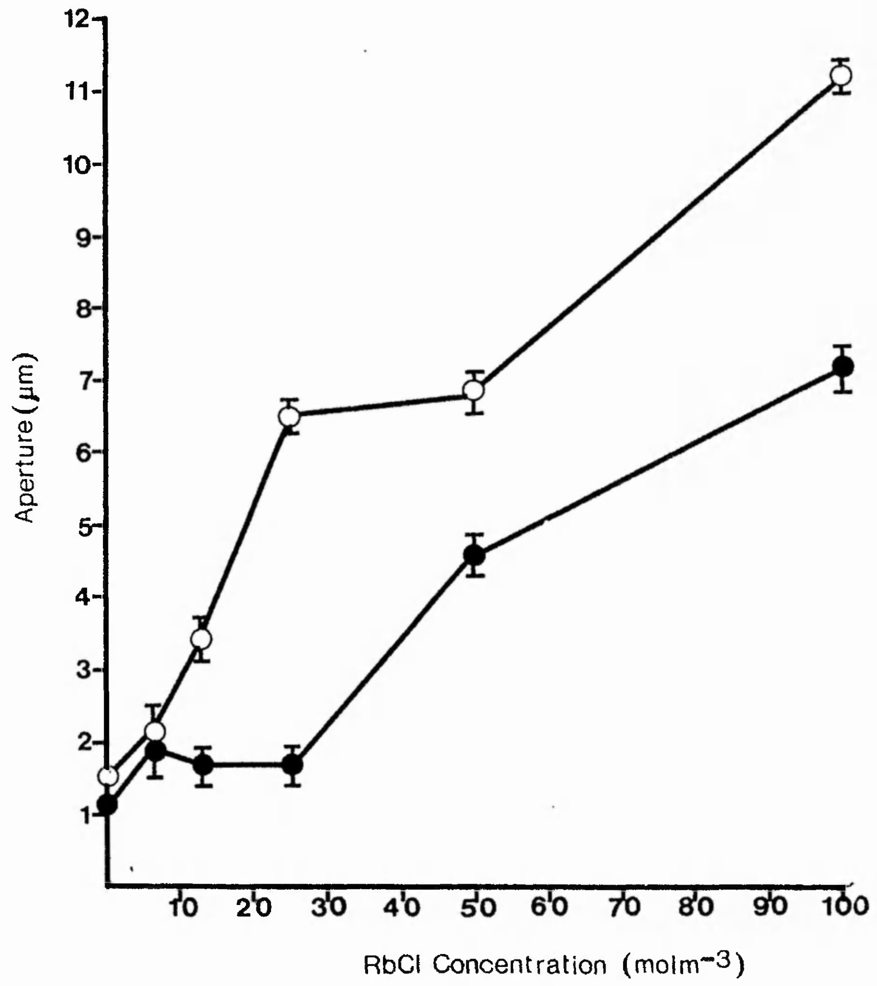
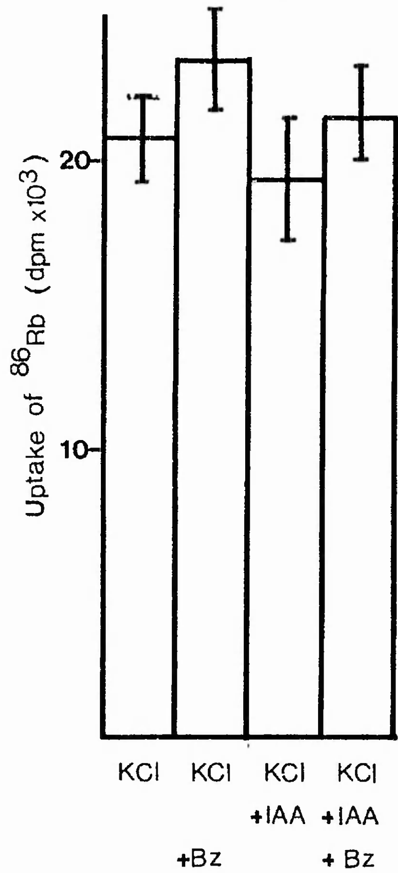


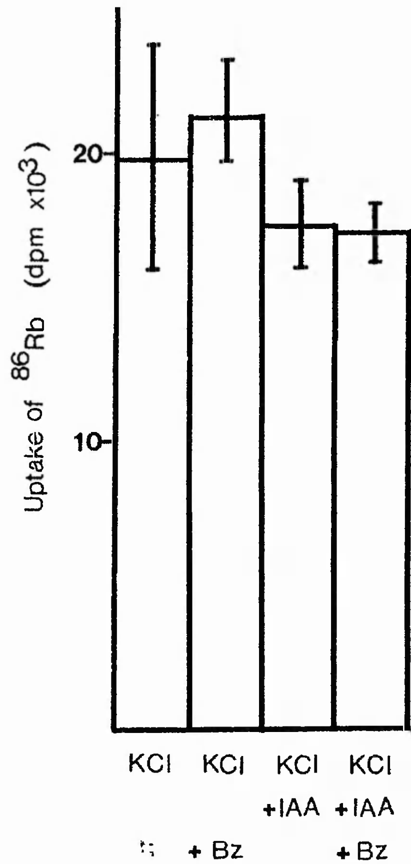
FIGURE 6.5 Uptake and retention of ^{86}Rb by epidermal peels at pH 6 and 50 mol m^{-3} KCl in the presence and absence of both 10^{-4} M IAA and 10^{-4}M bentazone, and comparison with the change in aperture under conditions designed to maintain i) stomatal opening and, ii) stomatal closure. Each bar represents the mean plus/minus twice the standard error where n = 9 for ^{86}Rb graph and n = 6C for Δ aperture graph.

Figures 6.5(i)



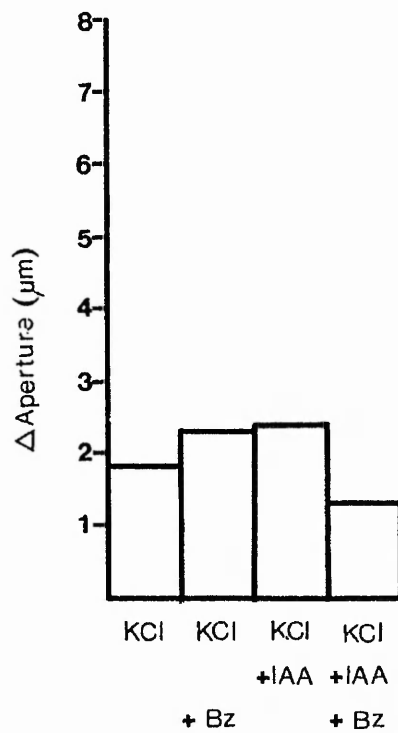
Light

Figures 6.5(ii)



Dark

Light

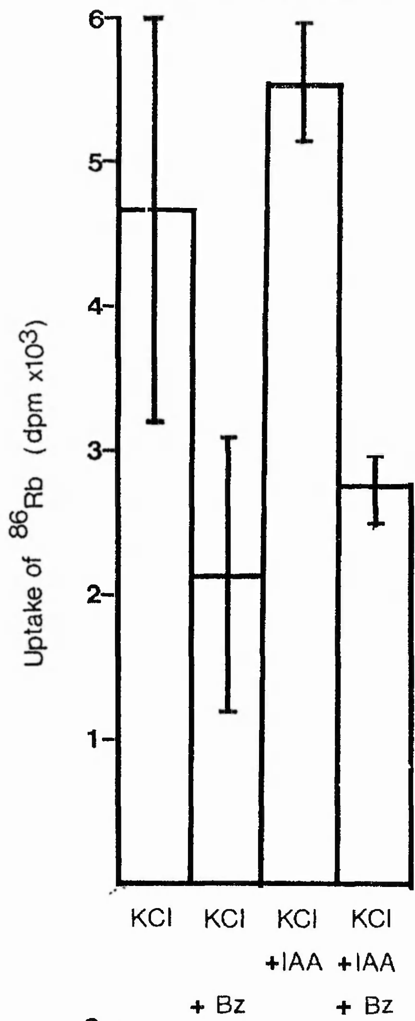


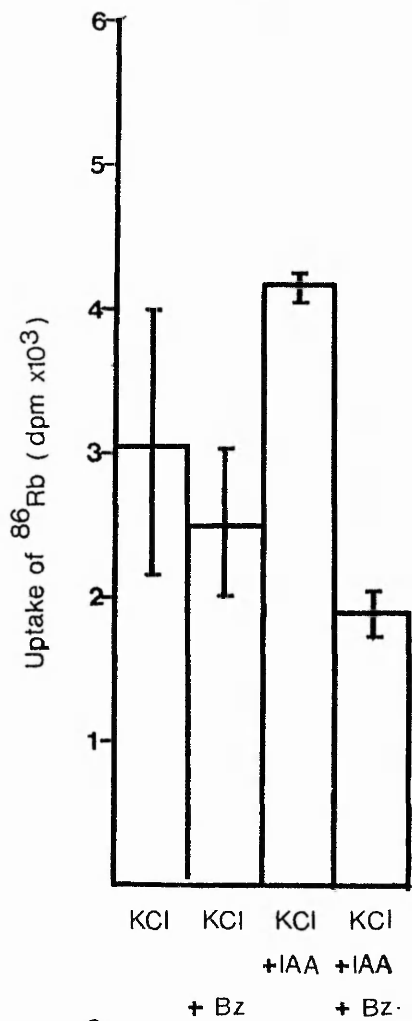
Dark

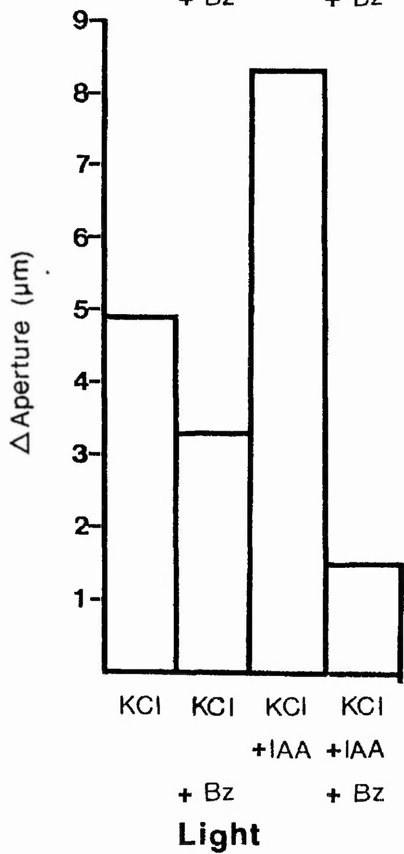
FIGURE 6.6 Uptake and retention of ^{86}Rb by epidermal peels at pH 4 and 50 mol m^{-3} KCl in the presence and absence of both 10^{-4}M IAA and 10^{-4}M bentazone, and comparison with the change in aperture under conditions designed to maintain i) stomatal opening and, ii) stomatal closure. Each bar represents the mean plus/minus twice the standard error where n = 9 for ^{86}Rb graph and n = 60 for Δ aperture graph.

Figures 6.6 (i)

Figures 6.6 ii)







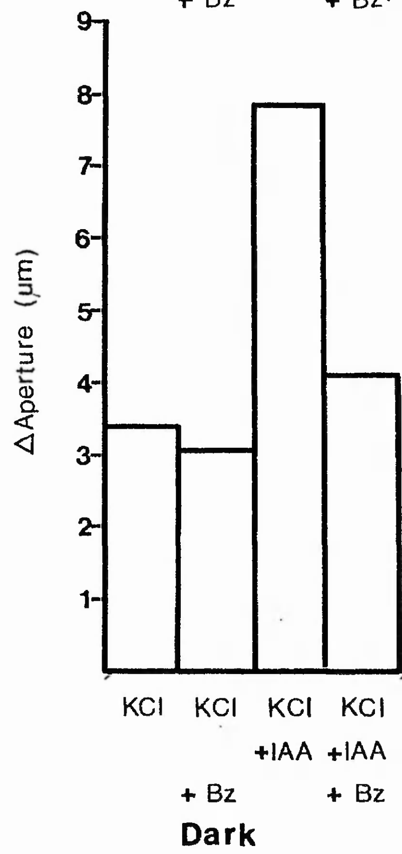
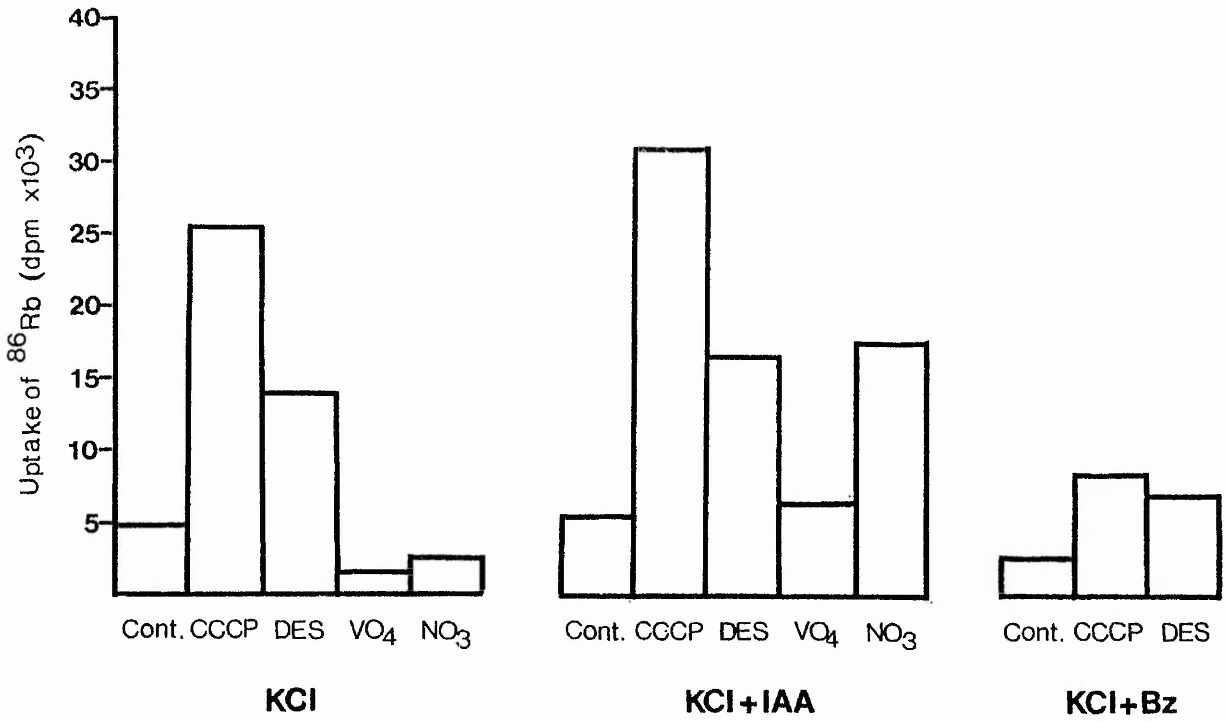


FIGURE 6.7 The effects of CCCP (10 um), DES (20 um), VO_4 (50 um) and NO_3 (50 mol m^{-3}) on stomatal aperture and ^{86}Rb uptake and retention at 50 mol m^{-3} KCl and pH 4 in the presence or absence of 10^{-4}M bentazone and 10^{-4}M IAA under conditions designed to maintain stomatal opening. Each bar represents the mean taken from a single experiment where n = 3 for ^{86}Rb uptake graph and n = 45 for Δ aperture graph.

