STUDIES ON THE EFFECT OF HERBICIDES ON THE PHYSIOLOGY OF *CHENOPODIUM ALBUM*

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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ABSTRACT

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Penetration of ^{14}C-bentazone into intact C. album leaves is found to be very slow, particularly if applied when stomata are closed. However, incorporation of the oil adjuvant Actipron improves uptake under these conditions and overrides any time of day differences. Moreover, independent IRGA investigations show that photosynthesis and transpiration in this species are inhibited concurrently by bentazone and that maximum stomatal closure occurs in advance of complete inhibition of CO_2 assimilation. Inclusion of Actipron reverses this response and it is suggested that the normally poor bentazone penetration may result in a major portion remaining on the leaf surface where significant photosynthetic inhibition may be caused by stomatal closure. Actipron may enhance bentazone movement to the mesophyll, thus by-passing any stomatal contribution to the herbicidal mode of action.

The mechanism of bentazone action on C. album stomata is shown by epidermal peel studies to be complex. Normal responses to potassium ions, ambient CO_2 concentration and exogenous nucleotides are disrupted by the herbicide causing differential opening and closure. These effects are discussed in relation to those produced by other chemicals with stomatal activity and in the context of a potential anti-transpirant use for bentazone and its derivatives.

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DECLARATIONS

1) The observations in this study are, except where otherwise stated, entirely the work of the author.
2) The author has not received, and has not been registered for any other higher degree awarded by a University or the Council of National Academic Awards during the course of this work nor has any part of it been previously submitted in partial fulfilment for the award of a higher degree.
3) The author has attended conferences and programmes of study relevant to the present investigation.
4) Due acknowledgements have been made for the assistance given during the course of this work and in the presentation of the thesis on which it is based.

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Aspects of the investigations outlined in chapters 2-5 are at present in preparation for future publication.

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LIST OF ABBREVIATIONS

ABA	-	Abscisic acid.
C.E.	-	Counting efficiency.
c.m.c.	-	Critical micelle concentration.
CMU	-	3-(4-chlorophenyl)-1,1-dimethylurea.
c.p.m.	-	Counts per minute.
C.R.	-	Channels ratio.
2,4-D	-	2,4-dichlorophenoxyacetic acid.
DCMU	-	3-(3,4-dichlorophenyl)-1,1-dimethylurea.
DNP	-	Dinitrophenol.
d.p.m.	-	Disintegrations per minute.
ECLD	-	Epicuticular lip distance.
IRGA	-	Infra-red gas analyser.
IAA	-	Indole acetic acid.
LDR	-	Leaf diffusive resistance.
MCPA	-	(4-chloro-O-tolyloxy) acetic acid.
MCPB	-	4-(4-chloro-O-tolyloxy) butyric acid.
NAA	-	α - naphthyl acetic acid.
NOXA	-	β - naphthyloxyacetic acid.
PAR	-	Photosynthetically active radiation.
PEPC	-	Phosphoenol pyruvate carboxylase.
PMS	-	Phenazine metho sulphate.
p.p.b.	-	Parts per billion by volume.
p.p.m.	-	Parts per million by volume.
RubPC	-	Ribulose-bis-phosphate carboxylase.
2,4,5-T	-	2,4,5-trichlorophenoxy acetic acid.

CHAPTER 1

INTRODUCTION

1.1. Herbicidal control of weeds - a general introduction

A weed is considered to be a plant growing where it is not wanted. Although by this definition any plant can be a weed, the more obvious weeds tend to have certain characteristics responsible for their widespread and troublesome occurrence. For example, they establish themselves rather than being planted, are difficult to eradicate, compete successfully with other plant species, are adaptable and can exploit many habitats. Weeds often form extensive populations, which can interfere with agricultural and horticultural activities, due to their very efficient reproductive processes and many are able to survive temporarily in unfavourable conditions. Moreover, the activities of man in draining, liming, fertilising, cropping and the use of chemical control, may all alter the relative abundance and distribution, favouring one species at the expense of another. Their presence in a crop not only reduces yield but may also affect the quality of the product or interfere with mechanical harvesting.

Weeds can therefore cause considerable expenditure in agriculture and thus much time and money has been dedicated to the production of herbicides, which are chemicals that will kill weeds without seriously damaging the crop. Obviously it is important to know the characteristics of individual weeds before suitable herbicides can be selected, and these will also determine both the method and rate of application. However, for a chemical to be effective it must first enter plant tissues to reach its site of action. Thus consideration must be given to the anatomy and physiology of the weed and other factors affecting penetration of the herbicide into the plant.

1.2. Leaf surface characteristics in relation to herbicide uptake

The structure of the leaf surface and its wettability are important factors governing the uptake of foliar applied chemicals. These properties vary with the leaf age and development or the

environment in which the plant is grown. For example, young leaves generally have thinner cuticles than older ones (Schieferstein and Loomis, 1959; Leon and Bukovac, 1978) and consequently, absorption usually decreases with age (Crafts and Foy, 1962). However, cuticular structure and development is often affected by light intensity (Skoss, 1955; Reed and Tukey, 1982) and quality (Hull, 1970), photoperiod (Wilkinson, 1966), temperature (Whitecross and Armstrong, 1972; Reed and Tukey, 1982) and the water status of the plant (Daly, 1964; Hunt and Baker, 1982). Similarly, the structure and frequency of stomata may also be changed according to growth conditions (Palevitz, 1981; Wild and Wolf, 1980). Leaf wettability is further influenced by a number of factors, including leaf orientation (Holly, 1976), the hairiness and corrugation of the surface (Fogg, 1948; Al-Jaff, Cook, Carr and Duncan, 1982) and the chemical and physical nature of the surface wax (Martin and Juniper, 1970).

Foliar penetration of herbicides is generally considered to occur via the cuticle, although under certain conditions the stomatal apparatus has been implicated (Crafts and Foy, 1962; Sargent and Blackman, 1962; Franke, 1964). Since the cuticle is largely lipoidal, non-polar molecules will penetrate more readily than polar. The former may move along routes associated with wax formation, while polar compounds may traverse more slowly through pectinaceous pathways (Martin and Juniper, 1970).

The leaf surface structure and the composition and ultrastructure of the epicuticular wax also influence the retention and penetration of aqueous solutions (Silva Fernandez, 1965; Holloway, 1969). There is increasing evidence that the cuticle provides the chief barrier to the penetration of water soluble compounds due to the hydrophobic nature of the wax (Schönherr, 1976 a and b) and the rigid structure of the highly cross-linked matrix (Martin and Juniper, 1970; Kollatukudy, 1980). Indeed, in the absence of stomata diffusion is very slow and may not occur at all if high levels of cutin are present (Silva Fernandez, 1965). However, whilst the structure (Amelunxen, Morgenroth and Picksale, 1967), quality (Baker and Bukovac, 1971) and composition (Martin and Juniper, 1970) of epicuticular wax are highly variable there is no

doubt that these factors will influence leaf wettability. The term contact angle, defined as the angle between the surface of the leaf and the tangent plane of a water droplet at the circle of contact between air, liquid and leaf (Fogg, 1947), is a useful way of characterising the wettability of leaves. High contact angles caused by the trapping of air between repellant projections, are a function of the epicuticular wax (Holloway, 1969).

Whilst stomatal penetration by aqueous solutions of pesticides has been reported (Skoss, 1955; Currier, Pickering and Foy, 1964) it is generally considered that movement through the stomatal pore does not occur (e.g. Sargent and Blackman, 1962) particularly at normal surface tensions(Schönherr and Bukovac, 1972). Stomatal penetration has been observed when the surface tension was sufficiently decreased by the addition of a surfactant (Dybing and Currier, 1961), whilst none was found when the stomata were completely closed. However, inside the pore, the solution must still traverse some form of cuticle. The cuticle within the pore is thinner, more permeable and more hydrated than that covering the outer surface (Currier, et al, 1964), and may also be more polar in some species (Norris and Bukovac, 1968).

Measurements of relative stomatal densities and stomatal diffusive resistances have shown good correlation with herbicide uptake in some studies (e.g. Sands and Bachelard, 1973). However, other authors have found uptake to be related to stomatal frequency but not to leaf diffusive resistance. For example, penetration of ^{14}C-triclopyr into leaves of Lithocarpus densiflorus was significantly less through the adaxial surface, which has no stomata, than through the abaxial surface which has stomata (King and Radosevich, 1979). However, diffusive resistance measurements of the leaf discs used showed stomatal resistances to be significantly higher than for intact leaves. This was taken to indicate at least partial closure of the stomata perhaps making entry through the pore unlikely.

Many leaf surfaces are covered with unicellular hairs which may have a specialised function and are known as trichomes. Absorption of herbicides via trichomes is also thought to occur

(Currier and Dybing, 1959; Hull, 1970). Indeed, studies of Pteridium aquilinum growing in exposed or shaded areas have shown that, whilst the latter had thinner cuticles, uptake was greater in the former which have greater trichome and stomatal density (Cook, Carr and Duncan, 1979). Ectodesmata i.e. special channels which connect the cuticle with the living protoplasts of the cell, are apparently numerous in both guard and trichome accessory cells (Franke, 1961; 1964; 1971) and it has been postulated that these structures may account for observations of enhanced penetration (King and Radosevich, 1979; Al-Jaff, et al, 1982). However, the nature of ectodesmata is unknown and Norris and Bukovac (1968) have suggested that preferential uptake sites may result from localised differences in the quality and orientation of the epicuticular wax.

In conclusion, many conflicting statements have been made with regard to foliar penetration of herbicides. Studies which implicated stomatal involvement have often failed to take account of more recent discoveries concerning the unusual physiology of these structures. Little is known of trichome function and authors have tended to favour direct penetration across the cuticle as the most likely route of uptake. However, it is important to note that penetration into a leaf may vary according to the species and the chemical involved. Hence there is no general rule which can be applied to all cases.

1.3. Non-biotic factors affecting foliar penetration of herbicides

1.3.1 Herbicide formulation: Increased polarity of a chemical reduces the efficiency of penetration through lipoidal matrices, such as the cuticle, and a suitable balance of polar/apolar groups of the molecule is considered to be essential in enhancing uptake (van Overbeek, 1956). Indeed, structural changes in the ring or side chain of phenoxyalkyl herbicides have been shown to markedly alter their effectiveness. For example, the high susceptibility of Vicia faba to MCPA has been related to rapid cuticular penetration and subsequent translocation, whereas resistance to MCPB was associated with its virtual confinement to the cuticular wax of the treated leaves (Kirkwood, Dalziel, Matlib and Somerville, 1972).

Furthermore, additional halogen substitution of these compounds has generally favoured penetration in the lipodal phase of the cuticle. Thus, modification of molecular structure which results in increased lipid solubility will often enhance foliar penetration (Bukovac, 1976).

In general, the addition of surfactants or wetting agents enhances penetration and the effectiveness of foliar-applied herbicides (Staniforth and Loomis, 1949). Holly (1976) has suggested several ways by which the reduced surface tension, afforded by surfactants, may affect penetration. These include increasing the area of contact by enhanced wetting, eliminating the air films between the solution and plant surface, acting as cosolvents or solubilising agents in cuticular penetration, easing entry through open stomata and subsequent movement through intercellular spaces, and acting as humectants by retarding the drying of the solution. It is believed that surfactants act by forming micelles with their hydrophilic groups towards the inside, and the enhanced penetration may be due to solubilisation of the cutin by these aggregates with subsequent removal of the wax (van Overbeek and Blondeau, 1954). Alternatively, it has been proposed that the surfactant may interact with the cutin matrix, altering its charge characteristics and swelling properties, and hence, the membrane resistance to the diffusion of herbicides (Bukovac, 1976).