Figure 6.7



Δ Aperture (μm)

10
9
8
7
6
5
4
3
2
1
0
-1
-2

Cont. CCCP DES VO4 NO3

KCl

Cont. CCCP DES VO4 NO3

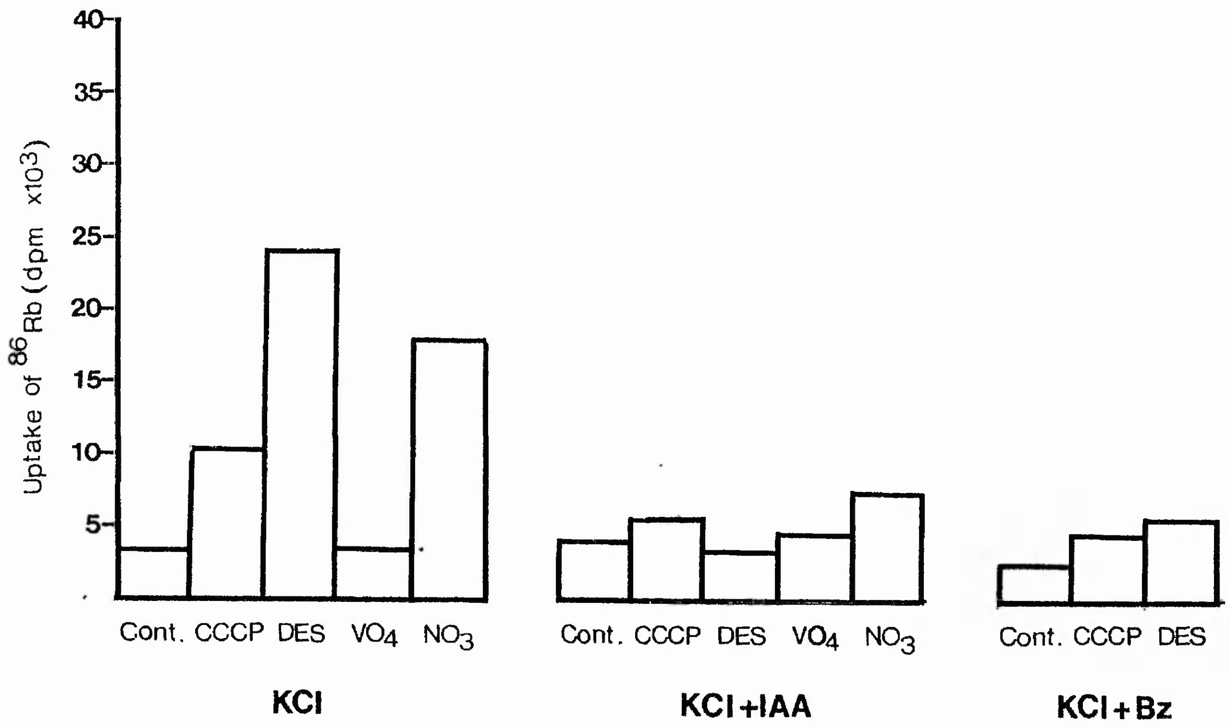
KCl+IAA

Cont. CCCP DES

KCl +Bz

FIGURE 6.8 The effect of CCCP (10 µm), DES (20 um), VO_4 (50 µm) and NO_3 (50 mol m^{-3}) on stomatal aperture and ^{86}Rb uptake and retention at 50 mol m^{-3} KCl and pH 4 in the presence or absence of 10^{-4}M bentazone and 10^{-4}M IAA under conditions designed to maintain stomatal closure. Each bar represents the mean taken from a single experiment where n = 3 for ^{86}Rb uptake graph and n = 45 for Δ aperture graph.

Figure 6.8



Δ Aperture (µm)
10
9
8
7
6
5
4
3
2
1
0
-1
-2
Cont. CCCP DES VO_4 NO_3
KCl
Cont. CCCP DES VO_4 NO_3
KCl+IAA
Cont. CCCP DES
KCl+Bz

6.4 DISCUSSION

The apparent lack of correlation between aperture and ^{86}Rb content of epidermal peels under conditions designed to maintain opening at pH 6 may be due to inefficiency of washing at pH 6. MacRobbie (1981) suggested that in order to remove free-space activity before counting, the rinse solutions should be around pH 3.9. However, when pH 6-incubated peels were rinsed at pH 4 little change in ^{86}Rb content was observed (results not shown). At pH 4, ^{86}Rb content followed apertures quite well (Figure 6.6), indicating that reduction in aperture by bentazone and stimulation by IAA could reflect the ability of potassium to be taken up and retained by the guard cells. Mixing the two compounds did reduce uptake somewhat but no more than bentazone alone, suggesting that a factor other than potassium movement may be involved in the interaction. The effect observed with CCCP is difficult to explain as CCCP which increases membrane permeability to protons (Leigh, 1986 unpublished data) would be expected to reduce the uptake of potassium as protons would be flowing back into the guard cell. It may be possible that other changes occurred in the membrane when CCCP was introduced into the system which prevented potassium leakage thus enabling the stomata to remain open. However, this would in itself not explain the stimulation of aperture and ^{86}Rb content. This may also be the case in the presence of IAA and bentazone although the change in aperture may not reflect this due to the already high rate of potassium uptake (Figures 6.7 and 6.8). DES strongly inhibited stomatal opening, however uptake and retention of ^{86}Rb was in most cases higher than that of the control. It has been shown that DES has an ability to reduce mitochondrial ATP production as well as acting on ATPase activity (Balke _et al_, 1979). It may be that DES inhibition of mitochondrial ATP production produces an energy deficiency in the guard cell which reduces other metabolic processes which are important to stomatal movement and that the ATPase inhibition is minimal allowing some accumulation of ^{86}Rb to continue. Vanadate has been shown to inhibit plasmalemma ATPase activity (Brummel, 1986) which may

explain the reduction of aperture, it also prevented IAA stimulation bringing aperture and ^{86}Rb uptake in line with that of potassium alone. This would suggest that IAA activity was centred on the ATPase's on the plasmalemma not the tonoplast as tonoplast ATPase's are unaffected by VO_4 (Brummel, 1986). This inhibitor could have been a useful indicator of the point of action of bentazone, however, due to a lack of ^{86}Rb this experiment was not possible. Nitrate appeared to slightly reduce aperture and ^{86}Rb content under conditions designed to maintain opening in the presence of potassium alone, and reduced aperture slightly in the presence of IAA, but here the amount of ^{86}Rb was increased. As NO_3 is a possible inhibitor of the tonoplast ATPase (Rea <u>et al</u>, 1986) the action is likely to be on the balancing ion, i.e. it may reduce malate release and therefore proton release from the plasmalemma, as this is a two stage effect this process may take longer to alter the stomatal balance producing less pronounced effects than the other inhibitors. Under conditions designed to maintain closure NO_3 increased the aperture slightly in the absence of IAA and decreased aperture in its presence in both cases increasing the amount of ^{86}Rb detected. This increase in ^{86}Rb uptake may be a result of potassium being taken up to balance ionic changes which would normally be accounted for by exchanges across the tonoplast which have been blocked due to nitrate inhibition of tonoplast ATPase activity.

6.5 CONCLUSIONS

Few conclusions can be drawn from these studies as the small number of replicates does not enable detailed statistical analysis to be performed. These experiments have however shown that rubidium affects stomata similarly to potassium and therefore is a good candidate for use as a tracer ion (Figure 6.4) and have indicated that changes in potassium uptake may be implicated in bentazone and IAA alterations of stomatal movement. These results also suggest that changes in potassium uptake may be linked to ATPase activity and energy within the guard cell therefore supporting the theories of other workers (e.g. Fujino, 1967; Moody <u>et al</u>, 1978; Kasamo, 1979b; Outlaw, 1983; Edwards <u>et al</u>, 1985; Shimazaki <u>et al</u>, 1986).

CHAPTER 7: DISCUSSION

7.1 A CRITICAL EXAMINATION OF TECHNIQUES USED IN THIS STUDY

The stomata of C. communis have been shown to be extremely sensitive to environmental and chemical changes, both within the plant and externally. Stomata have been shown to be sensitive to environmental factors such as light (Hsiao et al, 1973; Sheriff, 1979; Mansfield, 1983; Roth-Bejerano et al, 1985), CO_2 (Meidner et al, 1959; Pallas, 1965; Pallaghy, 1971; Travis et al, 1979; Blackman et al, 1984; Morison, 1985), humidity and water stress (Sheriff, 1979; Das et al, 1982; Mansfield, 1983; Ball et al, 1984) and temperature (Hofstra et al, 1969; Raschke, 1970; Spence et al, 1984). The stomatal response to each of these environmental factors is often inter-related, for example, at temperatures above 35°C the stomata appear to lose their ability to respond to CO_2 and any correlation between CO_2 assimilation and aperture is also removed (Raschke, 1970; Spence et al, 1984). Chemicals which are known to alter stomatal responses include endogenous plant growth regulators such as ABA (Jarvis et al, 1980; MacRobbie, 1981; Wilson, 1981; Pemadasa, 1982; Blackman et al, 1983), IAA (Pemadasa,1982; Govil, 1985), Cytokinins (Das et al, 1976; Goring et al, 1984), ethylene (Madhavan et al, 1983) and gibberellins (Horton, 1971; Goring et al, 1974). Other chemicals which affect stomatal movement include the fungal toxin, fusicoccin, gaseous pollutants, herbicides and metabolic inhibitors.

It is therefore apparent that when studying such sensitive cells it is extremely important to control as many factors as possible. With systems which include the extremely sensitive stomata of C. communis standardisation of techniques is vital to the investigation. It was for this reason that the early part of the study was devoted to developing techniques which allowed the stomata to function "normally" giving reproducible results.

Before standardisation of experimentation could begin healthy unstressed plants of C. communis had to be produced. Seed was obtained from Dr. Colin Willmer at Sterling University and the growth

conditions used were outlined in a communication from him. These growth conditions produced plants which looked healthy and which easily yielded clean, viable epidermal peels. Throughout the investigation peels were taken from the abaxial leaf surface using watchmakers forceps. Care was taken during the removal of peels although the procedure was not as precise as that outlined by Weyers et al (1983). In order to ascertain the viability of these peels neutral red staining was routinely employed. Care was taken not to leave the peels for too long in the stain as it may itself cause cell damage. Peels were examined immediately after removal from the stain to prevent degeneration prior to the estimation of viability. The estimation of epidermal peel viability obtained from neutral red staining (see Chapter 2) indicated that the peeling technique consistently produced epidermes suitable for experimentation.

Once suitable peels had been produced the conditions under which they were treated had to be standardised with respect to the environmental conditions outlined in the earlier part of this discussion. Throughout the investigation lighting was provided by Phillips 40W fluorescent tubes (cool white) at a photon flux density of 50 μmol $m^{-2}s^{-1}$ (PAR). These fluorescent tubes were regularly checked and cleaned, and the flux density was measured prior to the start of each experiment. The Petri dishes containing the peels were kept at a constant temperature by a water jacket around the incubation tank which was supplied with water by a circulator. When low CO_2 was required air was passed over a 20 cm column of soda lime which was replaced regularly to ensure that it absorbed CO_2 as efficiently as possible. For high CO_2-air laboratory air was used, and although this may have varied slightly in concentration it was always high enough to produce CO_2 responses. To this extent environmental conditions were controlled as much as was possible using the available equipment.

As a safeguard, all peels were pretreated to open or close for 1 hour prior to experimentation. If in this time the peels failed to show the 'normal' opening or closing response the experiment was abandoned.