The pH of an applied solution affects the ease of cuticular penetration since it influences the polarity of the cuticle and the penetrant (van Overbeek, 1956), although the primary influence is considered to be on the penetrant (Norris and Bukovac, 1972). Penetration of weak organic acid herbicides, such as 2,4-D is significantly affected by pH (Sargent, 1965). At low pH such compounds are largely undissociated and hence readily partition into the lipoidal phases of the cuticle and the plasmalemma (Simon and Beevers, 1952). Furthermore, with the addition of ammonium salts, penetration may be enhanced at high pH (Horsfall and Moore, 1962). The magnitude of pH enhancement obtained may well be related to the inherent phytotoxicity of the active ingredient and the concentration at which it is used (Hull, 1970).

Finally, the method of application of herbicides is extremely

important to the rate of uptake since this will determine both the droplet size and the efficiency of cover. Similarly, the volume of diluent applied with the chemical may significantly affect the degree of control obtained (Merrit and Taylor, 1976).

1.3.2. Environmental factors: Since absorption is governed by both metabolic and non-metabolic components, temperature will indirectly influence absorption by altering the general metabolic rate (Sargent, 1965). For example, a four-fold increase in foliar penetration of ^{14}C-dalapon was observed when the post-treatment temperature was 43°C as opposed to 26°C (Prasad, Foy and Crafts, 1967). Furthermore, pre-treatment growth temperature may also affect rate of uptake. For example, Phaseolus vulgaris grown at 15°C showed few damage symptoms in response to 2,4-D application, until transferred to 25°C whilst plants grown at 5°C exhibited marked symptoms upon transfer to 15°C (Kelly, 1949). This may reflect increased uptake due to an increase in physiological activity, although reduced viscosity and increased fluidity of the cuticle at higher temperature cannot be excluded.

Light generally enhances the foliar penetration of solutes and this may be due to a direct effect on the energy mechanisms for uptake associated with photosynthesis (Kylin, 1960). However, Currier and Dybing (1959) consider that light promotes absorption by inducing stomatal opening, thus increasing photosynthesis and the export of carbohydrates from the leaf. Furthermore, although penetration reaches optimal levels at relatively low light intensities, light-enhanced uptake of 2,4-D (Sargent and Blackman, 1965) and NAA (Greene and Bukovac, 1972) can be negated by inhibitors of the Hill reaction indicating that absorption may be dependent on a supply of ATP.

High humidity, moisture on the external surface and low moisture tension within the plant, favour rapid foliar uptake (Clor, Crafts and Yamaguchi, 1962). This may be attributed to the swelling of the hydrophilic groups of the cutin matrix pushing the apolar wax units apart (van Overbeek, 1956). Under these conditions, the pores would be filled with water and the pectin highly hydrated, promoting absorption of water-soluble herbicides via the apoplast (Crafts, 1961; Foy 1964). Conversely, water stress and low humidity generally results in reduced uptake (van Overbeek, 1956; Currier

and Dybing, 1959), although absorption of 2,4-D, for example, may be unaffected by moisture stress and reduced efficacy of this herbicide under such conditions has been correlated with lower translocation (Basler, Wood and Meyer, 1961).

1.4 Stomatal physiology and the influence of herbicides

1.4.1. Stomatal structure: Stomata are the pores on aerial plant surfaces responsible for gas exchange. In higher plants stomata may occur on both leaf surfaces or only on one, but the pores are almost always surrounded by specialised cells known as guard cells. Typically, 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 and Hepler, 1976). The type of thickening varies with species and growth conditions to give guard cells of different shapes (Palevitz, 1981). Elliptical guard cells, as in Allium and Pisum spp., result from thickening of the paradermal and ventral walls in a fan-like pattern that radiates away from the pore (Singh and Srivastava, 1973; Palevitz and Hepler, 1976). Grasses, however, have guard cells which are dumbell-shaped. The wall around the constricted middle of each cell is heavily thickened, to give the characteristic shape, by cellulose microfibrils which are orientated axially to the pore (Ziegenspeck, 1944).

1.4.2. Stomatal movement: The guard cells may be surrounded by specialised subsidiary cells or by many undifferentiated epidermal cells, in which case the stomata are said to be anomocytic (e.g. Chenopodium album). Stomatal movement depends on the relative turgor pressure of the two cell types and the physiology of this phenomenon has been reviewed several times in recent years (e.g. Raschke, 1975; 1979; Jarvis and Mansfield, 1981). Thus, stomatal opening occurs when guard cells have high turgor pressure, relative to the surrounding cells, resulting from accumulation of large quantities of solutes (Hsiao, 1976). Many early workers considered these solutes to arise when starch, formed during photosynthesis and present in large quantities in most guard cells, was broken down into soluble sugars (e.g. Lloyd, 1908). Starch metabolism is mediated by pH and it was suggested that fluctuating inter-

cellular CO_2 concentration could control the amount of solute present (Levitt, 1967). However, certain members of the Liliaceae, Iridaceae and Amaryllidaceae, have functional guard cells which contain no starch (Steinberger, 1922; Allaway and Setterfield, 1972; Schnabl, 1977; Schnabl and Zeigler, 1977) and the "starch-sugar" hypothesis has fallen into disrepute.

It was Japanese workers who first suggested that potassium was the major turgor inducing cation involved in stomatal opening (Imamura, 1943; Fujino, 1967), and the work of Fischer (1968) confirmed that Vicia faba epidermal peel stomata could be induced to open only when K^+ was present in the bathing medium. Subsequent studies have shown that during opening guard cells of all species investigated accumulate large quantities of K^+ from the surrounding cells (Willmer and Pallas, 1973; Dayanandan and Kaufmann, 1975), and the concentration of this ion in turgid guard cells may be as high as 448 mol. $m.^{-3}$ in Commelina communis (Penny and Bowling, 1974). Furthermore, in V. faba it has been shown that when K^+ is actively taken into the guard cell H^+ is extruded with a stoichiometry of 1:1 (Raschke and Humble, 1973), thus maintaining the electrical balance across the guard cell membrane without uptake of a counter-ion. In the same species, Allaway (1973) found that increased levels of malate in open guard cells would balance about half the potassium ions. Chloride ions in the incubation medium have been shown to balance the other half (Raschke and Schnabl, 1978) whilst, in the absence of Cl^-, malate may increase sufficiently to account for all the accumulated potassium (van Kirk and Raschke, 1978).

This interchangeability of anions has also been reported in other species. For example, Commelina spp. also accumulate malate (Pearson and Milthorpe, 1974; Travis and Mansfield, 1977) or chloride (Penny, Kelday and Bowling, 1976) in the guard cells during stomatal opening, whilst in Zea mays, chloride has been shown to account for about 40% of accumulated potassium (Raschke and Fellows, 1971). However, guard cells clearly have the ability to produce large quantities of malate if required. Guard cell starch disappears as stomata open and this may provide the carbon skeletons for malate synthesis (Outlaw and Manchester, 1979). Furthermore,

PEPC (Phosphoenol pyruvate carboxylase) activity sufficient for the necessary malate accumulation has been demonstrated in V. faba (Outlaw and Kennedy, 1978; Outlaw, Manchester and Di Camelli, 1979), C. communis and Tulipa gesneriana (Willmer, Pallas and Black, 1973) and C. cyanea (Thorpe, Brady and Millthorpe 1978) and $^{14}CO_2$, fixed by isolated guard cells, is incorporated into malate in these species (Allaway, 1976; Willmer and Rutter, 1977; Thorpe and Millthorpe, 1977; 1979). Lesser amounts of other organic anions may also accumulate in C. communis (Willmer, 1981) whilst in starch-free species, such as Allium cepa, malate is present (Schnabl and Zeigler, 1977), but its source or its influence on stomatal movements are at present unknown.

Studies in stomatal physiology have involved relatively few plant species, mainly due to the difficulty of obtaining suitable epidermal peels. For this reason care should be exercised in the extrapolation of the available data to other species, particularly in view of the great differences already observed in structure and metabolism.

1.4.3. Influence of the environment: Assimilation of CO_2 by stomata is limited by a need to maintain the internal water status of the plant. Thus, stomata will respond to a variety of environmental stimuli to cause either opening or closure. Cowan (1977) has explained these responses on the basis of a hydraulic loop with both feedback and feedforward control which is consistent with the hypothesis that stomata are primarily sensitive to internal water status and intercellular CO_2 concentration. Such models are complex, but nevertheless important in the elucidation of the role of stomata in the whole plant, and serve as a reminder that individual responses should not be considered in isolation.

Stomata generally respond to light by opening and the action spectrum of this response shows that the optimum wavelengths are primarily in the blue region with a lesser peak in the red (Kuiper, 1964). The blue light response is similar to that found elsewhere in green plants and fungi (Smith, 1975) and is thought to be mediated by, as yet, an unidentified flavin receptor, which controls a wide range of physiological activities. The pigment is unresponsive to red light and experiments with metabolic inhibitors

have indicated that this wavelength may provide a source of energy for stomatal opening via cyclic photophosphorylation in the guard cell chloroplasts (Humble and Hsiao, 1970; Willmer and Mansfield, 1970; Raghavendra and Das, 1972).

Although guard cell chloroplasts of some species have recently been shown to contain both photosystem I and II (Zeiger, Armond and Melis, 1980; Outlaw, et al, 1981), most evidence suggests that light is not the primary source of energy in stomatal movements. For example, guard cells of certain Paphiopedilum spp. have no chloroplasts (Nelson and Mayo, 1975; Rutter and Willmer, 1979) yet function normally. Even stomata containing chloroplasts are apparently unable to photosynthetically reduce carbon dioxide (Raschke and Dittrich, 1977) and only traces of essential Calvin cycle enzymes, such as RubPC (Ribulose bis Phosphate Carboxylase) have been detected in isolated epidermes (Outlaw, Manchester DiCamelli, Randall, Rapp and Veith, 1979). Furthermore, the ratio of mitochondria to chloroplasts may be 4 times greater in guard cells than in the mesophyll (Allaway and Millthorpe, 1976) and, in Helianthus annuus, oxygen has been shown to be essential for stomatal opening in light or darkness (Couchat, Lasceve and Audouin, 1982) indicating that the energy source may be respiratory. It has therefore been postulated that guard cell chloroplasts may function as environmental sensors to either light (Outlaw, et al, 1981) or CO_2 (Melis and Zeiger, 1982).

The most convincing evidence against light as the energy source is the opening response of stomata to reduced CO_2 concentrations. This occurs independently of light and may explain night opening of stomata in the Crassulaceae (Neales, 1970). Similar responses occur in other species, particularly if the reduction in CO_2 concentration coincides with an increase in temperature (Mansfield, 1976). Ambient CO_2 concentration is detected within the guard cell (Mouravieff, 1956) and it has been proposed that the cytoplasmic level of malate, and therefore guard cell pH, is proportional to ambient CO_2 concentration (Raschke, 1979). Thus, stomatal responses may be controlled by the effect of these factors on PEPC and malic enzyme activity. Alternatively, Travis and Mansfield (1979 a) have suggested that both light and

CO_2 may affect stomatal movement by exercising control more directly on the rate of K^+ exchange by guard cells.

Temperature also influences stomatal movement, primarily as a result of interaction with the leaf intercellular air space CO_2 concentration (Meidner and Mansfield, 1968). Photosynthetic CO_2 compensation point rises with increased temperature causing reduced stomatal apertures and this has been suggested as the cause of midday closure of stomata in some species (Heath and Orchard, 1957). However, in many species midday closure does not occur and temperature has generally been found to affect the rate, rather than the degree of stomatal opening (Loftfield, 1921; Stalfelt, 1962).

Stomata in intact leaves of well-watered plants close in response to increases in the leaf water vapour pressure deficit (Rich and Turner, 1972; Davies and Kozlowski, 1974) as do those in isolated epidermes (Lange, Lösch, Schulze and Kappen, 1971). This direct response to humidity may result from increased water loss due to transpiration, causing a decrease in guard cell turgor and stomatal closure (Maier-Maercker, 1979). However, in non-stressed plants stomatal responses to humidity are relatively small compared to those induced by other environmental factors (Lösch and Tenhunen, 1981).

The degree of stomatal opening is relatively unaffected by small reductions in leaf water potential but once a critical level is reached small increases in water deficit will cause marked stomatal closure (Hall, Schulze and Lange, 1976). Exogenously applied abscisic acid (ABA) also causes stomatal closure (Mittelheuser and van Steveninck 1969; Cummins, Kende and Raschke, 1971; Kriedemann, Loveys, Fuller and Leopold, 1972) and its concentration rises in water-stressed leaves (Hsiao, 1973; Milborrow, 1974). Furthermore, ABA induces reversible stomatal closure and inhibits stomatal opening in isolated epidermes. This has led to the hypothesis that ABA acts as a messenger in a feedback loop controlling water loss (Dubbe, Farquhar and Raschke, 1978; Mansfield, Wellburn and Moreira, 1978) although the bulk of ABA synthesis occurs in mesophyll cells (Loveys, 1977) and its concentration in rewatered plants returns to normal up to 2 days before complete stomatal

recovery (Beardsall and Cohen, 1977).

Other plant hormones, principally auxins and cytokinins, have also been found to influence stomatal movement. Cytokinins stimulate transpiration in whole plants (e.g. Meidner, 1967; Cooper, Digby, and Cooper, 1972) and stomatal opening in isolated epidermes (Jewer and Incoll, 1980; 1981) invoking a rapid response (Kuraishi, Mashimoto and Shiraishi, 1981) which may be antagonistic to ABA (Das, Rao, and Raghavendra, 1976; Biddington and Thomas, 1978). Similarly, IAA has recently been shown to cause stomatal opening, and interact with ABA-induced stomatal closure, in isolated epidermes of C. communis (Pemadasa, 1982; Snaith and Mansfield, 1982). However, very high auxin concentrations (i.e. 10^{-2}-10^{-1}M) were required in these experiments and, although such concentrations have been reported in young leaves (Wrightman, 1977) the amount available to stomata of mature leaves in vivo is unknown. Thus, hormonal interactions may be involved in the control of stomatal responses to environmental change but the mechanism is uncertain.

1.4.4. The influence of herbicides on stomatal movement: Several investigations have used metabolic inhibitors to determine the energy dependence of stomatal movement. In Commelina spp. DNP (2,4-Dinitrophenol), an uncoupler of ATP formation in oxidative and photophosphorylation, caused closure of open stomata (Raghavendra and Das, 1972) and inhibited ion-induced opening in the light but not in the dark (Willmer and Mansfield, 1970). In general, inhibitors of cyclic electron flow, such as salicylaldoxime, gave similar responses whilst catalysts of this process, for example PMS (phenazine-metho-sulphate), stimulated opening, (Raghavendra and Das, 1972). Conversely, inhibitors of non-cyclic electron flow, like DCMU (3-(3,4-dichlorophenyl)-1, 1-dimethylurea), had little effect if intercellular CO_2 concentrations were regulated (Allaway and Mansfield, 1967; Willmer and Mansfield, 1970).

Commercially available herbicides often have less specific modes of action and consequently have been shown to initiate a variety of stomatal responses. Stimulated transpiration was observed in Phaseolus vulgaris after root application of urea, triazine and uracil herbicides (Van Oorschot, 1970) and similar

effects have been reported for alachlor in Zea mays and Solanum tuberosum (Ashley, 1974; Ashley and Vavrina, 1976). Foliar applications of paraquat and 2,4,5-T have been shown to reverse normal stomatal behaviour in several tree species causing excessive daytime transpiration and wilting as a result (Rao, Swamy and Das, 1977). Little information is available regarding the mechanism of these responses, which were considered by the authors to be important factors in herbicide phytotoxicity.

Other herbicides have been found to cause stomatal closure. Picloram reduced transpiration slightly in Quercus turbinella (Davis, 1978), whilst nitrofen and oxyfluorfen induced stomatal closure in Portulaca oleracea as a result of increased membrane permeability (Gorske and Hopen, 1978). Several phenoxyacetic acid herbicides also inhibit stomatal opening. In the case of 2,4,5,-T and MCPA, this is thought to be a result of disturbance of the CO_2 balance of the leaf whilst for 2,4-D, NAA and NOXA the cause may be more direct (Pemadasa and Jeyaseelan, 1976; Pemadasa, 1979). These authors suggested interference with membrane permeability, starch hydrolysis or potassium accumulation as possible explanations. Glyphosate caused stomatal closure in Phaseolus vulgaris which directly resulted in a reduction in photosynthesis (Shaner and Lyon, 1979). This compound was also found to induce stomatal cycling under certain environmental conditions.

In a more comprehensive study into the influence on stomata of several herbicides of different types, Das and Santakumari (1975) correlated the observed responses with the susceptibility of a species to a particular chemical. Generally, foliar application of herbicides to susceptible species stimulated transpiration and increased stomatal aperture whilst the converse was found for tolerant species. These observations suggest that the effects of herbicides on stomata may be a major factor in their mode of action or, at least, a useful guide when determining selectivity.

In conclusion, the effect of herbicides on stomata has received scant attention considering their importance as potential sites of preferential uptake. Effects of herbicides on transpiration are generally considered secondary to the inhibition of photosynthesis. However, it is evident that herbicides must first

enter the leaf to reach the sites of photosynthesis before inhibition of this process can occur. Consequently, whatever the route of uptake, stomata are the first vulnerable cells to come into contact with foliar applied chemicals.

1.5 Control of Chenopodium album by bentazone

1.5.1. Chenopodium album weed characteristics: On a worldwide basis, C. album is one of the five most common weeds (Coquillat, 1951) and grows well in arable soils extracting large quantities of nutrients, particularly nitrate for which it has a very high reductase capacity (Chu, Sweet, Lozbun and Kaplan, 1980). For example, it has been shown that doubling the soil nitrogen content in Beta vularis may result in double the weight of C. album with no change in crop yield (Scott and Wilcockson, 1976). This aggressive competition for nutrients may well reflect the more extensive root development in this weed than is found in many crop species (Williams, 1964; Bassett and Crompton, 1978).

C. album is an hermaphrodite which produces small clusters of flowers from July to September. Each plant yields an average of 3,000 seeds (Hill, 1982) which display somatic polymorphism to give 4 seed types of different size, shape, colour and germination characteristics (Harper, 1977). Germination, which may be stimulated by the presence of fertilisers such as potassium nitrate (Henson, 1970), occurs predominantly during May and August in the U.K. at depths of 0.5 to 3.0 cm although in dry, well aerated soils seeds may germinate at a depth of 8 cm (Hanf, 1974). However, they will remain viable for up to 40 years in unfavourable conditions (Toole and Brown, 1946) conveying considerable advantage on the species in the ever-changing agricultural environment.

Once established, C. album shows remarkable tolerance to shading as found within the crop stand, competing successfully for light by rapid stem extension and the production of large leaves (Noguchi and Nakayama, 1978). These features have been shown to result from phytochrome-mediated response to low red/far red ratios such as those observed under canopy cover (Morgan and Smith, 1978; 1981; Child, Morgan and Smith, 1981).

In structure, C. album is a typical dicotyledonous annual

angiosperm. The leaves are long-stalked with unequal forwardly-directed teeth, oval to lanceolate in shape (Hanf, 1974) and display a pronounced endogenous circadian rhythm of nyctinastic movement (Andersen and Koukkari, 1979). Both leaf surfaces have stomata (Metcalf and Chalk, 1950), the pores of which are small and sunken, with stomatal opening characteristically involving a widening of the apparently wax-free ante-chamber (Taylor, 1979) with relatively little change in pore-width. The epidermes also exhibit many trichomes which give the young plant a silvery bloom (Brian and Cattlin, 1968). These cells are about 80 µm in diameter and are believed to have a specialised function typical of halophytes (Luttge, 1974). They are also known as "bladder cells" and accumulate considerable quantities of excess salt from the plant (Osmond, et al, 1969). It is not known if salts are transported back from these cells to the mesophyll but this seems unlikely since they collapse as the leaf matures.

The aerial parts of the plant are usually covered with a layer of epicuticular wax which consists mainly of C26 and C28 alcohols, and C28 aldehydes (Allebone, et al, 1970). Wax quality and quantity are apparently unrelated to the prevailing environmental conditions, although the abaxial leaf surface has more wax than the adaxial (Taylor, 1979). C. album epicuticular wax differs from that of many other weed species in that it contains a substantial proportion of aldehydes which have been shown to strongly impede penetration of water and hydrophilic molecules (Baker and Bukovac, 1971).

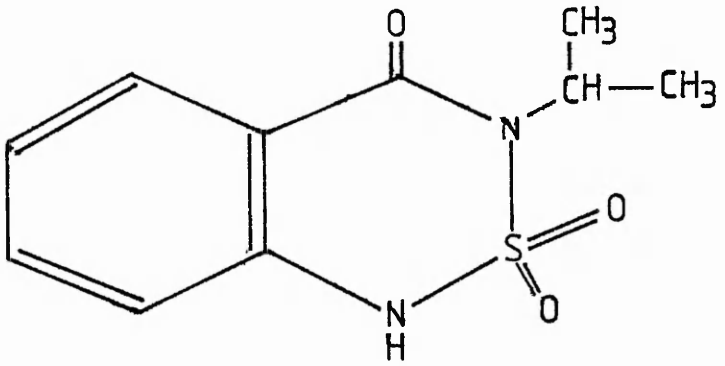
There is considerable phenotypic variability within C. album populations (Cole, 1962) resulting in the production of biotypes which are resistant to herbicides, particularly atrazine. Repeated spraying of Zea mays crops with this chemical in the USA has reduced populations of susceptible S-biotypes of C. album to the advantage of resistant R-biotypes which have lower germination rates and slower early growth (Marriage and Warwick, 1980). As a rule, this would result in an inability to compete with faster growing S-biotypes but post-emergence applications of atrazine leave only R-biotypes which grow at normal rates as they mature and hence are still a problem weed. Resistance is not due to

differential absorption or metabolism but occurs at the chloroplast thylakoid level (Radosevich, 1977; Machado, Bandeen, Stephenson and Jensen, 1977) and does not result from altered penetration of atrazine into this organelle in R-biotypes (Machado, et al, 1978). However, whilst R-biotypes are often resistant to other herbicides with similar mode of action to atrazine they are occasionally more susceptible (Arntzen, Pfister and Steinback, 1982). For example, atrazine-resistant C. album biotypes have been shown to be slightly resistant to DCMU but more susceptible to bentazone, the herbicide used in the present study.

To summarise, C. album is endowed with a wide variety of characteristics which enable the species not only to compete successfully but also to overcome the effect of herbicide applications. In consequence, it has become widely distributed, occupying many habitats, adaptation to which has been enhanced by a high genetic variability within the species.

1.5.2. Current knowledge of bentazone activity: Early reports on bentazone showed that the herbicide selectively inhibited CO_2 assimilation in some species when applied at a rate of 1 kg. $ha.^{-1}$ (Retzlaff and Fischer, 1973). Furthermore, studies of Cyperus serotinus have shown that photosynthesis is inhibited by bentazone in both intact plants and isolated chloroplasts (Mine and Matsunaka, 1975). This inhibition is similar to that induced by DCMU (Böger, Beese and Miller, 1977) and is more pronounced at pH 6 than at pH 8 (Retzlaff, Hilton and St. John, 1977). In addition, bentazone has been found to induce the formation of shade-type chloroplasts in Raphanus sativus seedlings with consequent changes in the ultrastructure and chemical composition (Lichtenthaler, 1979; Lichtenthaler, et al, 1980; Grumbach and Drollinger, 1980; Meier, et al, 1980). All these observations suggest that the primary effect of bentazone is the disruption of the photosynthetic apparatus. However, Mine and Matsunaka (1975) consider that as foliar applications produce a much quicker inhibition of photosynthesis than root treatments, another rapid contact effect may play an important role in the complete mode of action of this herbicide.