The environmental conditions and growth conditions employed throughout this investigation vary little from those used by many other stomatal physiologists. However, the treatment of C. communis epidermes differed in two important ways. Firstly, this investigation did not use a buffer whereas most workers have used MES buffer when working with C. communis (Willmer et al, 1969; Pemadasa, 1982). Secondly, the incubation medium used in these studies included 0.1 mol m^{-3} $CaCl_2$. These changes to accepted practice were made in view of the considerations outlined in the following text. In this study the pH of the solutions were precisely set using minimal quantities of KOH and HCl. These solutions were constantly mixed throughout the treatment time by the bubbling of air or low CO_2-air through the solution. The ratio of solution volume to peel area was high and there was little chance of extreme changes in local pH. The pH was monitored throughout the experiments and no change was detected. It was therefore considered that in view of the warnings of Meidner (1981) that called for caution in the use of MES buffer, as it could lead to epidermal peel damage, it was unnecessary to add another variable, such as MES buffer, to an already complex system. Low concentrations of $CaCl_2$ were added to the incubation medium as in some species such as V. faba (Pallaghy, 1970) it had been shown to increase membrane selectivity for potassium. In a recent review MacRobbie (1987) also indicates that calcium increases the membrane specificity for potassium in C. communis. However, other workers had found that calcium could suppress stomatal opening in C. communis and at concentrations as low as 1 mol m^{-3} Ca^{2+} could actually abolish opening (Willmer, et al, 1969; MacRobbie, 1987). In view of these findings, tests were run on the experimental system in the presence and absence of calcium. The results of these tests were given in Chapter 2 and showed that in the absence of buffer calcium ions did not appear to suppress stomatal opening. However, in the presence of MES buffer calcium ions did suppress stomatal opening in the manner described by Willmer et al (1969). This may indicate an interaction of MES on the stomatal system. Later investigations (Chapter 3) also showed that MES buffer altered the effect of bentazone on

stomatal aperture. As the effect of bentazone in the absence of buffer was in some agreement with the effect observed in whole plants by Cobb et al (1983), it would seem possible that the buffering system could be altering the 'normal' stomatal response. It was felt that these findings supported the decision not to use MES buffer in these investigations. It should be noted that in circumstances where peels were kept in treatment conditions for longer periods of time than those used in this investigation, buffers may be of more importance and under such circumstances tests on the necessity of a buffering system would need to be repeated. The decision not to use buffer meant that there were no problems with the use of low concentrations of $CaCl_2$ in the incubation medium and this may have been an advantage as it meant that the guard cell plasma-membrane was likely to have a higher specificity for potassium ions.

Once the solutions had been decided upon it was necessary to see whether or not the stomata on the epidermal peels responded in the expected manner to parameters such as potassium concentration. The potassium response curves obtained (Chapter 2, Figure 2.2) proved to be comparable with those obtained by Travis et al, (1979). This would suggest that the tissue used in this investigation was of a similar standard to that used by previous workers. Neutral red staining after experimentation demonstrates that the peels maintained a sufficiently high viability throughout the experimental period. Thus, results obtained in the experiments are likely to be due to treatments rather than to degeneration in the quality of the peels themselves. Cobaltinitrite staining of the potassium in the guard cells showed that, as in the studies of Squire et al (1972), Pemadasa et al (1977) and Pemadasa (1982), stomatal opening could be associated with an increase in the potassium content of the guard cells. This confirmed once more that the incubation techniques employed in this investigation allow the stomata to behave in a similar manner to that observed by previous workers. This staining technique was only used to detect the presence or absence of potassium in the guard cell, it was felt that the technique could not

give any quantitative data on the amount of potassium present. The response to the stain tended to be all or nothing and thus could give no indication of whether one treatment slightly altered the amount of potassium accumulation by the guard cell. Data of this type was produced by Pemadasa (1979) however, it was felt that few conclusions could be drawn from observations such as these.

Treatment chemicals were always freshly made up prior to experimentation. Both IAA and bentazone were initially dissolved in absolute alcohol before being diluted with distilled water. Stock solutions were made up so that the concentration of ethanol was less than 5% (v/v). The concentration of ethanol reaching the treatment solutions was therefore less than 1.25% and this appeared to have no effect on the viability of epidermal peels. As with the basic incubation media, the pH of these chemicals was adjusted using minimal amounts of KOH and HCl.

The range of pH used in this investigation was within the range used by other workers, the lowest pH being pH 4.0. MacRobbie (1987) claimed that guard cells could survive at pH 3.9 for long periods of time and this treatment was not likely to alter the properties of the guard cell wall. Similarly, the results in this investigation show no guard cell damage or loss of function due to treatment at pH 4.0.

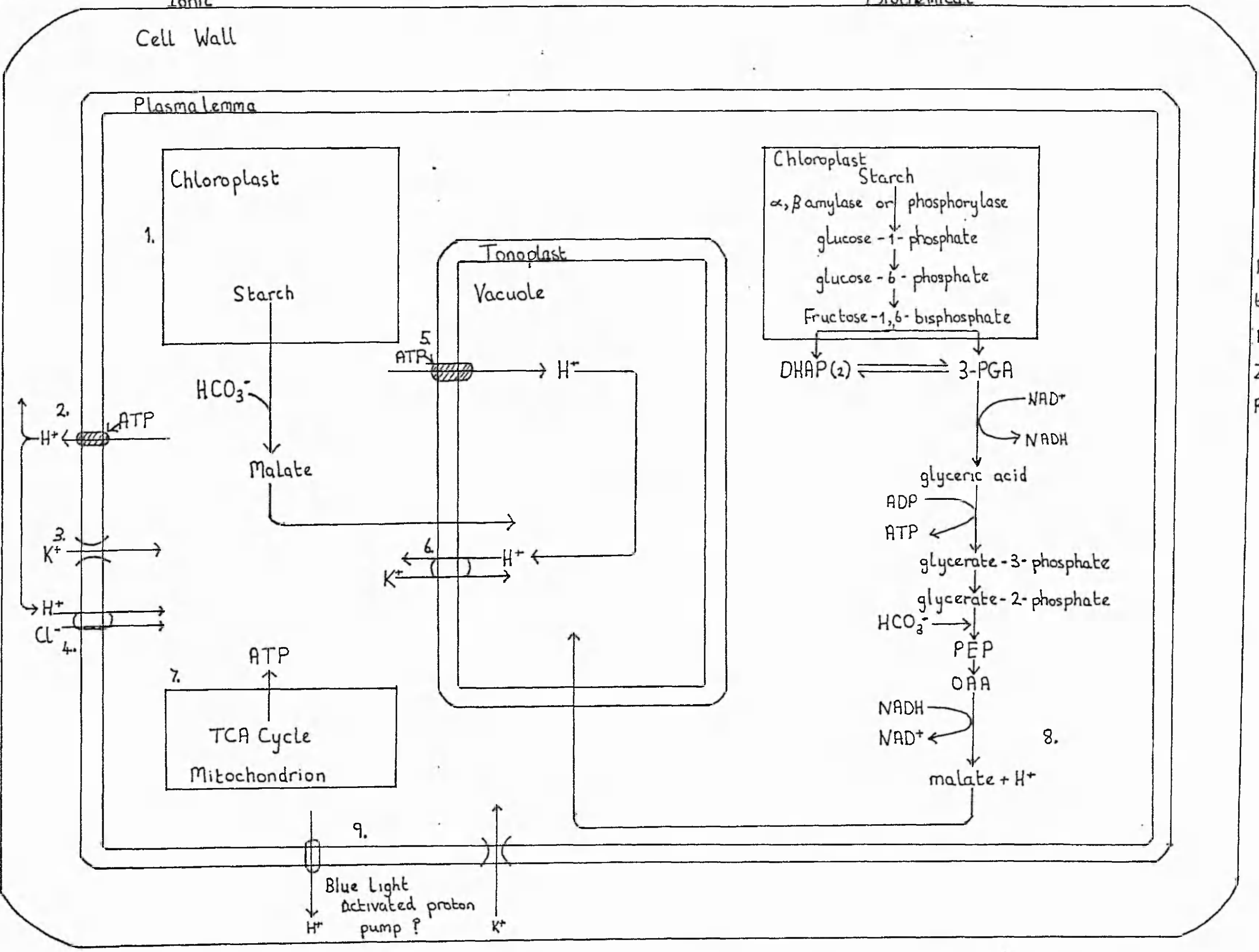
Once the environmental conditions and the incubation media had been standardised and shown to give reliable reproducible results it was possible to commence more detailed studies on the epidermal peels.

The malate dehydrogenase assay was performed as set out in Chapter 5. The NAD-dependant enzyme was chosen for this study as Birkenhead et al (1986) claimed that little of the NADP-dependant enzyme could be found in guard cell protoplasts of C. communis. Each enzyme preparation took a large number of peels. Initial studies were carried out to assess the optimal number of peels for the assay. The preparation of the enzyme was carried out in an ice bath, however, the centrifugation was not carried out in a temperature controlled microfuge. This may have lead to some loss

of enzyme activity, however, the centrifugation was similar for each preparation and so variations between treatments would not have been caused by this. The enzyme was assayed at a temperature of 20°C which was controlled within the spectrophotometer cuvette holder. The protein assay was done according to the method outlined by Schleif et al (1981). This particular version of the Lowry protein assay was chosen as it was designed for work on bacterial cultures and was sensitive to low levels of protein. It should be noted that the enzyme was assayed directly after extraction so that no denaturation occurred due to storage of the enzyme.

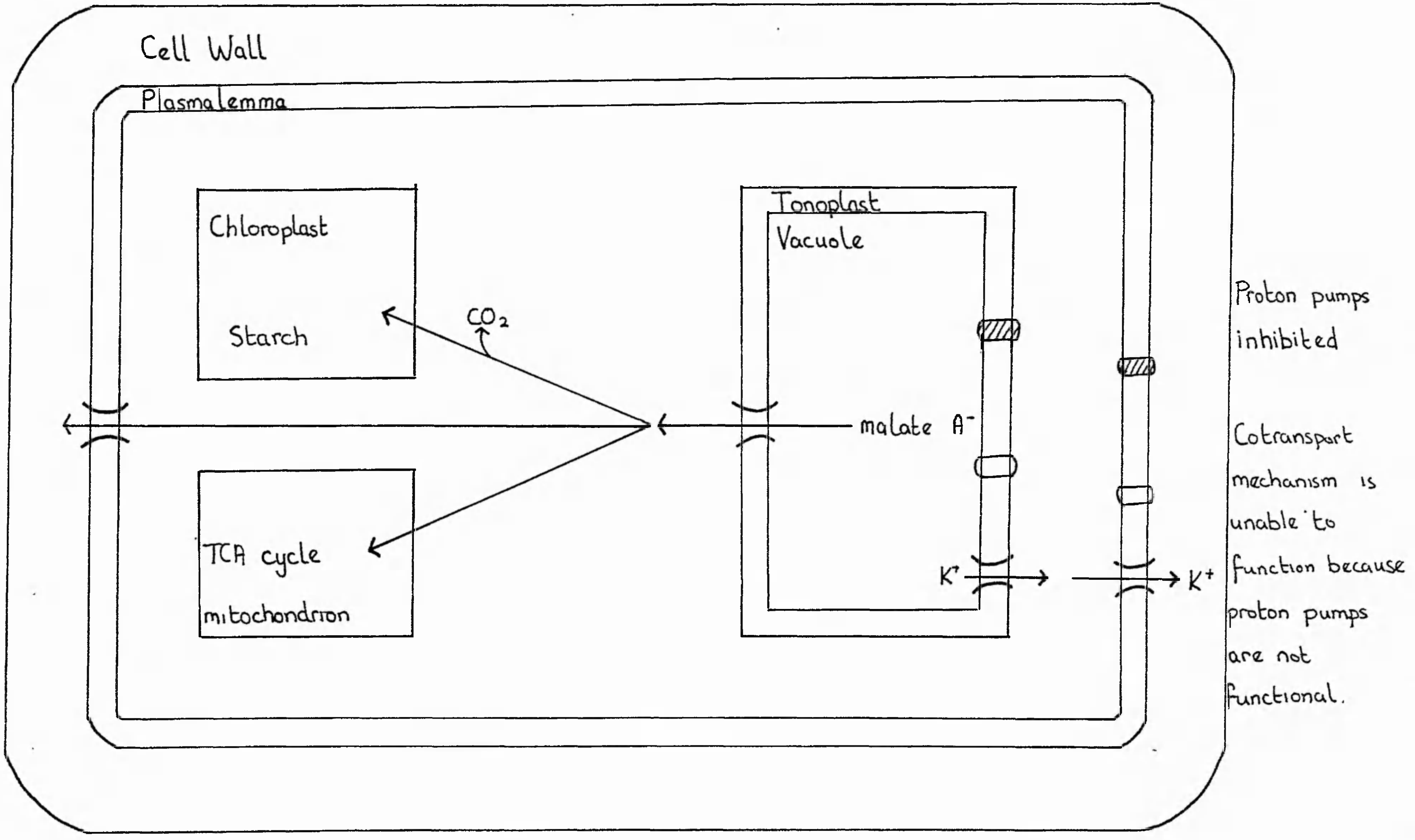
The radiolabel studies were designed to find out whether IAA or bentazone had an effect on the movement of potassium ions into epidermal peels. The isotope of rubidium, ^{86}Rb, seemed the most logical choice of radiolabel as it had been used successfully by several workers in previous studies (Fischer et al, 1968; Mansfield, 1970; Weyers et al, 1979; MacRobbie et al, 1980; MacRobbie, 1981 and 1982). Ideally, it would have been preferable to have used an isotope of potassium as Pallaghy and Fischer (1974) did in their studies on stomatal opening and ion accumulation by guard cells of V. faba. However, the half-life of most potassium isotopes is far too short for experiments of the type used in this investigation. It was possible to show that rubidium could be used to stimulate stomatal opening in a similar manner to potassium (Chapter 6, Figure 6.4) and therefore it was considered to be a good alternative. The techniques used are outlined in Chapter 6. Čerenkov counting was chosen as the method of scintillation counting since ^{86}Rb produces β-particles of sufficiently high energy to permit its use. In order to get peels to take up sufficiently high amounts of ^{86}Rb it was necessary to add 1×10^8 dpm of ^{86}Rb to the test solution. This is a higher level than that used by MacRobbie (1980). When incubations were carried out at pH 4, the radioactive count in the peels appeared to have some relationship to stomatal aperture (Chapter 6, Figure 6.6), however, at pH 6 this was not the case. MacRobbie (1981) suggested that the best pH for post-incubation washing was pH 3.9. When pH 6 incubated peels were

Figure 71.



Adapted from the schemes of Assmann and Zeiger (1987) and Raschke (1987).

Figure 7.2. Stomatal Closure



washed at pH 4 no improvement was seen with respect to the correlation of ^{86}Rb uptake and stomatal aperture. Therefore, as it can be seen the method used still requires improvement.

Thus, bearing the above in mind the basic, epidermal peel techniques were shown to be reliable and to give reproducible results. Therefore studies of malate dehydrogenase and rubidium uptake may add to our understanding of guard cell function in the presence and absence of bentazone and auxin.

7.2 OVERALL INTERPRETATION OF THE FINDINGS IN RELATION TO THE LITERATURE

Figures 7.1 and 7.2 summarise the events thought to be involved in stomatal opening and closure respectively. It is hoped that with reference to the literature and the results of the study it may be possible to explain the responses of the stomata, or at least some of them, to bentazone and IAA. On Figure 7.1 eight possible sites of action for these chemicals have been postulated, some of which have been directly studied in this investigation and others which may require further scrutiny in the light of the findings and current literature.

One of the first areas where bentazone might be expected to have an effect is at the chloroplast (1). Bentazone is known to inhibit photosynthesis and rapidly inhibits CO_2 fixation in isolated chloroplasts (Mine et al, 1975). This action is due to the ability of this herbicide to block photosynthetic electron transport between photosystems I and II and is strictly dependant on the amount of bentazone reaching the chloroplast (Retzlaff et al, 1978). In connection with the transport of bentazone into cells it has been shown that it can be more readily absorbed by cells at lower pH values, i.e. below pH 6 (Retzlaff, 1979). Thus, in the light, bentazone may be expected to reduce stomatal aperture by inhibiting photosynthesis and reducing the production of ATP by photophosphorylation. In this investigation it has been shown that at low potassium concentrations in the light bentazone stimulates stomatal opening but at higher concentrations of potassium suppression of aperture is observed (Figure 3.2 (i)). In darkness

however, bentazone was shown to be able to stimulate stomatal opening over the whole potassium concentration range studied (Figure 3.2 (ii)). In darkness photosynthesis could not occur and the ATP required for stomatal opening would have to be produced by oxidative phosphorylation alone, whereas in the light photophosphorylation is an additional source of ATP and is likely to be responsible for the ability of stomata to open wider in light than in darkness (Assmann and Zeiger, 1987). The suppression of stomatal opening in the light at high potassium concentrations may therefore be due to bentazone preventing or reducing the photosynthetic activity and thus limiting the amount of ATP produced by photophosphorylation and consequently available for active transport processes. This explanation assumes that there is some photosynthetic activity in guard cells. In their review of guard cell bioenergetics Assmann et al (1987) proffered evidence for photosynthetic activity in guard cells by showing that the major pigments and enzymes were present and that the photosystem II inhibitor DCMU could reduce stomatal opening in the light. The action of bentazone on photosynthesis and photophosphorylation however does not provide a full explanation of its ability to alter stomatal movement. The proposed action of bentazone on photosynthesis does not explain its ability to stimulate stomatal opening at low potassium concentrations in the light or over the whole potassium concentration range in darkness. Also the fact that the ability of bentazone to stimulate stomatal opening increases with decreasing pH (Figures 3.4 (i) and 3.4 (ii)) is not consistent with the observation that under conditions of low pH increased amounts of bentazone would reach the chloroplast. At pH's as low as pH 4, however, photosynthesis would not operate since the enzyme systems involved work optimally around pH 7.5 (Retzlaff et al, 1979) and therefore no input of energy from photophosphorylation would be expected under these conditions.

Another possible site of action for bentazone is stimulation or inhibition of plasmalemma bound pumps (2). MacRobbie (1987) in her review states that it is now generally accepted that salt

accumulation in plant cells is driven by primary proton extrusion at the plasmalemma which is powered by ATP. This proton extrusion is thought to generate an electrical driving force (inside membrane negative) for cation entry, plus a pH gradient across the plasmalemma with the cytoplasm being most alkaline. This pH gradient can be dissipated by the synthesis of malate in the cytoplasm or by chloride uptake by cotransport with one or more proton as shown in Figure 7.1. MacRobbie (1987) claims that evidence for this primary proton pump lies in the sensitivity of potassium and chloride uptake to the fungal toxin fusicoccin. Another chemical which is thought to stimulate plasmalemma bound ATPase pumps in IAA. Such action of IAA is often presented in the explanation of its stimulation of coleoptile elongation. There are certain similarities between the molecules of bentazone and IAA and bentazone has been shown to be active in the coleoptile bioassay (Miller et al, 1986). It is considered therefore, that bentazone may have some auxin-type activity and it is therefore possible that it could have as a site of action the plasmalemma bound ATPase pump. Before any work on this could be performed the response of stomata to IAA had to be established. At pH 6 it was found that IAA had little effect on stomatal aperture and really only started to show slight activity at 10^{-3}M which is an abnormally high concentration of IAA. These results were in agreement with those of Govil (1985). Others such as Livne et al (1965) came to the conclusion that IAA had little to do with stomatal movement. Pemadasa (1982), however did show an involvement of IAA in stomatal movement in his investigations into the apparent disparity between the opening of stomata on the abaxial and adaxial epidermes of C. communis. He showed that IAA was able to eliminate the disparity with respect to both opening and potassium accumulation. In this investigation it was possible to show IAA stimulation of stomatal aperture at levels more like those expected within the plant at pH values below pH 6. It was possible to demonstrate that the sensitivity of the guard cell to both bentazone and IAA was increased at low pH. This complies with the work of other researchers who have shown that both IAA (Kaldeway,

1984) and bentazone (Retzlaff <u>et al</u>, 1979) have increased activity at low pH. In both cases this was likely to be related to the dissociation of the molecules as both molecules are most easily transported in the lipophillic (undissociated) form. Having established that IAA had an effect on stomatal movement it was then necessary to see whether there was an interaction between the two chemicals which might suggest a similar site of action. Such an interaction was found at pH 4 (Figure 4.2 and 4.3). It was therefore considered possible that bentazone may have a site of action at the plasmalemma ATPase pump. An inhibitor of plasmalemma-bound ATPase's was included in a brief study in the absence of bentazone and/or IAA and was shown to reduce the uptake of ^{86}Rb, in the light, as would be expected. In the presence of IAA this was not as apparent showing that IAA may have been able to overcome some of the inhibition caused by vanadate or that it was able in some other way to increase the transport of ^{86}Rb across the plasmalemma. Unfortunately, there was too little ^{86}Rb left to complete the study and it was not possible to speculate further on the link between bentazone and plasmalemma bound ATPase pumps.

Another site of action which was considered was that bentazone may in some way have altered the selective permeability of the plasma-membrane itself (3). As discussed earlier it is already thought that calcium ions can alter the specificity of the membrane and it is possible that bentazone may have a similar effect. In his review Raschke(1987) has suggested that calcium may be able to stimulate ion efflux and thus exert some control on stomatal movement. However the activity of calcium ions on stomatal movement is most often linked with ABA effects on stomata. DeSilva <u>et al</u> (1985) showed that the action of ABA is dependant on the presence of calcium ions. Davies and Mansfield (1987) in their review suggest that ABA could increase the passage of calcium through the plasmalemma and that these ions could bind to a small protein, calmodulin, which can activate certain enzymes in the plant cell. If bentazone, like calcium ions, stimulated ion efflux then stomatal closure would be likely to occur, and where bentazone was shown to cause stomatal closure the amount of ^{86}Rb in the cells was reduced

(Figure 6.6). In the presence of the inhibitor CCCP, which increases proton efflux, the amount of ^{86}Rb uptake into the guard cells was increased as would be expected assuming the model shown in Figure 7.1 is correct. Bentazone greatly reduced this ^{86}Rb uptake and the aperture in the presence of CCCP, again demonstrating a possible increase of ion efflux from the guard cell. Thus, the ability of bentazone to cause stomatal closure under some circumstances may be due to an ability to increase the efflux of potassium from the guard cell. This efflux may be due to a general increase in membrane permeability allowing pH and charge gradient to be dissipated or due to bentazone causing a specific leakage of potassium. It should be noted that in the light (Figure 6.6) IAA does not cause a reduction in the uptake of ^{86}Rb suggesting that the effects of the two molecules vary in some respects.

Bentazone could also act as a carrier across the membrane (4). Bentazone is a complex molecule which may move across the membrane taking some ions with it. It could carry ions such as protons and chloride back into the guard cell to help maintain a balance and the rate at which bentazone caused the movement of these ions could dictate whether it led to stomatal opening or closure. At present this is totally hypothetical but it may be an important area of study in the future.

In recent years, more importance has been placed on the role of an ATPase proton pump on the tonoplast (5). Such a pump has recently been found and characterised. Unlike the plasmalemma pump it is not inhibited by vanadate (Rea et al, 1986, unpublished). It is possible that the apparent reduction of the inhibition of vanadate by IAA could have been due to IAA-stimulation of the tonoplast pump. However, what role bentazone could have at such a pump is unknown. The stimulation of this pump would increase the uptake of potassium into the vacuole and may therefore increase opening. The inhibitor potassium nitrate was suggested by Rea et al (1986) to inhibit the tonoplast ATPase pump. In the light nitrate was shown to inhibit ^{86}Rb uptake, although the addition of IAA alleviated this (Figure 6.7) and in darkness nitrate increased ^{86}Rb uptake in the absence of IAA and addition of IAA had no effect.

Again, due to lack of ^{86}Rb, no experiments were performed in the presence of bentazone. These results suggest a difference in the opening mechanisms in light and darkness and they are more likely to be explained by nitrate uncoupling photophosphorylation than by action on a tonoplast pump. However, this does not totally rule out a role for the tonoplast pump, it just highlights the need for further investigation. Again, it is possible that bentazone may be involved as a carrier across the tonoplast possibly increasing the uptake of potassium and extrusion of protons (6). In this position bentazone could act in two ways to maintain stomatal opening at low potassium values. Firstly, bentazone could increase the uptake of potassium and secondly, it could block the extrusion of potassium therefore concentrating it further in the vacuole.

Bentazone could also be responsible for disrupting the production of ATP by the mitochondria (7). This would lead to the alteration of many metabolic processes within the cell and also to a reduction in the amount of ATP available to the proton pumps. The inhibitor DES was included in a brief study. This inhibitor can inhibit potassium absorption by decreasing mitochondrial ATP production in addition to inhibiting membrane bound ATPase activity (Balke et al, 1979). In the light, DES caused a reduction in stomatal aperture (Figure 6.7), however, it actually increased ^{86}Rb uptake in the absence of both IAA and bentazone and in the presence of IAA. In the presence of bentazone, the increase in ^{86}Rb uptake was marginal and unlikely to prove statistically significant in a larger experiment. In darkness (Figure 6.8) DES stimulated ^{86}Rb uptake in the absence of IAA and bentazone but had no effect on uptake in the presence of either chemical. Again, as in the light, it reduced stomatal aperture. In all cases, this demonstrates the importance of ATP in stomatal movement. In the light however, the production of ATP can occur by photophosphorylation and it may be this which allows the uptake of potassium in the absence of both chemicals and in the presence of IAA. However, bentazone may act at the chloroplast (1) and prevent this ATP being produced. In darkness where photophosphorylation is unimportant there is no stimulation of ^{86}Rb uptake in the presence of

either IAA or bentazone. However, ^{86}Rb uptake still occurs in the absence of both chemicals. This in itself suggests that potassium uptake is not totally reliant on the ATPase pump. However, it would appear that in the absence of ATP bentazone and IAA either block the route for potassium uptake or stimulate the efflux of potassium from the cell.