Bentazone is the approved common name of 3-isopropyl-1, (H)-2,1,3,-benzothiadiazin-(4),3,H-one-2,2-dioxide:-



and, like many other herbicides, it has been shown to block non-cyclic photosynthetic electron transport between the proposed quencher Q and the plastoquinone pool on the reducing side of photosystem II (Pfister, Buschmann and Lichtenthaler, 1975; Suwanketnikom, Hatzios, Penner and Bell, 1982). This type of herbicide may interfere with electron transport and/or uncouple ATP formation in photochemical reactions. On this basis, attempts have been made to classify compounds into the following categories (Moreland and Hilton, 1976; Moreland 1977):-

a) electron transport inhibitors.
b) uncouplers.
c) energy transfer inhibitors.
d) inhibitory uncouplers (multiple types of inhibition).
e) electron acceptors.

The effect of bentazone may be reversed by exogenous applications of sucrose (Mine and Matsunaka, 1975). Consequently, this herbicide is designated as a simple electron transport inhibitor by Moreland (1980), who suggests that sufficient ATP is still generated via glycolysis, oxidative phosphorylation, or cyclic photophosphorylation if an adequate supply of respirable substrate is available. However, there have been no direct studies of photosynthetic ATP production as influenced by bentazone, whose chemical structure is related to the thiadiazoles which are considered to act as multisite inhibitory uncouplers (Moreland, 1980). Hence, the nature of bentazone action at the thylakoid level is at present unclear.

The mechanism of bentazone selectivity is generally considered to arise from differential metabolism by resistant and susceptible species (Mine, Miyakado and Matsunaka, 1975; Retzlaff and Hamm, 1976). For example, photosynthesis is inhibited in both resistant Triticum aestivum and susceptible Sinapis alba. Inhibition is at a maximum in T. aestivum after 3 hours whilst S. alba does not recover. In T. aestivum the level of active bentazone in the leaf is also at a maximum after 3 hours but with subsequent metabolism the majority is converted to its 6- and 8-hydroxy derivatives. These compounds, which have negligible phytotoxicity when applied to S. alba are found in only small quantities in susceptible species (Mine, et al, 1975).

The efficacy of bentazone has been found to be variable under different climatic conditions (Andersen, et al, 1974), and at different times of day (Doran and Andersen, 1976). Furthermore, Taylor (1979) has shown that bentazone phytotoxicity in C. album is dependent on the prevailing environmental conditions both before and after application. Penetration of ^{14}C-bentazone is also greatly influenced by climatic conditions (Nalewaja and Adamczewski, 1977; Dannigkeit, 1977a) a factor which may be overcome by the addition of surfactants (Nalewaja, et al, 1975). Although bentazone is usually foliar applied, uptake occurs through both root and shoot, and is virtually the same in both resistant and susceptible species (Zaunbrecher and Rogers, 1973). Differential absorption, however, has been suggested as a possible contributor to bentazone selectivity (Börner, 1979). Furthermore, Hayes and Wax (1975) have shown that a sensitive variety of Glycine max, PI 229 342, absorbs twice as much through the leaves as the tolerant variety Clark 63.

Once in the plant bentazone is translocated to varying degrees. For example, Mine and Matsunaka (1975) found that soil applications resulted in rapid translocation to shoot and leaves in C. serotinus whilst after foliar applications to various other species translocation varied between 6-24% (Wills and McWhorter, 1972). However, bentazone is foliar-applied commercially and the former authors were studying translocation under flooded field conditions more likely to facilitate fast, long-distance transport

in the xylem as a result of transpirational pull. Translocation after foliar application appears to be via the phloem since it is multidirectional (Mahoney and Penner, 1975) and is considerably influenced by temperature. For example, for one variety of Glycine max a temperature rise of 10°C increased translocation from less than 20% to 62% (Wills, 1976). However, most studies have shown that at normal temperatures translocation of bentazone is very low.

The common agricultural practice of applying pesticides as tank mixtures highlights some interesting features of bentazone in combination with other chemicals. For example, bentazone has been found to interact with the herbicide diclofop causing increased injury to Glycine max (Woldetatios and Harvey, 1977). Furthermore, in mixtures containing organophosphate or carbamate insecticides bentazone may reduce G. max fresh weights by 50% although neither compound is significantly phytotoxic when applied alone (Campbell and Penner, 1982). Finally, interference with translocation of glyphosate, by bentazone has also been reported (Sprankle, Meggitt and Penner, 1973; 1975). These antagonistic properties may occur frequently when pesticides are mixed and, whilst the mechanisms are unknown, they should be considered when physiological studies are undertaken.

1.5.3. Bentazone and Chenopodium album control: Bentazone is a contact herbicide for controlling broad-leaved weeds in legumes and graminaceous crops chiefly Glycine max (Luib and van der Weerd, 1972) and Oryza sativa (Mine, Hino, Ueda and Matsunaka, 1974). It was also used successfully in the U.K. for Phaseolus vulgaris until the warm dry summers of 1975 and 1976 when C. album was found to have highly variable susceptibility to bentazone (King, 1976). Control was improved by the addition of an oil-based adjuvant, Actipron (BP Chemicals Ltd.), and it was suggested that C. album became resistant to bentazone in warm dry weather due to the production of a thick waxy cuticular layer which impaired herbicide penetration (King, 1976). The improved control in the presence of Actipron was considered to be caused by increased coverage, resulting in more cuticular penetration (King and Handley, 1976).

It has been shown that C. album susceptibility to bentazone

is stongly influenced by the environmental conditions both before and after application (Taylor, 1979). Susceptibility was not correlated with leaf surface wax production (Taylor, Davies and Cobb, 1981) and studies of isolated epidermal peels showed that bentazone induced a rapid light-dependent stomatal opening response in this species (Taylor, Cobb and Davies, 1980). The stomatal apparatus of C. album has been further implicated as a potential preferential site for uptake of applied chemicals by scanning electron microscope observations which have revealed the presence of apparently wax-free ante-chambers within the guard cell complex (Taylor, 1979; Taylor, et al, 1980). These wax-free areas may present less of a barrier to penetration than the remainder of the leaf which is covered with thick projections of epicuticular wax.

1.6 Aims of the investigation

This study is essentially a continuation and extension of the work of Taylor (1979) in this laboratory. Susceptibility of C. album to bentazone is apparently unrelated to surface wax production and so the aim here is to further characterise the effect of the herbicide on leaf physiology in this species. Initially it is intended that this will involve studies in two specific areas, namely C. album whole leaf photosynthesis and stomatal physiology.

1. Chenopodium album leaf photosynthesis

Plants are unique in their ability to convert solar energy into chemical energy via the process of photosynthesis in their leaf cell chloroplasts. Hence, plant growth is finely attuned to photosynthetic performance. However, many herbicides including bentazone, effectively kill weeds by inhibiting photosynthesis in susceptible species. Thus the quantitative data relating whole leaf photosynthetic activity to herbicide treatment is a vital pre-requisite to any study of a weed/herbicide interaction.

Net photosynthesis by intact leaves can be successfully monitored using suitably constructed cabinets that fully enclose a leaf, which may then be subjected to controlled conditions for photosynthesis, namely temperature, relative humidity and light

intensity with a constant flow of clean air of known composition over the leaf. The air exposed to the photosynthesising leaf is then accurately and routinely analysed for carbon dioxide content, using a sensitive Infra Red Gas Analyser (IRGA). Thus, net uptake of carbon dioxide by the leaf is indicative of photosynthetic performance (Gaastra, 1959).

Once fully operational this approach may be used to fully characterise photosynthesis by C. album leaves exposed to a variety of experimental conditions, including herbicides such as bentazone. In this way precise interactions between the weed and the herbicide may be established. Initially this will be used to determine the influence of the environment on light saturation of untreated whole leaf photosynthesis. This information will enable a study of bentazone interference with C. album whole leaf photosynthesis to determine:-

1. the speed of herbicidal action.
2. the response to different bentazone concentrations.
3. the effect of Actipron.

One major pre-requisite for photosynthesis is a constant supply of atmospheric carbon dioxide entering the leaf intercellular air spaces. However, here a basic dilemma exists, because for each molecule of carbon dioxide that enters the leaf it is estimated that at least 100 molecules of water are lost by transpiration through open stomata (Raschke, 1975). Indeed excessive water loss reduces stomatal opening by a variety of mechanisms (e.g. Mansfield, Wellburn and Moreira, 1978), with an imposed reduction in photosynthetic rate. Hence photosynthesis, and leaf physiology in general, are closely controlled by stomatal aperture. Thus, measurement of stomatal resistance to gaseous exchange in intact leaves may be used to monitor the physiological state of a plant under various experimental conditions at any time. The investigation will therefore include routine measurement of stomatal resistances using a porometer to ascertain further effects of bentazone treatments.

2. Studies of C. album stomatal physiology

Previous studies in this laboratory have indicated that bentazone may have a profound effect on C. album stomatal apertures

and may enter the leaf via this apparatus (Taylor, 1979). Thus, further investigations using whole plants and epidermal peels will attempt to ascertain:

a) How bentazone influences stomatal apertures in isolated peels and intact leaves.

b) Whether bentazone interferes with stomatal ion uptake.

c) If the stomatal apparatus plays an important role in herbicide penetration in this species.

Initially this will involve characterisation of the previously observed response to the herbicide (Taylor, Cobb and Davies, 1980), and it is expected that this will then be investigated further using autoradiography aided by the donation of ^{14}C-bentazone from BASF A.G. Limited.

CHAPTER 2

Changes in Chenopodium album leaf diffusive resistance following treatment with bentazone at different times of day

2.1 Introduction

Previous work in this laboratory has indicated that an effective control of Chenopodium album by bentazone is dependent on the prevailing environmental conditions before and after spraying, as well as the physiological age of the plants at the time of application (Taylor, 1979). Furthermore, sensitivity to environmental conditions is due to factors other than wax production (Taylor, Davies and Cobb, 1981), and stomatal involvement has been implicated in the herbicidal action of bentazone in this species using abaxial epidermal peels (Taylor, Cobb and Davies, 1980). However, the use of isolated leaf epidermal peels is open to several criticisms, especially when the data is extrapolated to the whole plant. Hence, the purpose of this study is to describe the leaf diffusive resistance (LDR) of C. album leaves grown under constant environmental conditions in the presence or absence of field-rate bentazone, using an automatic diffusive resistance porometer.

2.2 Materials and methods

2.2.1. Plant growth conditions: Seeds of C. album, obtained from B & S. Weed Seed Suppliers (Notts.) were used for all experiments. These were germinated on filter paper and planted in 8 cm. plastic pots containing a 1:1 mix of John Innes No. 2 and J. Arthur Bowers potting compost. The pots were placed in a Fisons growth cabinet (model 600G3, type TTL) under a 16 hour photoperiod (Atlas Gro-lux and Warm White tubes) at a photon flux density (P.A.R.) of 200 μ E. $m.^{-2}$ $S.^{-1}$. Seedlings were watered daily from below, fed every 2 days with a liquid fertiliser (2N:1P:1K), and maintained at a mean day temperature of 20°C and 12°C (minimum) at night. After 3-4 weeks growth in these conditions the second pair of fully-expanded leaves were sufficiently large to cover the modified sensor head cup of the

PLATE 1

Typical *C.album* plants used throughout this study

a) Complete batch in growth cabinet

b) Close-up

porometer (i.e. 1.7 cm. diameter) as shown in Plate 1.

2.2.2. Diffusive resistance porometry - theory:

1. Leaf diffusive resistance: Leaf diffusive resistance or conductance cannot be measured directly but can be calculated from measurements of transpiration at known environmental conditions. Transpiration rate (E) of a leaf is directly related to the humidity gradient between the site of evaporation in the leaf and the ambient air ($C_i - C_e$) and the leaf resistance to diffusion of water vapour (R_{H_2O}) so that:

$$R_{H_2O} = \frac{E}{C_i - C_e} \qquad (1.)$$ (Jarvis, 1971)

R_{H_2O}, in s. cm.$^{-1}$, includes a stomatal and a cuticular component. The latter is usually very small (e.g. Holmgren, Jarvis and Jarvis, 1965) and is neglected. The internal humidity (C_i) is usually considered to represent the saturation concentration at leaf temperature (Jarvis, 1971). The difference in molecular diffusion rate of water vapour and CO_2 is accounted for as:

$$R_{CO_2} = 0.64 \; R_{H_2O}$$

With R_{CO_2} being the diffusive resistance to CO_2 uptake of stomata and substomatal cavity (Jarvis, 1971). The factor is only valid in the absence of turbulent diffusion or pressure gradients (Jarvis, 1971).