Bentazone may not exert its effect solely at membrane sites or by altering metabolic energy ratios. It may have a more biochemical role and act on enzyme systems. This is more likely to be a secondary effect of the compound as the responses to bentazone have been shown to occur quite rapidly (Figure 4.4). The enzymes system which was chosen for study was malate dehydrogenase which is responsible for the conversion of oxaloacetic acid to malate (8). As has been discussed in earlier chapters, malate is thought to be an important balancing ion in stomatal movement and may also provide some of the protons which are extruded from the cell. One of the explanations offered by Pemadasa (1982) for the ability of IAA to overcome the disparity between adaxial and abaxial stomata was that IAA could increase malate production in the adaxial stomata. However, in this investigation IAA alone was never seen to increase the specific activity of malate dehydrogenase greatly and at pH 6 actually appeared to reduce the specific activity (Figures 5.3 (i) and 5.3 (ii)). This would apparently conflict with the findings of Pemadasa (1982) however, it should be noted that in this investigation abaxial epidermes were used and not adaxial epidermes. Pemadasa (1982) noted that IAA-induced malate production was much less at higher KCl regimes. This fits in with the view of Raschke and Schnabl (1978) who suggested that malate and chloride ions are competitive anions for potassium ions and one begins to play a progressively more dominant role as the other becomes limiting. It is also possible that the increase of malate in the guard cells observed by Pemadasa (1982) may have been due to an alteration of malate efflux or metabolism rather than of malate production. If this was the case, no enhancement of malate dehydrogenase activity would be expected. Indeed, if malate was

retained at high enough levels negative feedback could cause a reduction in malate dehydrogenase activity. The action of bentazone on the specific activity of malate dehydrogenase appeared to correlate a little better with stomatal aperture (Figures 5.1 and 5.2). At low KCl concentrations (pH 6), bentazone was shown to enhance the specific activity of malate dehydrogenase whilst at higher KCl concentrations (>50 mol m^{-3}) the specific activity was either reduced (Figure 5.1) or similar to that of the control (Figure 5.2). These results concur with the findings of Raschke et al (1978) which were discussed above. In these experiments, the specific activity of malate dehydrogenase correlated well with the stomatal apertures. However, in the set of experiments performed under conditions designed to maintain stomatal closure the stimulatory effect of bentazone on stomatal aperture was only seen at low KCl levels. This reflected problems with the growth cabinets. These problems were not likely to be overcome during the remaining time of study and therefore these experiments could not be repeated with plants of a similar standard to those used in the initial part of this investigation. The results of this study seem to suggest that bentazone only affects the specific activity of malate dehydrogenase under opening conditions. This may reflect differences in the mechanisms of stomatal opening in light and darkness. Outlaw (1987), in an overview of carbon metabolism in guard cells, suggests that the mechanism of malate formation in guard cells on opening requires the release of phosphoglycerate molecules from the chloroplasts which have come from the breakdown of starch as shown in Figure 7.1. In darkness there may be a reduced availability of phosphoglycerate as photosynthesis is not occurring and the guard cell starch is not rapidly broken down. Therefore, this would mean that there would be no requirement for enhanced malate dehydrogenase activity in darkness. The results of the malate dehydrogenase study show that bentazone can alter the specific activity of the enzyme but do not yield any real explanation as to how bentazone alters stomatal movement. They also demonstrate that bentazone and IAA do not behave similarly with respect to malate dehydrogenase although they do show that the

chemicals can work together to produce an effect different to the single chemical effect (Figures 5.3 and 5.4). It would therefore seem that bentazone has some auxin-like activity, but also acts on systems that would not normally be affected by IAA. It should be noted that no studies on the effect of either chemical on purified malate dehydrogenase were carried out as part of this investigation. The apparent effects of the specific activity of malate dehydrogenase observed in this study may therefore have been caused by the chemicals causing changes in other systems which in turn affect the activity of malate dehydrogenase. For example, increased demand on malate by the mitochondria or increased transport of malate into the vacuole would remove any chance of negative feedback on malate dehydrogenase and may therefore lead to higher activity. Also the alteration of the activity of other enzymes, for example, PEP carboxylase would affect the availability of oxaloacetic acid and therefore the activity of malate dehydrogenase. The effect of the chemicals on the biochemical pathways involved with stomatal movement is still unclear and warrants further investigation.

There are other possible sites of action for bentazone in the guard cell including the postulated blue light receptor (9). However, the exact identity of the blue light receptor or receptors in plants still remains to be established. The view of Zeiger, Iino, Shimazaki and Ogawa (1987) is that the blue light receptor is most likely to be a flavin which probably stimulates a plasmalemmal ATPase.

There is much about stomatal movement which is still unknown and therefore it is impossible to state conclusively how a stomatally active chemical has its effect. Bentazone has been shown to alter several factors involved in stomatal movement. It has been shown to alter the uptake of ^{86}Rb by the guard cells and this may be linked with an ability to alter energy availability or by direct action on ATPase pumps. Bentazone has also been shown to alter the specific activity of malate dehydrogenase and may therefore be

able to alter the ionic balance by changing malate levels. In some of its actions bentazone has acted similarly to IAA and an interaction has been shown to exist between the two chemicals at pH 4. However differences between the two chemicals were demonstrated in the limited inhibitor study and the responses of malate dehydrogenase activity. Therefore although bentazone may have some IAA-type activity it also acts on different systems or sites to IAA at other times. The speed of bentazone effect suggests at least some effect on the membrane either by action on the ATPase pump or by altering the membrane permeability. The enzymatic effects of the chemical would however serve to prolong the effects of bentazone on the guard cell.

7.3 SUGGESTIONS FOR FURTHER STUDY

This investigation has shown many areas which merit further investigation when studying the effect of bentazone on stomatal movement, for example, the apparent differences in bentazone action under light and darkness may suggest some photosynthetic action and therefore more emphasis may need to be put on photosynthesis in guard cell chloroplasts. Chloroplasts could be extracted from guard cell protoplasts and could be used in experiments to show their photosynthetic ability. Other chloroplasts could be removed from guard cells which have been treated with bentazone. These two chloroplast extracts could be compared for photosynthetic competence and structure. Such a study may give a clearer picture of the role of photosynthesis in stomatal movement as well as showing any action of bentazone.

Photophosphorylation is one possible source of metabolic energy in the cell and oxidative phosphorylation is the other. As it is known that metabolic energy is utilized during stomatal opening it may be necessary to monitor the changes exerted by bentazone on mitochondrial respiration as well as photosynthesis. Assays could also be performed on the amount of ATP available in the guard cells of untreated and bentazone treated peels.

The increased activity of both bentazone and IAA at low pH was explained by increased uptake of the chemicals. However, in this investigation this was never experimentally proven. Therefore, in any future investigation this would need to be studied using radiolabelled bentazone and IAA. Studies into the location of bentazone and IAA within the cell would also be of use as they could give vital information on the sites of action of these chemicals.

Another important area for further study is that of malate concentration. This investigation showed that the specific activity of malate dehydrogenase could be altered by bentazone, although further replication of the study would be required for conclusive statistical data. However, it was not shown how this affected the malate concentration within the guard cell. Malate is considered to be an important balancing ion in stomatal movement. However, it can also be used in mitochondrial respiration and under certain circumstances it can be leaked from the guard cell (Raschke, 1987). The present investigation has not shown what happens to any extra malate produced by increased activity of malate dehydrogenase neither has it demonstrated any direct action of bentazone on purified enzyme. The inclusion of such a study would demonstrate whether the action of bentazone was direct or indirect. Should the action of bentazone be shown to be indirect it would be necessary to perform further enzyme studies to ascertain where the effect of bentazone was exerted. Such studies may include assays of PEP carboxylase activity as this is another key step in the production of malate within the guard cell.

Many more inhibitor studies need to be performed. Those included in this study gave a valuable insight into guard cell action. However, these studies were extremely limited and would not withstand statistical analysis. The use of selective inhibitor studies would yield more constructive data on the plasmalemma and tonoplast ATPase pumps. They would also enable more detailed investigation of the roles of photophosphorylation and oxidative phosphorylation in stomatal movement. It may even be possible to use

inhibitors to block specific enzyme pathways and thus look at the effects of bentazone on single systems or part systems.

In this investigation the activity of bentazone has been compared with that of IAA as this was the plant growth regulator with which it appeared to compare best. Other workers have shown that there is an interaction between ABA and IAA (Davis and Mansfield, 1987). It may be useful to study the action of other plant growth and their interactions with IAA and see how these results compare with those obtained in the bentazone study. Such investigations may give a greater understanding of the processes and controls involved in stomatal movement.

Another area in which this study has raised questions is the use of buffers for stomatal investigations. Although it is important to consider the stability of pH during investigations it is equally important to ensure that the buffer used does not in any way affect the stomata physically or metabolically. This investigation has raised doubts about the use of MES buffer and a comprehensive screen of buffers for use in stomatal studies would prove extremely useful for future investigators.

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pH DEPENDANCE OF AUXIN-INDUCED STOMATAL OPENING

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Stomatal movement is extremely sensitive to both the physical and chemical environment at the leaf surface and many exogenous and endogenous factors have been reported to alter pore size. One such factor is the endogenous plant growth regulator indol-3yl-acetic acid (IAA) which is known to have a widespread role in plant growth and development. The action of IAA on stomatal movement has not been unequivically demonstrated, since at the commonly used pH range of 6 to 7, concentrations as high as 10^{-2}M IAA are required to open stomata. In this investigation abaxial epidermes of Commelina communis were used to study the response of stomata, in the presence of 50 $molm^{-3}$ KCl, to several concentrations of IAA (10^{-3}M - 10^{-6}M) over a pH range of 4 to 7. At pH7 there was little response to IAA even at the highest concentration of 10^{-3}M. However, as pH decreased the stomata became more responsive to lower concentrations of IAA, to the extent that at pH4 10^{-3}M IAA inhibited opening, indicating a supraoptimal response. These responses to IAA were observed in both light and darkness. Neutral red staining revealed that over the experimental period guard cells remained viable at all pH values. A time-course study at pH4 with 10^{-4}M IAA showed that IAA had a marked opening effect within one hour, indicating a rapid action on guard cells at physiologically active concentrations in both light and darkness. This study has therefore demonstrated that IAA can rapidly open stomata on abaxial peels of C.communis and its effectiveness is governed by the pH of the bathing medium.

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Physicochemical and Biophysical Panel Symposium Movement of Pesticides in Plants—Student Meeting

The following are summaries of papers presented at a meeting of the Physicochemical and Biophysical Panel on 12 February 1985 at the Society of Chemical Industry, 14 Belgrave Square, London SW1X 8PS. The papers so presented are entirely the responsibility of the authors and do not reflect the views of the Editorial Board of Pesticide Science.

The Uptake, Translocation and Metabolism of Fluazifop-butyl

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Fluazifop-butyl (butyl (*RS*)-2-[4-(5-trifluoromethyl-2-pyridyloxy)phenoxy] propionate: ICI plc) is a selective postemergence herbicide for the control of annual and perennial grasses in broadleaved crops.[1,2] The uptake, translocation and metabolism of this herbicide was investigated in the susceptible annual grass *Setaria viridis* L. Beauv.

Young 3–4 leaf plants exhibited symptoms of herbicide damage 3 days after a foliar application of fluazifop-butyl at half the recommended field rate (250 g ai. in 200 litres ha^{-1}). The mid and basal portions of mature leaves appeared dark brown and flaccid, whilst developing leaves showed signs of chlorosis. Shortly afterwards, internode elongation ceased and necrosis of the shoot apical meristem was evident. The plants appeared brown and desiccated after 10 days and were dead within 3 weeks.

Herbicide uptake and translocation from the second, third or fourth leaf of 3–4 leaf plants was studied using fluazifop-butyl uniformly ^{14}C-labelled in the phenyl ring (specific activity 0.795 GBq $mmol^{-1}$.[3] In each case the mid portion of the leaf was protected by a polyethylene cover and the plants sprayed with formulated fluazifop-butyl (250 g a.i. ha^{-1}). [^{14}C] fluazifop-butyl was subsequently applied to the unsprayed area as $5\times0.2\mu l$ droplets. Unadsorbed ^{14}C was removed by washing the treated leaf at each assessment time and uptake and translocation were quantified by liquid spectrometry over a 10 day period.

Table 1. Translocation of [^{14}C]fluazifop-butyl from leaf 3

	Recovery of [^{14}C]fluazifop-butyl as % of ^{14}C translocated (h after application)			
	8	72	120	240
Leaves 1 & 2	16.35	12.64	12.35	12.57
Leaf 4	25.96	39.56	48.24	56.29
Apical meristem	37.50	35.16	27.06	21.56
Roots	20.19	12.64	12.35	9.58

Uptake by leaf 3 was rapid and approximately 60% of the applied dose had entered the leaf tissue within 24 h. Further uptake was slow, reaching a maximum of 70% after 120 h. Most of the ^{14}C remained in the treated portion of the leaf, whilst less than 3% moved to either the base or tip. Translocation was limited reaching only 1.8% of the applied dose after 72 h. Thirty-eight per cent of this translocated activity accumulated in the shoot apical meristem within 8 h (Table 1) and this tissue appeared necrotic after 72 h. Subsequent translocation of ^{14}C was predominantly to the developing foliage.

Uptake of ^{14}C by the immature leaf 4 was more rapid than by the older leaf 2 and, furthermore, significant acropetal movement within this leaf was detected after 72 h. Transloca-

tion from both leaves was low (<2%) but the physiological status of leaf 2 as an assimilate exporter was reflected in the proportion of herbicide translocated from this leaf which was twice that translocated from leaf 4. Over half of the activity from leaf 2 accumulated in the younger leaves 3 and 4 whilst ^{14}C from leaf 4 moved primarily to the shoot meristem.

Fluazifop-butyl was hydrolysed rapidly to the free acid, fluazifop, within the treated foliage and further metabolism to acid conjugates occurred, although 72 h after application a proportion of unmetabolised fluazifop-butyl was still detectable. Concurrent analysis of the shoot apical meristem revealed only fluazifop and acid conjugates.

These observations indicate that fluazifop is the likely phytotoxic moiety of fluazifop-butyl. The primary site of action is shown to be the shoot apical meristem, in agreement with a recent study of the activity of this herbicide on *E. repens.*[4]

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Metabolically Active *Glycine max* Cells for the Study of the Activity and Movement of Herbicides

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The routine and successful mechanical isolation of cells from *Glycine max* first trifoliate leaves has been achieved. Plants of *G.max* (cv. Fiskeby V) were grown in a controlled environment at 25°C, under a photon flux density of $125 \mu mol\, m^{-2} s^{-1}$ (400–700 nm), and a total irradiance of $160\, W m^{-2}$ at the leaf surface. Cells isolated from 27-day old tissue provided maximum photosynthetic activity which approached $140 \mu mol$ of oxygen evolved mg^{-1} chlorophyll h^{-1} and these rates compared favourably with those cited for enzymic isolation techniques.[1,2] More than 70% of the isolated cells were intact as measured by fluorescein diacetate ultraviolet microscopy, and by enzymic conversion of cells to protoplasts.[3] *G.max* cells of maximum photosynthetic activity were then employed to study key metabolic pathways including respiration, protein, nucleic acid, and lipid metabolism. Once the metabolic competence of these cells was characterised the interaction of selected herbicides was investigated.

Data are presented to show the effects of 0.1–500 μM bentazone, ioxynil and atrazine on photosynthesis as measured by fluorescence (excitation wavelength of 420 nm and emission wavelength between 648 and 725 nm in a Turner model 112 filter fluorometer), and bicarbonate-dependent oxygen evolution (Hansatech oxygen electrode, at $500 \mu mol\, m^{-2} s^{-1}$, 25°C and 10 mM sodium hydrogen carbonate in medium buffered to pH 7.6. Fluorescence intensity rapidly increased on addition of >0.1 μM atrazine and ioxynil to the cell suspension, whereas the fluorescence response following the addition of up to 500 μM of bentazone was low. Atrazine caused an immediate and linear inhibition of oxygen evolution proportional to concentration, I_{50}=0.47 μM. Ioxynil treatment produced a short lag period prior to inhibition (5 min at 5 μM), whereas long lag periods were observed with bentazone (60 min at 10 μM) prior to any inhibition of oxygen evolution.

Atrazine was shown to enter the chloroplast rapidly. This highly lipophilic, water insoluble, and associated molecule was rapid in its action. Ioxynil-sodium, in contrast, showed a less potent and less rapid action than atrazine. As a highly water soluble salt, a balance between hydrophile and lipophile may account for the longer time required for its action. The movement of bentazone was slow, and the barriers to its entry into the cells are considerable under these pH conditions. It is therefore suggested that both ioxynil-sodium and bentazone are less specific in their action, and may inhibit other more sensitive areas of cell metabolism in addition to their effects on photosynthetic electron transport. It is proposed that further studies with isolated *G.max* cells will provide clearer evidence for the mechanisms of action of these herbicides.

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Uptake and Movement of M&B 34790 and M&B 30755 in Cotton and Certain Weed Species

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In greenhouse trials both M&B 34790, (*RS*)-1-(3,4-dichloro-α-methoxybenzyl)-*N,N'*-dimethylimidazole-4,5-dicarboxamide, and M&B 30755, 1-(3,4-dichlorobenzyl)-*N,N'*-dimethylimidazole-4,5-dicarboxamide, showed differential herbicidal activity towards cotton, velvet leaf (*Abutilon theophrasti* L.) and barnyard grass (*Echinochloa crusgalli* L.). M&B 34790 was more selective and M&B 30755 more active as indicated by the phytotoxic symptoms which were indicative of contact-type herbicides. Cotton was susceptible to M&B 34790 but not M&B 30755; velvet leaf was susceptible and barnyard grass tolerant to both compounds.

Uptake and translocation studies were carried out using ^{14}C-ring labelled herbicides. After a seven day treatment period neither M&B 34790 nor M&B 30755 moved out of the treated leaves in any significant amounts; there was no evidence of herbicide metabolism during this period. Both compounds however, moved apoplastically within the treated leaves particularly in the case of M&B 34790. The levels of water/octanol partitioning, wax retention and accumulation around the application sites indicated that M&B 30755 was the more lipophilic compound.

The primary site of action of M&B 34790 and M&B 30755 appeared to involve the photosynthetic system and membrane permeability. In-vitro and in-vivo studies indicated that both compounds inhibited photosynthesis as expressed by oxygen evolution from isolated leaf discs. In-vitro inhibitory action of M&B 30755 was correlated with the ability of the herbicide to associate with isolated chloroplasts.

The selectivity of these herbicides appeared to involve differences in the efficiency of movement to the chloroplasts reflecting differing lipophilicity and association with cell or chloroplast membranes.

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1,8-Naphthalic Anhydride as a Herbicide Safener for Cultivated Oats

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The effect of the safener, 1,8-naphthalic anhydride on the uptake and translocation of diclofop-methyl (methyl (*RS*)-2-[4-(2,4-dichlorophenoxy)phenoxy]propanoate), in oat seedlings (*Avena sativa* cv. Margam) was investigated.

Oat seeds dusted with naphthalic anhydride (2% by wt.) were sown in a sandy loam and grown in a controlled environment. At the 1.5 leaf stage a 2.5μl droplet containing radiolabelled [*dichlorophenyl*-U-^{14}C]dichlofop-methyl was applied to the adaxial surface of each first leaf.

For uptake studies 0.03 μCi of *RS*-($\pm$)-diclofop-methyl (specific activity 19.8 mCi g^{-1}) was applied to each plant. Sampling times were 18, 42, 64 and 96 h after treatment. For translocation studies three levels of R-(+)-diclofop-methyl (specific activity 27.5 mCi g^{-1}) were applied. After 96 h shoots were divided into four sections. In both experiments, surface ^{14}C was removed from treated zones by washing and radioactivity in the remaining plant material was assayed by combustion and scintillation counting.

The results indicated a steady uptake of herbicide at the four sample times. After 96 h, approximately 80% of the applied ^{14}C was still present in the seedlings after surface deposits had

been removed. The uptake of herbicide by safened plants was consistently higher than controls though differences were small and not statistically significant.

Less ^{14}C (expressed as a percentage of total ^{14}C taken up) was translocated to the various shoot sections in safened oats when compared with controls.

It may therefore be assumed that differences in herbicide uptake do not explain the safening of oat seedlings by naphthalic anhydride. However, the small differences in diclofop-methyl translocation shown by safened and control seedlings, may at least contribute to the effect.

Foliar Uptake of Some Linear Alcohol and Alkylphenol Polyoxyethylene Surfactants

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Most pesticide formulations for use as water-based foliar sprays contain surfactants that are added not only as formulants *per se* but also as adjuvants to enhance retention and spreading of deposits and to increase uptake of active ingredients thereby improving target coverage, foliar penetration and sometimes selectivity. However, despite their widespread use and proven effectiveness in commercial practice, very little is known about the role of surfactants in the penetration process or even their fate following application to plants.

^{14}C-labelled compounds were used to examine the uptake, movement and metabolism of dilute aqueous solutions (0.5–5.0 g litre^{-1}) of three nonionic polyoxyethylene surfactants (a homogeneous linear alcohol $C_{12}E_8$, an oligomeric linear alcohol mixture $C_{18}E_{8.5}$ and an oligomeric nonylphenol mixture $NP\,E_{5.5}$) following foliar applications to a range of plant species. Surfactant uptake was rapid showing a characteristic double exponential form of curve, the rate depending mainly on plant species. Penetration rates for waxy leaves (e.g. rape, peas) were usually much greater than those for non-waxy leaves (e.g. field bean, sugar beet). Of the three surfactants examined, $C_{12}E_8$ entered leaves most rapidly with up to 90% uptake within 2 h of application in some species. Most of the surfactant that entered the plant, however, remained in the treated area where it was metabolised. Both the rates and products of metabolism varied according to plant species. In some plants, significant degradation could be detected within 1 h of penetration whereas in others breakdown was less rapid with substantial amounts of unchanged surfactant still being present after 24 days. From preliminary experiments with $C_{12}E_8$ it would appear that metabolism occurs by two main routes, (i) by de-ethoxylation to yield less polar products and (ii) by conjugation, probably with sugars, to form polar metabolites.

The Role of Uptake and Movement in the Differential Activity of the Hydroxybenzonitrile Herbicides

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The hydroxybenzonitrile herbicides were introduced in the 1960s for the postemergence control of broad-leaved weeds in cereals.[1] In addition to selectivity between the crop and weed, some weed species also exhibit a differential response to ioxynil and bromoxynil salts. Such differences are particularly evident in *Viola arvensis* (Field Pansy) and *Matricaria inodora* (Scentless Mayweed) and the mechanisms of activity and selectivity have been investigated in a post-graduate study.[2] This presentation assesses the role of uptake and movement in the differential response of these species.

Three–four leaved *V.arvensis* and *M.inodora* were sprayed with ioxynil-sodium and bromoxynil-potassium at field rate (560 g a.i. ha^{-1} 200 litre ha^{-1}) using a hydraulic laboratory pot sprayer. Typical symptoms were chlorosis and rapid necrosis of treated and developing leaves in *M.inodora*, and limited chlorosis of treated foliage in *V.arvensis*. Quantitative analysis of these

symptoms revealed that *M.inodora* was moderately susceptible to ioxynil-sodium and acutely susceptible to bromoxynil-potassium, whilst *V.arvensis* was moderately resistant to ioxynil-sodium, and resistant to bromoxynil-potassium.

This differential response could not be explained by interspecific differences in retention and leaf surface wettability. Microdroplet application of [^{14}C]ioxynil-sodium and [^{14}C]bromoxynil-potassium to leaf 3 of 3–4 leaved plants revealed slow uptake of these unformulated salts. After 7 days, 8.3 and 1.5% of the applied [^{14}C]ioxynil-sodium and [^{14}C]bromoxynil-potassium respectively had penetrated *M.inodora* whereas 16.8% of [^{14}C]ioxynil-sodium and 3.9% of [^{14}C]bromoxynil-potassium had penetrated *V.arvensis*. Selectivity thus appeared to be independent of the rate of hydroxybenzontrile uptake. Ultrastructural studies revealed rapid cellular disruption in *M.inodora* which may have prevented further uptake of ioxynil and bromoxynil.

Although greater [^{14}C]ioxynil uptake occurred in *V.arvensis*, 88–99% remained in the treated leaf and there was no significant movement to the apex and developing leaves. In contrast, [^{14}C]ioxynil-sodium was more mobile in susceptible *M.inodora* in which up to 8.1% was detected in the apex and presumably interfered with shoot development. Similarly, [^{14}C]bromoxynil-potassium was more mobile in *M.inodora* than *V.arvensis*. In metabolism studies, no evidence was obtained for the breakdown of ioxynil-sodium by either species or bromoxynil-potassium by *M.inodora*. However two unidentified halogenated metabolites were extracted from bromoxynil-potassium treated *V.arvensis* which may contribute to reduced phytotoxicity in this species.

The hydroxybenzonitriles have a site of action in the chloroplast and inhibit photosynthesis by binding to the thylakoid membrane. In-vitro studies have shown that ioxynil binds more strongly to the thylakoid membranes of *M.inodora* and *V.arvensis*, and is a more effective inhibitor than bromoxynil.[3] Thus selectivity must occur before the hydroxybenzonitriles bind to the site of action. It is proposed that a combination of contact and translocated effects may enhance the activity of the hydroxybenzonitriles in *M.inodora*. In contrast, ioxynil-sodium has a contact effect only against *V.arvensis* due to the low level of translocation. Furthermore bromoxynil-potassium is metabolised by *V.arvensis* which may further reduce phytotoxicity.

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Phloem Translocation of Xenobiotics in Plants: a Physicochemical Approach.

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Most compounds known to be transported in phloem are either polar and non-ionised or weak acids, thus giving rise to the intermediate permeability[1] and the weak acid[2] hypotheses, respectively. This work examines these theories in more detail using non-ionised chemicals with a range of octan-1-ol/water partition coefficients (K_{ow}) and weak acids with various PK_a and K_{ow} values.

Five-week-old plants of *Ricinus communis* var. Gibsonii, grown in nutrient solution, were used as model plants because of the ease with which phloem exudate can be collected from incisions in the stem. [^{14}C]-labelled chemicals were either applied to roots via nutrient solution or injected into the hollow petioles of mature leaves. Phloem sap was collected at different points along the stem and the distribution of parent chemicals in the plant determined following extraction and thin layer chromatography.

Efficiency of translocation of non-ionised chemicals to shoots following uptake by roots was similar to that observed in barley.[3] For compounds of log K_{ow}~2, phloem and xylem concentrations were similar over 72 h of uptake and, after transfer of the plants to untreated nutrient solution, these chemicals rapidly disappeared from the phloem. For the more polar chemicals, oxamyl and aldicarb sulphone (log K_{ow}~−0.5), phloem concentrations rose over 72 h

to several times the xylem concentrations and changed little after transfer of the plants to untreated solution, indicating export in the phloem of chemical from the leaves. These results show that phloem is freely accessible to these chemicals, but only polar chemicals whose permeation rates through membranes are relatively slow are sufficiently retained in the phloem to be transported over long distances, as described by the intermediate permeability hypothesis. Further supporting evidence is that following petiole injection, only the polar chemicals were found in phloem though very little chemical was found in roots.