2. Calibration: Calibration of a diffusive resistance porometer, using perforated plates of known diffusive resistance, under the prevailing ambient conditions, produces a direct value for LDR without reference to equation (1.). The porometer used is similar in design to the one described by Kanemasu, Thurtell and Tanner (1969) and was obtained from Crump Scientific Products Limited.

Stomatal resistance is simulated by the use of plates drilled with many small holes. The "low value" plate used here consists of five measurement positions. These are covered by

a series of PVC sheets to give different resistance at each position. According to Kanemasu, et al, (1969) resistance (r) to water vapour diffusion through these points is given by:

$$r = \frac{L}{\alpha} = 4A \left(L_o + \frac{d}{8}\right) / nd^2$$

where:-

L = effective diffusion path length,
α = water vapour diffusivity in air,
L_o = the actual length of each hole,
A = area of probe aperture,
n = number of holes in calibration plate, and
d = diameter of holes.

(N.B. The term $\frac{d}{8}$ was incorrectly stated as $\frac{d}{4}$ in Kanemasu, et al, (1969) but later corrected).

The constructed calibration curve of resistance versus transit time takes the form:

$$r = r_o + \frac{t}{S}$$

Where t is the time lapse, S is the slope of the curve and r_o is the diffusive resistance of the vapour cup. With this curve it is possible to obtain values for LDR simply by measuring t, as r_o and S are constant at constant temperature (see Fig. 1).

2.2.3. Diffusive resistance porometry - practice: The porometer head consists of a cup which clamps onto the leaf, and a fan which draws dry air into the cup (see Fig. 2). The cup contains a humidity sensor and thermistors to monitor leaf and probe temperatures. Loss of water by the leaf increases the humidity within the cup at a rate dependent on the size of the cup and its aperture, which are constant, and therefore LDR is directly related to the rate of increase of humidity within the cup. The time taken for the humidity to rise between two set points on the humidity scale

FIGURE 1

Typical diffusive resistance porometer calibration curves

Curves obtained under different environmental conditions:

Open squares; 13°C, 47%RH.

Closed circles; 17·5°C, 60%RH.

Open circles; 23·5°C, 55%RH.

Closed squares; 25°C, 48%RH.

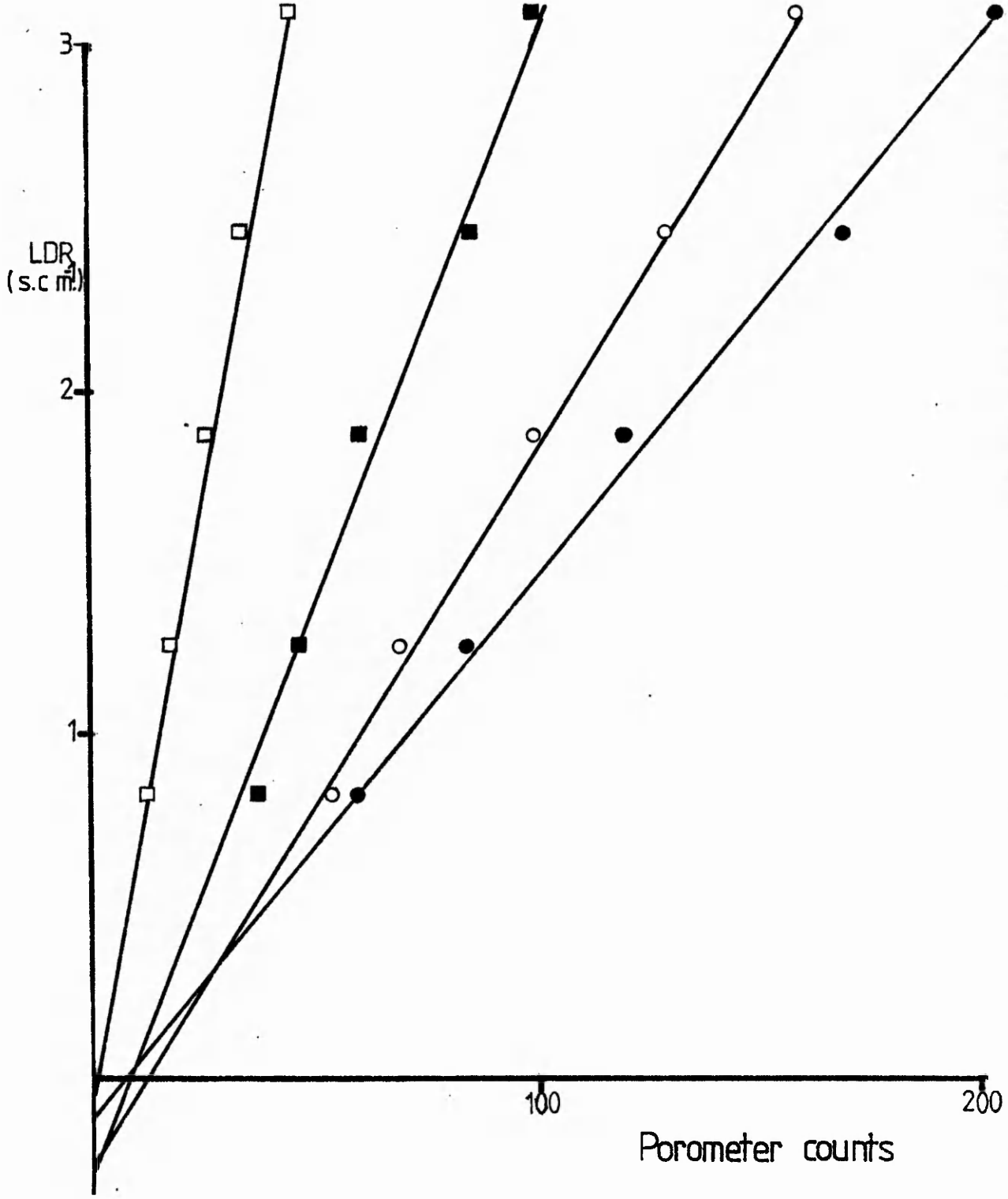
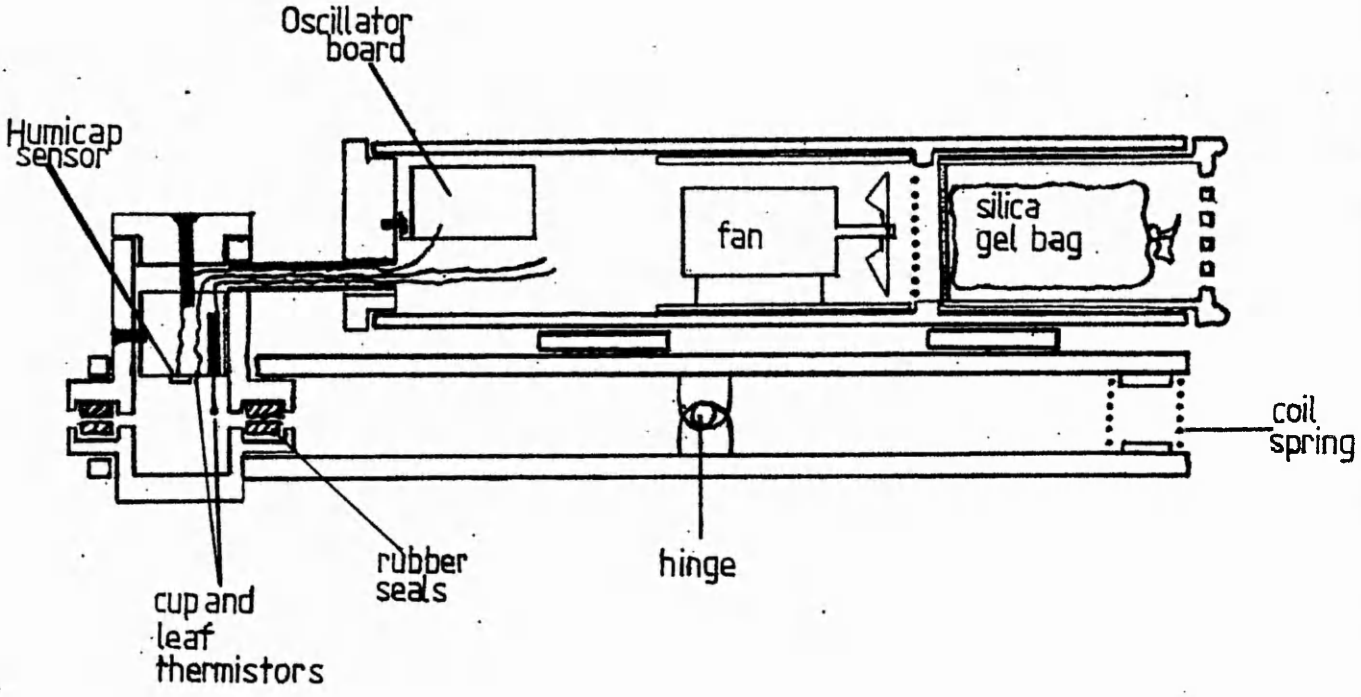

3
LDR
(s.c m.)
2
1
100
200
Porometer counts

FIGURE 2

Diffusive resistance porometer head and fan ($x\frac{3}{4}$)

Reproduced from manufacturer's manual.


Oscillator board
Humicap sensor
fan
silica gel bag
coil spring
rubber seals
hinge
cup and leaf thermistors

is determined by an electromechanical counter which is switched on electronically at the lower level and off at the higher level. When the counter stops, the fan automatically blows in dry air allowing the cycle of measurement to begin again. Counts can be converted to LDR from a graph of calibration plate LDR's versus counts as shown in Fig. 1.

2.2.4. Diffusive resistance porometry - precautions: The following precautions outlined by Morrow and Slatyer (1971), were observed during experiments:-

1. The cup was stored in dry air when not in use.
2. The cup was taken through several cycles prior to commencement of LDR measurements.
3. The air in the cup was dried to the same humidity between measurements.
4. The leakage rate of the empty cup was monitored, although it was found to be negligible in growth cabinet studies.
5. The cup and leaf temperatures were within 0.5°C.

In addition, the calibration plate was kept dry, condensation within the cup was avoided, and sponge rubber seals with closed pores were used to reduce the aperture area (McCree and van Bavel, 1977). The peculiarities of using the porometer in the growth cabinet also made the following extra precautions necessary:-

6. The porometer head was mounted in the growth cabinet on a clamp so that all plants were read at the same leaf orientation.
7. To avoid atmospheric changes measurements were taken with the cabinet door closed and the meter outside.
8. During transfer of the cup from one leaf to another care was taken to avoid undue disruption of the internal environment, and this time was kept to a minimum (~10s).
9. In the long-term experiments no leaf was measured more than twice in one day to avoid damage.
10. The porometer was calibrated before each set of readings commenced.
11. Measurements were taken in 30 minute blocks which allowed for a reasonable sample size of up to 20 plants, but avoided prolonged alteration of environmental conditions.

2.2.5. Measurement of C. album leaf diffusive resistance: To determine LDR at different times of day measurements were taken on random samples of 96 plants over a period of 10 days. For these studies the adaxial surface of the nearest fully expanded leaf to the apex was used, as preliminary observations showed an increase in LDR with leaf age. To cover the porometer cup with this leaf it was necessary to reduce the aperture to 1.7 cm (diameter) using stick-on rubber seals. Readings were taken for the whole 24 hour cycle as shown in Fig. 3.

For short-term herbicide studies at least 20 plants were selected for similar age, height and LDR and sprayed with field-rate bentazone (3 dm^3 in 280 dm^3 of water ha^{-1}; formulation BAS 3517H, containing 48% (w/v) a.i. bentazone) or left untreated to act as controls. Spray time was either 0930 - 0945h or 1215 - 1230h using a Binks-Bullows sprayer at 1 bar pressure. Leaf surfaces were dry within 30 mins after application and LDR measurements were taken at 0.5, 1.5, 2.5, 4.5 and 6.5 hour intervals. In the long-term experiments LDR of a population of 72 plants was measured for 6 days before and after herbicide application, with measurements taken between 0900 - 1030h and 1200 - 1330h. Fresh weights of control and treated plants were determined after 0, 7 and 11 or 14 days following treatment and the corresponding dry weights obtained by heating in an oven at 105°C overnight.

2.3 Results

As shown earlier, LDR is inversely proportional to the degree of stomatal opening, assuming that the internal leaf air spaces are saturated with water vapour and cuticular transpiration is negligible. Thus, the relationship obtained between LDR and time of day for intact C. album leaves (Fig. 3) infers a circadian rhythm of stomatal movement in this species when grown under the conditions described, with pre-dawn opening, pre-dusk closure and a tendency to close around midday. Minimum LDR, and hence maximum stomatal opening, is observed both before and after the midday closure period, whilst night-time LDR values are some 10-20 times greater, indicative of almost complete stomatal closure.

Figure 4 shows the long-term response of C. album LDR to

FIGURE 3

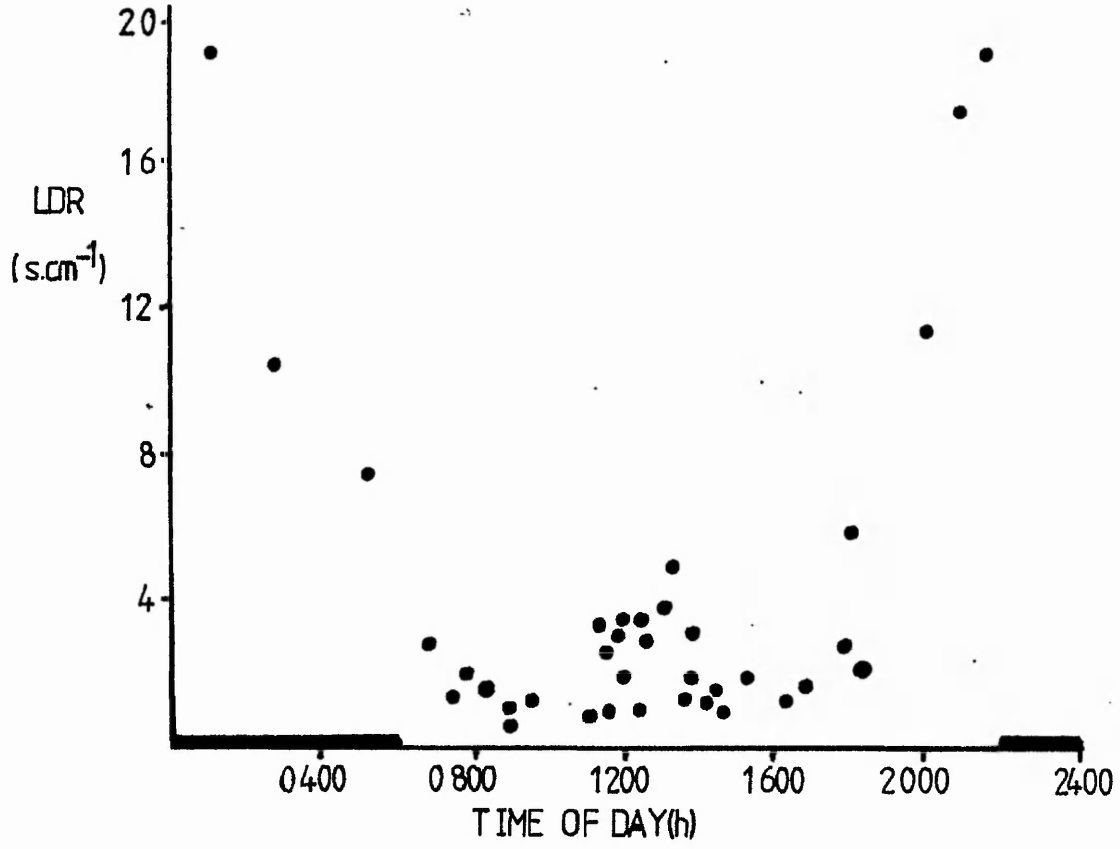
Relationship between Chenopodium album leaf diffusive resistance and time of day

Each point represents the mean of up to 20 adaxial measurements taken within a 30 minute period. A total of 96 plants were used over 10 days.

FIGURE 4

Long-term changes in C.album leaf diffusive resistance after application of field-rate bentazone at 0930h or 1230h

Each point is the mean of 20 readings on different plants taken at the times indicated. Bars representing standard error values are shown where they exceed the symbol. Closed circles; untreated plants, measured 0900-1030h. Open circles; untreated plants, measured 1200-1330h. Closed squares; bentazone sprayed 0930h, measured 0900-1030h. Open squares; bentazone sprayed 0930h, measured 1200-1330h. Closed diamonds; bentazone sprayed 1230h, measured 0900-1030h. Open diamonds; bentazone sprayed 1230h, measured 1200-1330h.


20
16
12
8
4
LDR
(s.cm⁻¹)
0400
0800
1200
1600
2000
2400
TIME OF DAY(h)

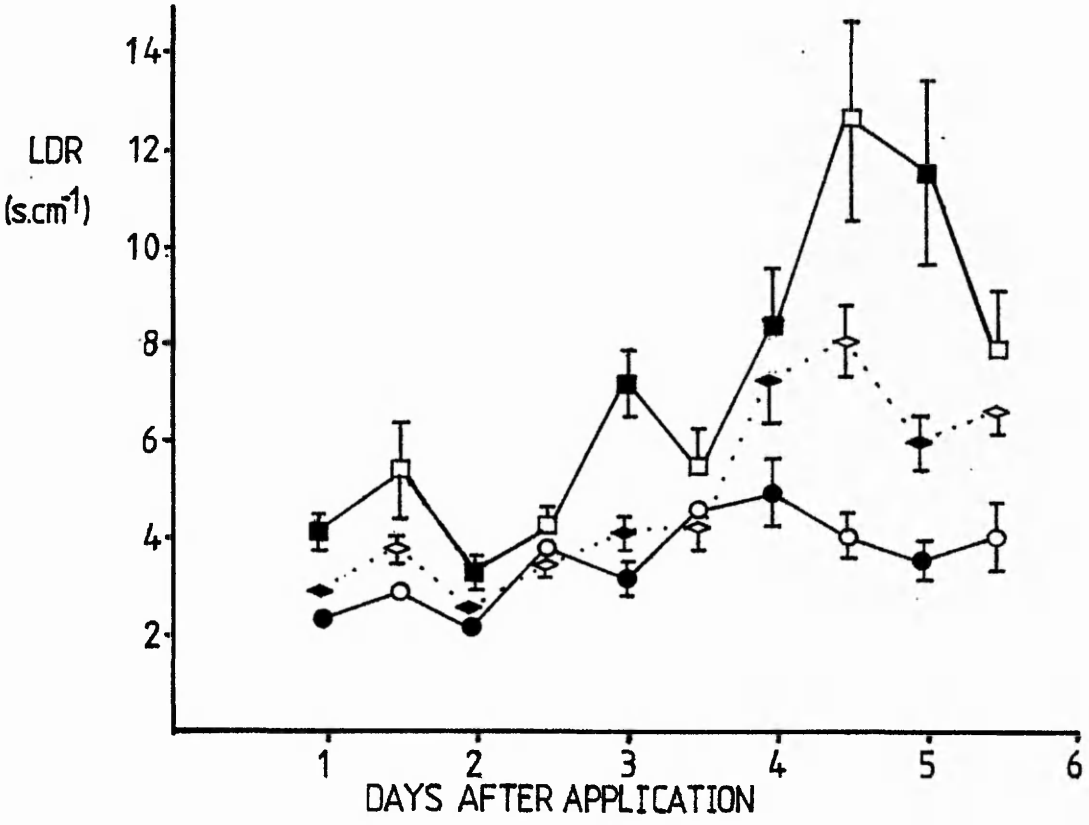

14
12
10
8
6
4
2
LDR
(s.cm⁻¹)
1
2
3
4
5
6
DAYS AFTER APPLICATION

FIGURE 5

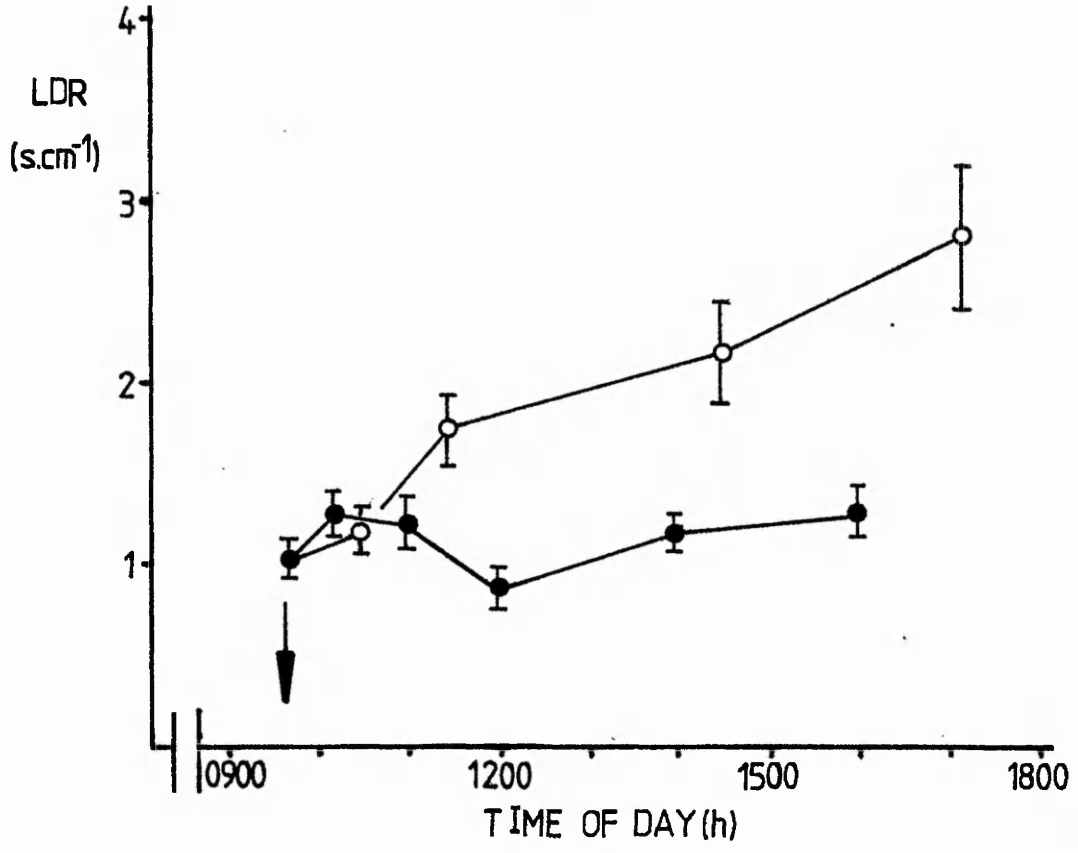
Short-term changes in C.album adaxial leaf diffusive resistance following the application of field-rate bentazone at 0930h

Each point represents the mean of 15 measurements of different leaves taken within a 30 minute period. Bars indicate standard error values. Arrow shows treatment time. Closed circles, untreated plants; open circles, field-rate bentazone.

FIGURE 6

Short-term changes in C.album adaxial leaf diffusive resistance following the application of field-rate bentazone at 1230h

Details as for figure 5.