Phenoxyacetic acids ($pK_a \sim 3$) with log K_{ow} between 1 and 3 were readily taken up and translocated in phloem to the apical leaf and the upper stem following petiole injection; strong retention in the stem rapidly depleted the phloem concentration down the stem so that little chemical was transported to the roots. Maleic hydrazide (pK_a 5.65, log K_{ow} -0.6) was not so well taken up from the petioles but was readily phloem translocated from the leaves and much less was retained by the stem, permitting appreciable amounts to reach the roots and external solution. A polar phenoxyacetic acid (log K_{ow} 0.1) was neither well taken up from the petioles nor well translocated in phloem. These findings can be considered in terms of the higher permeability of membranes to free acids compared to their anions which results in the accumulation of weak acids in plant compartments of higher pH.

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Movement of PP969 Injected into Apple Trees for the Control of Mildew and Scab

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Pressure injection of PP969, (5*RS*,6*RS*)-6-hydroxy-2,2,7,7, tetramethyl-5-(1,2,4-triazol-1-yl)octan-3-one, (Figure 1), an experimental fungicide produced by ICI plc., into Cox apple trees three weeks before bud-burst in 1983, initially placed a greater amount of chemical in trunk heartwood rather than sapwood. After bud-burst, PP969 moved into the xylem and was translocated into the tree crown where it gave good control of scab on leaves for 8–10 weeks. Extension growth continued to be protected throughout the season but spur leaves of some treated trees became infected. PP969 moved into young fruits but none was detectable at the end of the season; thus control of fruit scab would depend upon keeping leaves free from infection.

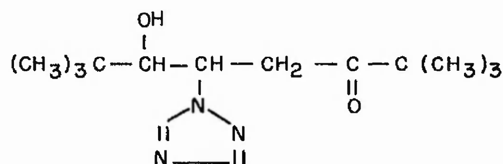

Figure 1. Structure of PP969, (5*RS*, 6*RS*) diastereoisomer

Whilst the PP969 initially placed in sapwood moved into the tree crown, movement of that in heartwood was restricted, possibly due to a combination of the binding of chemical to lignified tissue and of very limited water movement, so that little chemical was able to move from this area into sapwood and then into actively translocating xylem.

When PP969 was formulated as a viscous solution in water containing the gelling agent Kelzan and introduced into holes in trees without pressure, that proportion within sapwood was redistributed into the tree crown; however, the majority initially moved into heartwood and remained there.

The positioning of injection lances and timing of injection is critical in terms of achieving regular redistribution and effective delivery of the injected chemical to the target site.

Are Stomata Implicated in Herbicide Uptake and Movement?

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Leaf surface stomata are very sensitive to both internal and external stimuli and respond rapidly to a wide range of environmental and chemical conditions by opening and closure. Consequently, stomatal movement controls the leaf uptake of carbon dioxide (photosynthesis) and loss of water (transpiration), and may therefore exert considerable influence on herbicide uptake and movement. For example, transpiration is an essential factor for the uptake of soil applied herbicides through the roots,[1] and because stomatal movement will alter the rate of flow of the transpiration stream it will probably alter the rate of uptake of xylem-mobile herbicides. By altering photosynthetic rate, stomatal movement will also have control over phloem movement and therefore the long distance movement of herbicides which travel in the phloem either on their own or bound to photosynthate. In theory, therefore, any herbicide which is able to alter stomatal movement may play a role in controlling its own uptake and movement.

The activity of the herbicide bentazone has been shown to be extremely sensitive to environmental conditions. Originally the cause was thought to be connected with changes in the epicuticular wax composition and deposition.[2] Subsequently, however, it was found that the ability of bentazone to control *Chenopodium album* was dependent upon the temperature, light and humidity regimes in which plants were kept, both before and after bentazone application.[3] All of these environmental factors are able also to influence stomatal movement. Dunleavy[4] demonstrated that when plants of *C.album* with open stomata were sprayed with bentazone the leaf diffusive resistance increased indicating closure of open stomata by field rate bentazone. It was also shown that plants sprayed when stomata were open were killed more rapidly than those sprayed when the stomata were partially closed. Furthermore, studies using labelled bentazone showed that there was increased leaf uptake of bentazone when plants with open stomata were treated. Dunleavy[4] was also able to demonstrate that 1/10 field rate bentazone was able to increase transpiration. Therefore bentazone is able to open or close *C.album* stomata depending on bentazone concentration, time of day and prevailing environmental conditions.

This response was further characterised by in-vitro epidermal peel studies, and it was shown that bentazone could open closed stomata and close open stomata on *C.album* epidermal peels.[4] Further studies have shown that these responses are widespread and can be demonstrated in several species including *Vicia faba, Commelina communis, Beta vulgaris, Viola arvensis, Brassica napus, Pisum sativum, Galium aparine* and *Nicotiana tabacum*. Experiments have also been performed using isolated guard cell protoplasts from *C.communis* and it has been possible to demonstrate a rapid bentazone-induced swelling of guard cell protoplasts in darkness. This swelling demonstrates that the addition of bentazone effectively doubles the protoplast volume within a very short period of time. Experiments therefore indicate that there is a rapid membrane response to bentazone which further supports the responses obtained in whole leaves. Further work is now in progress to characterise the physiological nature of this response.

It has been inferred in the literature that some other herbicides may alter stomatal movement. Work is clearly needed to characterise and further substantiate these interactions, as stomatal movement appears to have a profound influence on herbicide uptake and long distance movement.

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THE CELL MEMBRANE AS A SITE FOR BENTAZONE ACTION

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ABSTRACT

Bentazone can induce rapid, concentration-dependent turgor changes in both light and darkness in isolated protoplasts (from Glycine max mesophyll cells and Commelina communis guard cells), abaxial epidermes (from C.communis) and etiolated coleoptiles (from Avena sativa). Hence, bentazone can alter protoplast size, stomatal apertures and coleoptile elongation, and each experimental system is sensitive to specific cations and may be inactivated by inhibitors of cytoplasmic ATP synthesis. A unifying scheme is proposed that interprets this data in terms of the cell membrane as a site of bentazone action, that is independent of any role in photosynthesis.

INTRODUCTION

Bentazone (3-isopropyl-1H-2,1,3-benzothiadiazine-4(3H)-one 2,2-dioxide) is a selective herbicide for the postemergence control of broadleaved weeds in several important crops including soybean, rice, peanut, pea, maize and beans. Studies conducted during the 1970s by several workers showed that this major herbicide inhibited electron flow at photosystem II, using isolated thylakoids (Pfister et al. 1975), chloroplasts (Mine & Matsunaka 1975, Böger et al. 1977) and cells (Retzlaff et al. 1979), and also inhibited photosynthesis in intact leaves (Penner 1975, Retzlaff & Hamm 1976, Potter 1977). However, subsequent studies in this laboratory clearly demonstrated that bentazone efficacy was particularly sensitive to environmental conditions both before and after application (Taylor et al. 1981), and this led to the hypothesis that bentazone could alter stomatal movement in Chenopodium album leaves (Dunleavy et al. 1982, Cobb et al. 1983) and isolated abaxial epidermes (Dunleavy & Cobb 1984 a and b). More recent observations (Nichols unpublished) have demonstrated that this response is not confined to C.album but is more widespread, suggesting that a feature of the bentazone molecule is an ability to alter stomatal movement, presumably by changing the permeability of the stomatal guard cell membranes.

The present study was conducted to examine further this membrane response in isolated protoplasts, epidermes and coleoptiles, in both light and darkness and to gain further insight into the role of the cell membrane as a site of bentazone action.

MATERIALS AND METHODS

Glycine max mesophyll cell protoplasts

Protoplasts were enzymatically prepared from mesophyll cells of first trifoliate leaves of Glycine max L. Merrill cv Fiskeby V using the method of Rees et al. (1985). Protoplasts were highly intact, c. 90% pure and capable of photosynthetic rates of up to 90 µmoles O_2 evolved.(mg.chlorophyll)$^{-1}$.hr^{-1}. For volume determinations protoplasts (2×10^6 ml^{-1}) were first incubated for 10 min at 25°C at a photon flux density (PFD) of 100 µmol.m^{-2}.s^{-1} (photosynthetically active radiation, PAR). An appropriate concentration of bentazone was then added to the reaction mixture (final concentration 0, 1, 10, 100 and 1000 µM) which was stirred (80 rpm), illuminated at 500 µmol.m^{-2}.s^{-1} and maintained at 25°C pH 7.6 for up to

90 min. Aliquots were withdrawn at regular intervals and protoplast diameters determined microscopically and, assuming the protoplasts to be spherical, converted to volumes using the formula $4/3\ \pi r^3$. Each bentazone treatment was replicated 4 times and mean protoplast volumes calculated ± S.E. (n = 40).

Commelina communis epidermes and guard cell protoplasts

Fully expanded leaves of Commelina communis L. were pretreated for 70 min on 25 ml 0.1 mM $CaCl_2$ and exposed to a photon flux density of 50 µmol. $m^{-2}.s^{-1}$ (PAR). Abaxial epidermes were carefully prepared, all visible contamination removed and the peels floated on pretreatment solutions for 60 min to induce either stomatal opening or closure. Opening was induced by the presence of 50 µmol.$m^{-2}.s^{-1}$ (PAR), 25°C, 100 mM KCl and < 50 µl/l CO_2, whereas closure was obtained by darkness, 25°C, > 500 µl/l CO_2 and the absence of KCl. Epidermal peels were then transferred to treatment solutions of varying KCl concentration ± 100 µM bentazone for 2 h and the environmental conditions maintained as described above. All solutions were maintained at pH 6.0 by the addition of KOH and pH remained constant throughout the period of study. Stomatal apertures were directly measured microscopically, each treatment was replicated 3 times and mean apertures calculated ± S.E. (n = 60).

Guard cell protoplasts were enzymatically isolated from abaxial epidermes according to the method of Fitzsimons and Weyers (1983). Protoplasts were incubated in buffer (pH 5.5) containing 10 mM KCl and 100 µM bentazone for up to 60 min at 25°C in darkness. Protoplast diameters were measured microscopically and mean volumes calculated ± S.E. (n = 30).

Coleoptile elongation in etiolated Avena sativa

Avena sativa L. cv Maris Tabard was grown in moist vermiculite for 3 d in total darkness at 25°C. Coleoptiles were removed in dim light, the apical 0.5 cm discarded and 1 cm segments preincubated on 25 ml 5 mM K_2HPO_4/KH_2PO_4 buffer (pH 6.0) for 45 min at 25°C in darkness. Segments were then transferred to 25 ml of incubation medium (buffer plus bentazone or IAA) and maintained in darkness at 25°C for up to 60 min. Fifteen segments were carefully removed at predetermined intervals and their lengths precisely determined using an overhead projector. Each treatment was replicated at least 3 times, mean lengths calculated ± S.E. (n = 45) and regression values determined where appropriate. In Fig. 3A, r = 0.69, control; r = 0.71, 1 µM; r = 0.71, 10 µM and r = 0.72, 100 µM bentazone.

RESULTS

The data presented in Figures 1, 2 and 3 clearly indicate an action of bentazone at the cell membrane of mesophyll and guard cell protoplasts, abaxial epidermes and etiolated coleoptiles. In each case the bentazone-induced responses were rapid and suggested large turgor changes within the treated cells.

Physiologically competent protoplasts isolated from G.max mesophyll cells showed a rapid increase in volume in the presence of bentazone (Fig. 1A). This response was concentration-dependent, in that 10 µM bentazone induced a near doubling in protoplast volume (197% of control values) after 30 min of incubation in the light (Fig. 1B). No strict light-dependency was evident in this response, since 10 µM bentazone was also found to cause protoplast swelling in total darkness (161% of control values after 30 min incubation). 100 µM bentazone induced a similar volume increase in protoplasts isolated from C.communis abaxial epidermes (Fig. 2A). This protoplast swelling in darkness was equally rapid but more transient, since

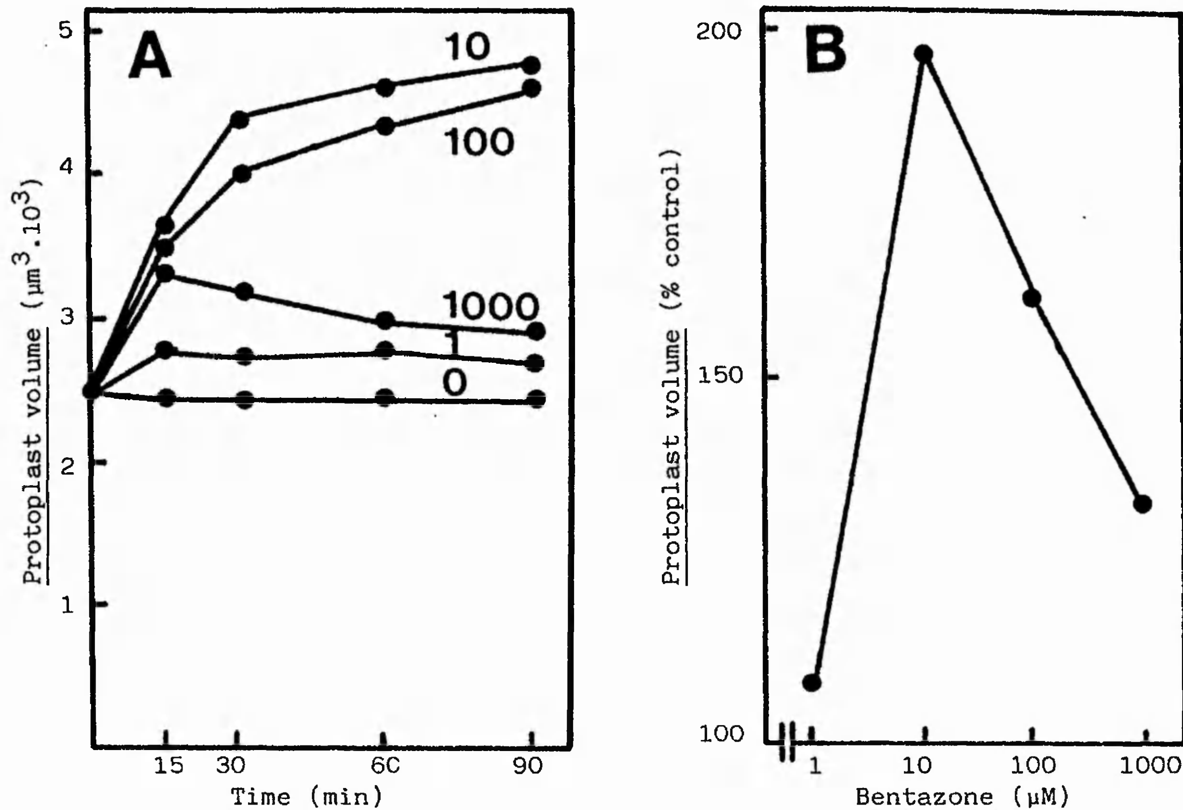

Fig. 1. Bentazone-induced protoplast swelling in isolated mesophyll cells from Glycine max incubated in the light, 25°C, pH 7.6 and in the presence of 0-1000 μM bentazone. Fig. 1A illustrates the time-course of protoplast swelling over a 90 min period, whereas Fig. 1B indicates a concentration-dependent response, expressed as % of control values after 30 min incubation.

volumes were observed to return to control values after 40 min of incubation, possibly implying a reversible response in this cell type. In both experimental systems little or no protoplast rupture was observed as a result of bentazone treatment. Furthermore, staining with neutral red, Evan's blue or fluorescein diacetate indicated a high degree of plasmalemma integrity suggesting fully functional cell membranes.