LDR
(s.cm⁻¹)
4
3
2
1
0900
1200
1500
1800
TIME OF DAY(h)

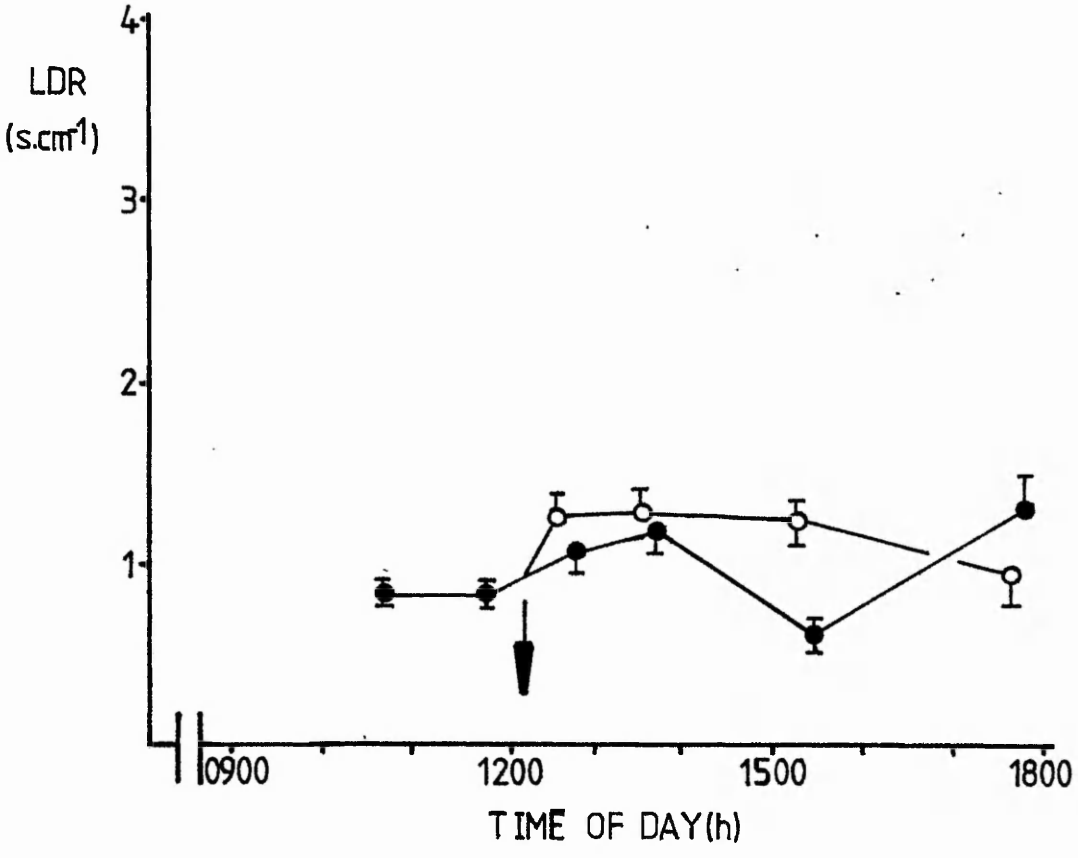
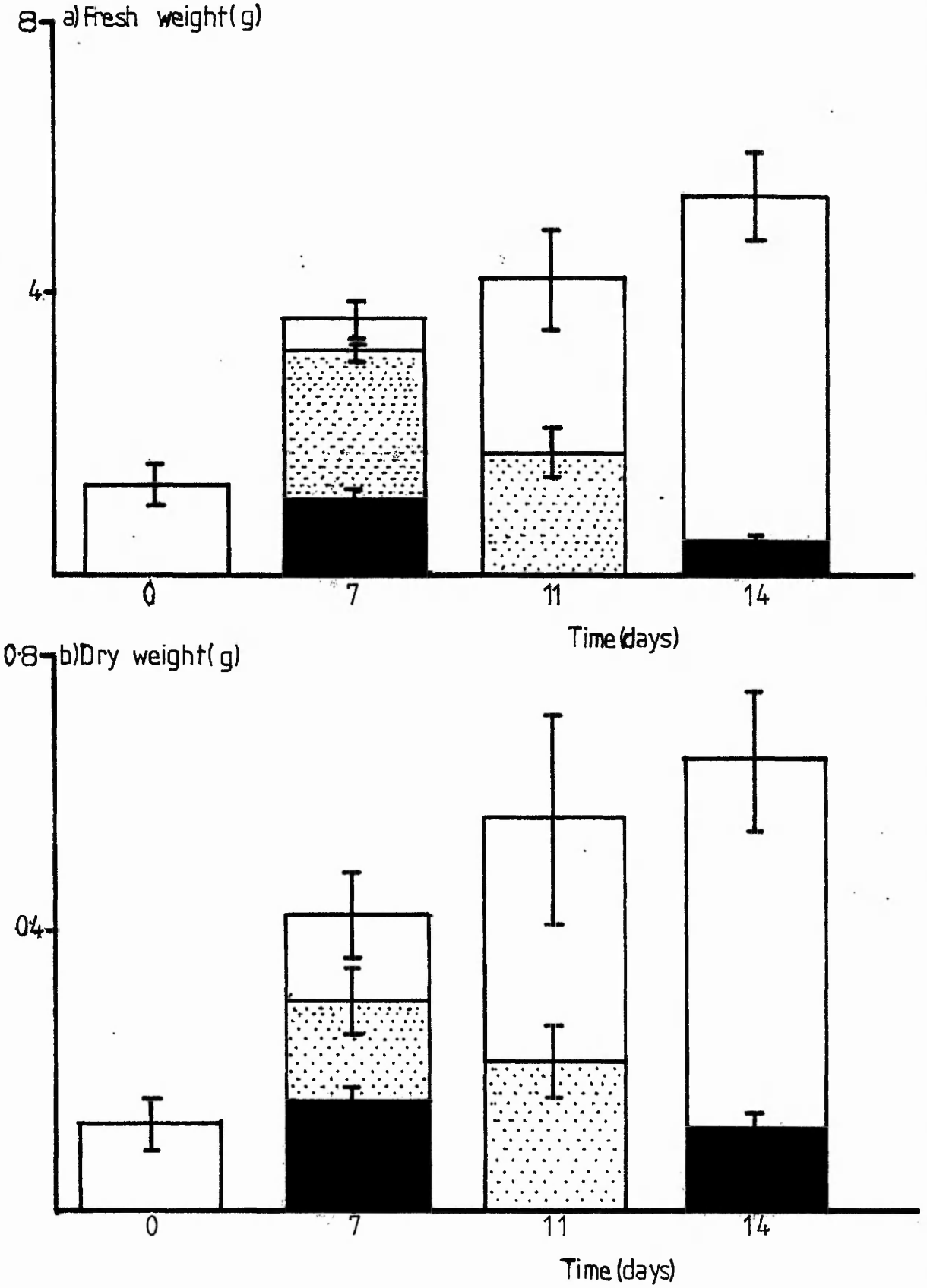

LDR
(s.cm⁻¹)
4
3
2
1
0900
1200
1500
1800
TIME OF DAY(h)

FIGURE 7

C.album shoot fresh and dry weights determined 0, 7 and 11 or 14 days after field-rate bentazone application

Results are expressed as mean weight ($g.shoot^{-1}$) and bars represent standard error values(n=8). Open histogram area, untreated plants; closed area, sprayed 0930h; shaded area, sprayed 1230h.


8
a) Fresh weight (g)
4
0
7
11
14
Time (days)
0·8
b) Dry weight (g)
0·4
0
7
11
14
Time (days)

bentazone application. Although untreated plants show midday closure throughout the period of observation, plants sprayed with field-rate bentazone at 0930 h or 1230 h show departures from this rhythm in two major respects. Firstly, treated plants show higher LDR values than the untreated, and secondly, the circadian rhythm in LDR is interrupted by bentazone application. Thus, plants treated at 0930 h show a marked increase in LDR 1 day after treatment and after day 2 the expected midday closure is lost. This is followed by a further increase in LDR culminating in the development of chlorotic symptoms 6 days after treatment. However, plants sprayed during the midday closure period (1230 h) show a more gradual increase in LDR, and only begin to deviate from untreated plants 4 days after bentazone application. These long-term differences in C. album LDR values following bentazone application are reflected in the shoot fresh and dry weight determinations (Fig. 7), i.e. phytotoxicity is more rapid following herbicide treatment on fully open stomata at 0930 h.

Short-term experiments, in which plants with low LDR were selected to give more rapid readings, and hence reduced disruption of the controlled environment, essentially confirm the results of long-term studies. Application of field-rate bentazone to C. album at 0930 h invoked a 3-fold increase in LDR within 8 hours (Fig. 4). This stomatal closure was absent in plants sprayed at 1230 h (Fig. 5) and, although LDR values were slightly higher than controls during the immediate post-application period, there was no significant difference by the end of the day.

2.4 Discussion

The data presented above clearly shows that when Chenopodium album is grown in a controlled environment, leaf adaxial stomata display a pronounced circadian rhythm of opening and closure (Fig. 3), and that LDR is altered by bentazone application (Fig. 4-6), as measured by porometry. Experiments with field grown C. album in June 1980 provided supporting data for the above although unstable weather conditions necessitated continual re-calibration of the porometer due to rapid changes in leaf external environment. The porometer head was shielded from these fluctuations

but readings were too variable to be conclusive. However, this study, and a previous one using epidermal peels (Taylor, Cobb and Davies, 1980), clearly shows that stomatal movement in C. album is affected by bentazone.

An apparent anomaly exists between C. album epidermal peels and intact leaf responses to bentazone, in that the former tissue exhibits an initial rapid stomatal opening (Taylor, Cobb and Davies, 1980), whereas the intact leaf shows increased LDR indicative of stomatal closure. This may reflect the artificial nature of epidermal peel studies, since the floating of abaxial peels cuticle upwards on test solutions bears little or no relation to the agricultural context of a herbicide sprayed onto a leaf adaxial surface. Furthermore, the presence of epidermal plus mesophyll cells in the intact leaf may modify a stomatal response, since the movement of compounds between the two cell types may be implicated in the control of stomatal movement in situ (e.g. Mansfield, Wellburn and Moreira, 1978). These considerations have prompted further studies to characterise the bentazone-induced response of epidermal peel stomata in C. album in order to resolve this apparent discrepancy (Chapter 4).

Although bentazone functions as a photosynthetic inhibitor (Mine and Matsunaka, 1975) it must first enter the plant to reach its chloroplastic site of action. The observation that C. album phytotoxicity is more rapid if bentazone is applied when the stomata are fully open and the leaves actively photosynthesising, than when applied during the midday closure period (Figs. 4-7), supports the hypothesis that bentazone action in this species is mediated via the stomatal apparatus (Taylor, et al. 1980; 1981). C. album leaf surfaces possess a dense covering of water-repellent epicuticular wax platelets, although guard cells, and their pronounced ante-chambers appear devoid of these structures (Taylor, 1979). Since the stomatal pore itself is narrow, it is possible that open stomata may present a larger guard cell surface area for bentazone penetration, than do partially closed stomata. Alternatively, the direct action of bentazone in causing C. album stomatal closure, implied by increased LDR, may also contribute significantly to phytotoxicity since this would ultimately lead to cessation of

photosynthesis and transpiration.

To invoke stomatal involvement in bentazone action would account for the data presented here, and may provide a unifying hypothesis for other observations in the literature regarding the effectiveness of this herbicide at different times of day. For example, Doran and Andersen (1976) found that whereas Glycine max was tolerant to bentazone irrespective of the time of day of treatment, Xanthium pensylvanicum and Abutilon theophrasti were more sensitive following application at 1400 h than at 0145, 0600 and 2230 h. Furthermore, A. theophrasti plants exhibit pronounced nyctinastic leaf movements which may account for this variable response (Andersen and Koukkari, 1978; 1979), although a daily rhythm in bentazone sensitivity still occurs at low relative humidity regardless of leaf movements (Koukkari and Johnson, 1979).