Guard cells function in vivo to control stomatal apertures by changes in their turgor. Hence, increased guard cell turgor, caused by potassium ion and water influx, induces stomatal opening, whilst decreased turgor, potassium ion and water efflux, results in stomatal closure. 100 μM bentazone was therefore added to functional C.communis abaxial epidermes for 2 h to examine the effect of turgor changes induced by bentazone in the incubation medium (Fig. 2B). In the light, where experimental conditions favoured stomatal opening, 100 μM bentazone induced a potassium concentration-dependent response. Below c. 80 mM potassium, bentazone caused stomatal opening relative to the control, whereas above c. 80 mM potassium, apertures were less than control values. On the other hand, 100 μM bentazone produced wider stomatal apertures than the control throughout the potassium concentration range when incubated in darkness. Thus, the

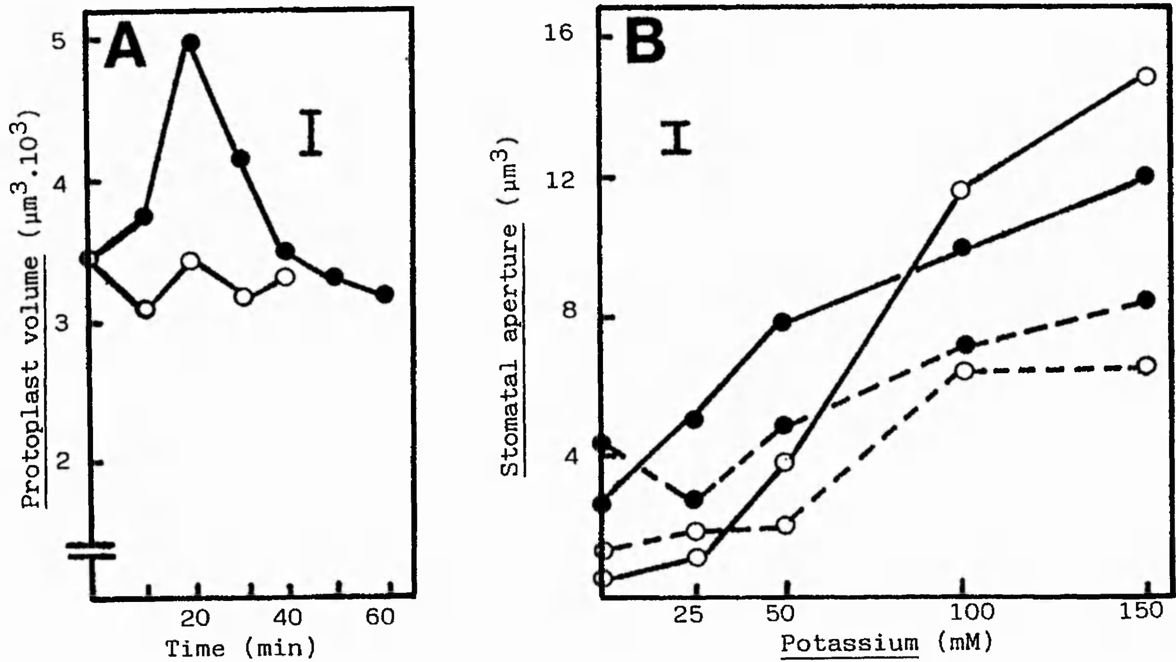

Fig. 2A. Bentazone-induced protoplast swelling in isolated guard cell protoplasts from Commelina communis incubated in darkness, 25°C and pH 5.5 for up to 60 min in the presence (●-●) or absence (O-O) of 100 μM bentazone. Fig. 2B. Effect of 100 μM bentazone on stomatal movement in abaxial epidermes of C.communis following 2 h incubation in either opening or closing conditions, at 0-150 mM KCl. Solid lines, opening conditions; broken lines, closing conditions; open circles, bentazone absent; closed circles, bentazone present.

bentazone-induced turgor changes observed with guard cell protoplasts in darkness (Fig. 2A) are reflected in wider stomatal apertures in dark-incubated epiderms (Fig. 2B).

Another well documented, rapid turgor response of plant cells is the elongation of etiolated Avena coleoptiles, particularly in the presence of indole acetic acid (IAA). Proton excretion from the coleoptile cells is thought to loosen the cell wall polysaccharides and the resultant influx of water causes an increase in cell length. Figure 3A clearly demonstrates that bentazone is able to induce coleoptile elongation in darkness in a concentration-dependent fashion. Indeed, 10 μM bentazone induced a three -fold increase in the rate of coleoptile elongation when compared to the control values, although it was not as active as IAA (Fig. 3B).

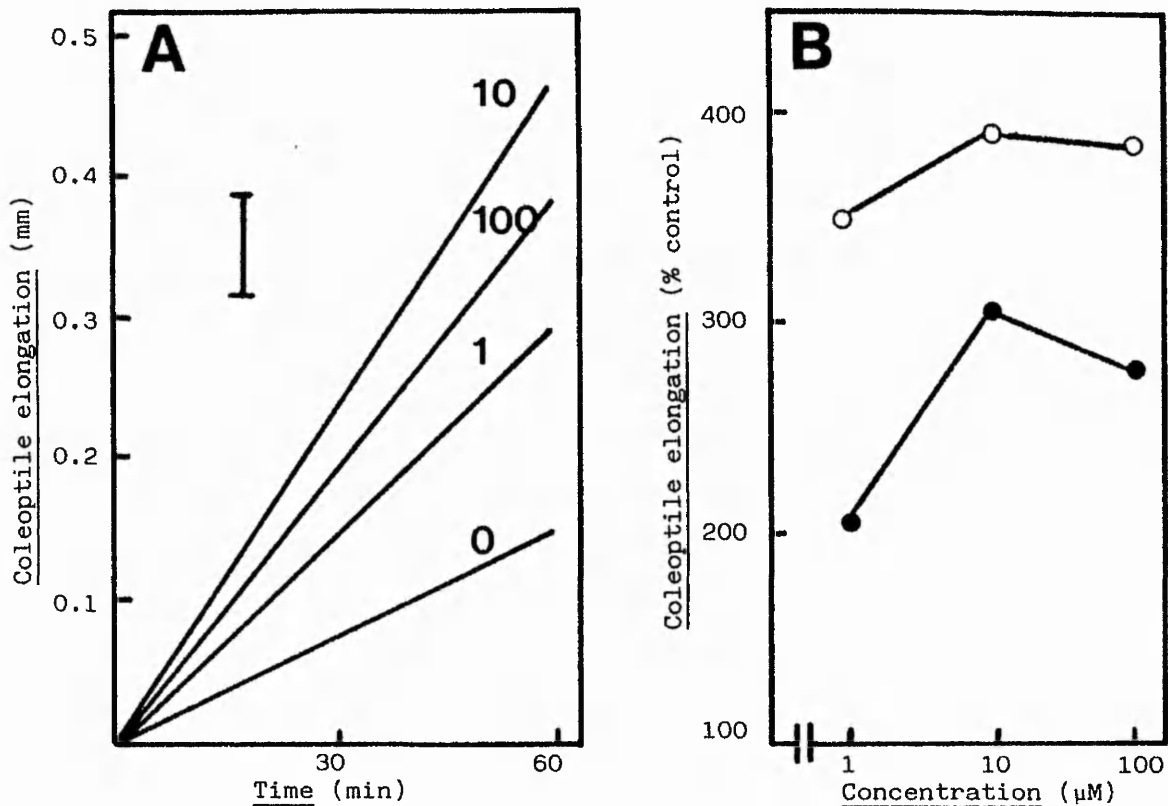

Fig. 3. Bentazone-induced coleoptile elongation in etiolated Avena sativa.
Fig. 3A. Time-course of the response in the presence of 0-100 μM bentazone.
Fig. 3B. Concentration-dependence of the response in the presence of bentazone (●-●) and auxin (O-O), expressed as % of control values after 60 min incubation.

DISCUSSION

Although there is abundant evidence in the literature designating bentazone as an inhibitor of photosynthetic electron flow at photosystem II (Moreland 1980), these findings clearly indicate a further action of bentazone at the plant cell membrane. Figures 1, 2 and 3 suggest that bentazone can induce rapid changes in the turgor of protoplasts, epidermes and coleoptiles in both light and darkness, and may therefore be considered to be independent of photosynthesis. Indeed, bentazone-induced swelling of G.max mesophyll cell protoplasts in darkness is particularly sensitive to inhibition by 10 μM rotenone and antimycin A (Rees, unpublished), from which it may be inferred that protoplast swelling has a requirement for ATP derived from mitochondria.

Protoplasts, epidermes and coleoptiles are all capable of rapid changes in cell turgor, mediated by the influx of cations and water into the cell in exchange for proton efflux, each possibly involving the action of membrane bound ATPases. Rees (unpublished) has demonstrated that G.max protoplast swelling is particularly sensitive to the magnesium ion concentration of the bathing medium and may be inhibited by compounds that prevent electron flow in mitochondria. Similarly, in stomatal movement potassium is the osmotically active cation (Fischer 1968) whose transport

into the guard cell may be prevented by several metabolic inhibitors (Outlaw 1983). Furthermore, coleoptile elongation in darkness has a requirement for calcium ions (Cohen & Nadler 1976) and is inactivated by inhibitors (see Davies 1973). Thus, there is good evidence in the literature for active cation transport at the cell membrane in each of the three experimental systems used in the study, although how bentazone may interact in each system remains to be established.

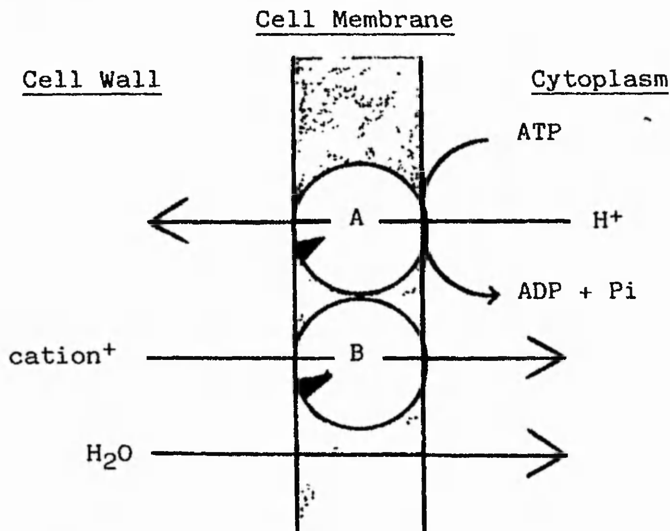

Fig. 4. A hypothetical scheme for bentazone action at the plant cell membrane. An ATPase proton pump (A) is envisaged to drive the efflux of protons in exchange for cations at a cation-specific transport site (B). One or both transport molecules may contain regulatory sites (◀) that are sensitive to bentazone and/or other regulatory molecules, such as IAA.

Since each bentazone-induced response is both rapid and concentration-dependent it is proposed that this herbicide may bind to active site(s) on the cell membrane altering cation and water flux, as represented in Fig. 4. In this scheme an ATPase proton pump is envisaged to drive the efflux of protons in exchange for cations at a cation-specific transport molecule. Bentazone could conceivably bind at one, both or at other sites on the membrane to alter cation influx and hence cell turgor. Evidence does exist, particularly with regard to coleoptile elongation, that the endogenous plant growth regulator IAA may induce increased cell turgor by binding to the cell membrane ATPase (eg Davies 1973). Furthermore, since bentazone can interact with auxin in this experimental system (Miller unpublished), an action of bentazone at a regulatory site on the cell membrane is conceivable. Indeed, the stoichiometry of cation transport in the presence or absence of bentazone, and other membrane active herbicides and regulators, is an active area of study in this laboratory, which may yield a greater understanding of bentazone and other herbicide actions at the cell membrane.

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