The observed circadian rhythm of C. album LDR (Fig. 3) has similar phase characteristics to those for nyctinastic leaf movements in this species (Andersen and Koukkari, 1979). Rhythmic movements of stomata, resulting from changes in guard cell turgor due to influx and efflux of potassium ions, have been observed in other species and may be a function of temperature on the leaf intercellular CO_2 concentration (Meidner and Mansfield, 1968). Leaf movements also involve active transport of large quantities of K^+ and it has been suggested that fluctuations in the permeability of plasma membranes to this ion may account for both types of circadian rhythm (Sweeney, 1979). Hence, in all the cases outlined above, variable efficacy of bentazone may depend on the oscillating physiological activity of the leaf with respect to potassium.

The involvement of rhythms in stomatal and leaf movements and the role of the stomatal apparatus in herbicidal entry, may be of wider significance in herbicide studies. Furthermore, the use of porometry, as shown in this study, may provide valuable data prior to the onset of phytotoxic symptoms (i.e. invisible injury), and so establish optimum times of day for application.

The present investigation has described the use of an automatic diffusive resistance porometer to demonstrate a circadian rhythm of LDR and, hence, stomatal movement in C. album grown under the conditions described. Treatment with field-rate bentazone was

found to induce a rapid increase in LDR and disruption of the circadian rhythm which was more apparent after application at 0930 h when stomata were open than at 1230 h when stomata were partially closed. Furthermore, this difference was reflected in plant damage suggesting that the stomatal apparatus may be involved in the action of bentazone on C. album.

CHAPTER 3

Uptake of ^{14}C-bentazone by C. album leaves following application at different times of day

3.1 Introduction

As many herbicides, including bentazone, control weeds by interfering with photosynthesis, their efficiency is often limited by their rate of penetration into the leaf to reach their chloroplastic site of action. Techniques used to investigate the uptake of foliar applied chemicals have included, for example, cathodoluminescence or X-ray diffraction in conjunction with scanning electron microscope observations (Hess, et al, 1974; 1975) and methods involving fluorescent dyes as tracers (e.g. Dybing and Currier, 1959). However, the most common and reliable way of monitoring herbicidal movement is by the liquid scintillation counting of radio-labelled herbicides.

Several workers have used ^{14}C-bentazone to show that uptake and translocation may be influenced by environment (Wills, 1976), formulation (Nalewaja and Adamczewski, 1977) or species (Dannigkeit, 1977a and b). However, no comprehensive study of the penetration of this herbicide into C. album leaves has been undertaken. Furthermore, studies into bentazone absorption have been limited to transport across the cuticle without any reference to possible sites of preferential uptake.

The aim of this investigation is to evaluate the relative rates of uptake of ^{14}C-bentazone applied to C. album intact leaves when stomata are either open or closed in the presence or absence of the oil adjuvant Actipron (B.P. Chemicals, U.K.). Microautoradiography is also used to determine any sites of preferential binding to isolated epidermes of this species, according to the method of Willmer and Rutter (1977) for uptake of L-(U^{14}C)-malic acid by Commelina communis epidermal peels. Such an approach may yield valuable information regarding the involvement of stomata in the uptake of bentazone by C. album.

3.2 Materials and methods

3.2.1 Determination of ^{14}C-bentazone uptake by C. album leaves:

Application of ^{14}C-bentazone: 4-6 week old C. album plants, grown as described in section 2.2.1., were removed from the growth cabinet at either 0930 or 2030 h, and LDR values of the youngest fully-expanded leaf were determined for each plant using a pre-calibrated porometer. To this leaf 5 x 0.5 µl drops of field-rate bentazone (3 dm^3 BAS 3517H in 280 dm^3) in 5% (v/v) aqueous ethanol, containing a total of 0.25 µC; of ^{14}C-bentazone in the presence or absence of 0.5% (v/v) Actipron, were applied equally spaced on the adaxial surface using a Burkard microsyringe. Four plants were used for each treatment and these were returned to the growth cabinet immediately after application. The treated leaves were excised after 0, 24, 48, 72 and 168 hours, and washed by dipping five times in 10 cm^3 of 0.5% (v/v) Actipron dissolved in 5% ethanol (v/v in water).

Measurement of ^{14}C-activity in washing solution: A 1 cm^3 sample of washing solution was pipetted into a vial, shaken with 10 cm^3 BDH cocktail T scintillation fluid (Toluene, 666 cm^3; Triton x-100, 332 cm^3; PPO, 5 g; POPOP, 0.15 g) and counted for emissions on a Packard (model 3330) liquid scintillation counter. The recorded counts were corrected for "quench" as described below, converted to d.p.m. and expressed as a percentage of the total applied dose recovered after zero time.

Measurement of ^{14}C-activity in treated leaf: The excised treated leaves were finely chopped with scissors in a scintillation vial and left to dissolve for 3 days in 1 cm^3 of 0.05 M Soluene 350. To this was then added 1 cm^3 H_2O_2 (20 vols.) to bleach the sample, followed 3 days later by 1 cm^3 0.05 M HCl to neutralise pH. 10 cm^3 BDH cocktail T scintiallation fluid were added, the mixture thoroughly shaken and counted for emissions. These values were corrected for "quench", converted to d.p.m. and expressed as a percentage of the dose recovered after zero time. Translocation was calculated as the amount of label not recovered in the washing solution or treated leaf.

Quench correction: Counted samples were corrected for "quench", due to plant material and the chemicals added in preparation, by use of

a chloroform quench correction curve. Into six vials were accurately weighed 0.00, 0.04, 0.08, 0.15, 0.25 or 0.45 cm^3 chloroform and to each was added 0.8 cm^3 high-purity ^{14}C-hexadecane of known specific activity. BDH scintillation cocktail T (10 cm^3) was then added and the samples counted for emissions at two different channel settings i.e.

Green Channel: Lower energy, 50; Upper energy, 1000; Gain, 6.8%.

Blue Channel: Lower energy, 150; Upper Energy, 1000; Gain, 6.8%.

Percentage counting efficiency (C.E.) was determined using the ratio of observed to predicted counts:

$$C.E. = \frac{\text{c.p.m. in green channel}}{\text{predicted d.p.m.}} \times 100$$

Channels ratio (C.R.) was determined by dividing the total blue channel counts by those in the green channel:

$$C.R. = \frac{\text{c.p.m. in blue channel}}{\text{c.p.m. in green channel}}$$

The quench correction curve (Fig. 8) was obtained by plotting C.E. against C.R. for each chloroform concentration. Conversion of c.p.m. to d.p.m. was obtained by reading off the observed C.R. on the curve to give the equivalent C.E. and hence total disintegrations.

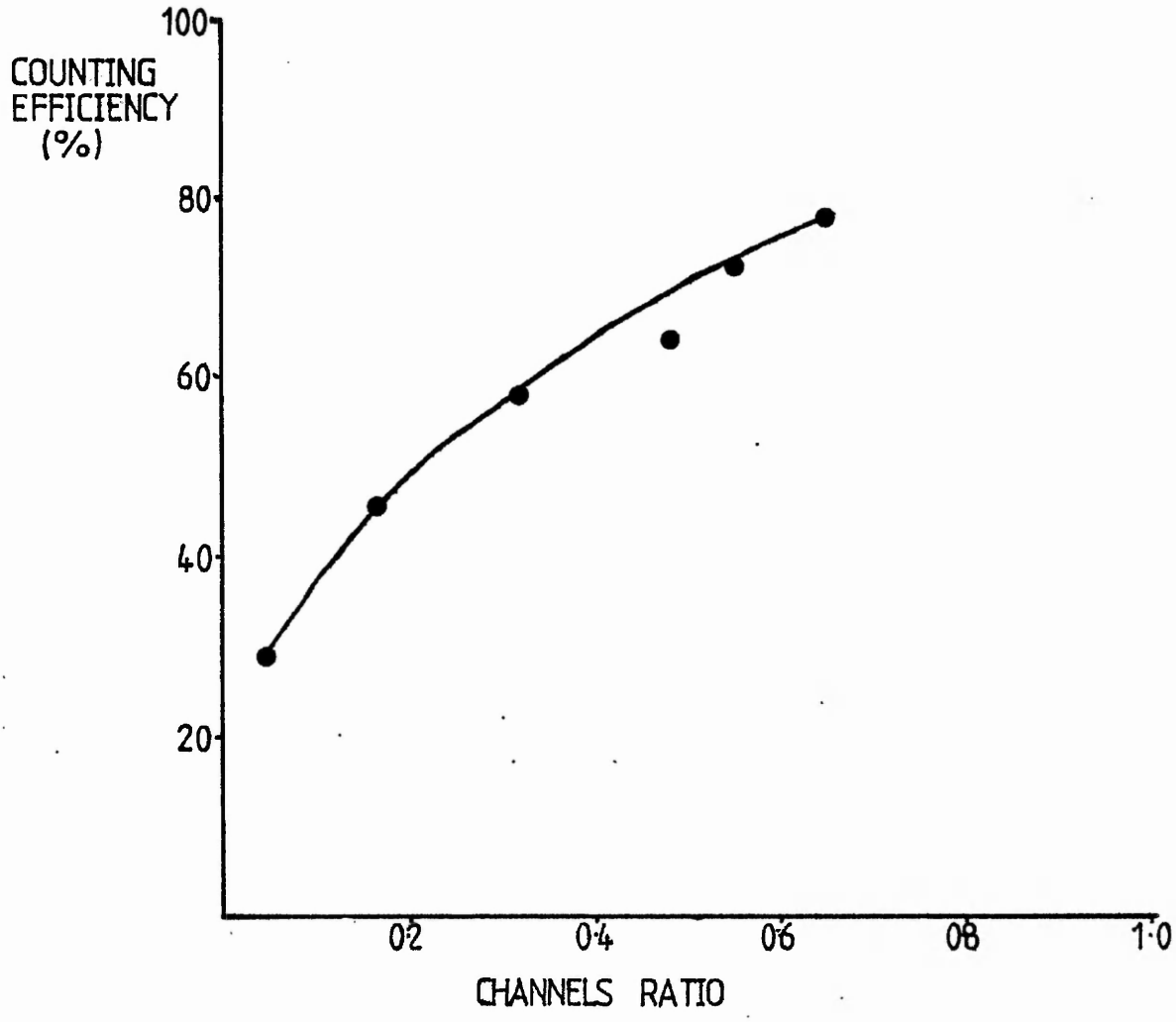
3.2.2. Microautoradiography of C. album epidermal peels after incubation on solutions containing ^{14}C-bentazone

Treatment of plant material: C. album epidermal peels, obtained as described in Chapter 4, were floated for 1 hour in darkness at 500 ppm CO_2 on 22.5 cm^3 of $10^{-4}M$ $CaCl_2$ pH 6.5, to induce stomatal closure, prior to the addition of 2.5 cm^3 of $10^{-3}M$ bentazone containing a total of 15 μC_i ^{14}C-bentazone. Peels were then

FIGURE 8

Chloroform quench correction curve

The relationship between channels ratio and counting efficiency for ^{14}C-hexadecane samples containing different amounts of chloroform. See text for details.


100
COUNTING
EFFICIENCY
(%)
80
60
40
20
0·2
0·4
0·6
0·8
1·0
CHANNELS RATIO

incubated for a further 2 hours in the same closing conditions, under which bentazone had been shown to cause stomatal opening (see Chapter 4). After this period the peels were washed twice on 10^{-4}M $CaCl_2$ then freeze-dried for 3 hours under vacuum (50 mm Hg). The dried peels, mounted on sellotape attached to a microscope slide, were pressed against specially cut frames of Ilford PANF film, clamped together and rolled up inside a large black polythene bag secured with strong elastic bands in a dark-room. These were then placed in a freezer and the resultant image developed after 1, 3 and 6 months.

Treatment of microautoradiographs: Exposed film was developed for $5\frac{1}{2}$ minutes in Paterson Aculux FX24 at 20°C, briefly washed in water and fixed for 10 minutes in Paterson Acufix. Photomicrographs of the prepared negatives were taken using a Leitz E.B. microscope at X30, X100 and X400 with AGFA DIA DIRECT film to produce negative slides from which prints were made. Similar photographs of the original epidermes, stained with safranin, were produced using EKTACHROME 100 ASA colour film.

3.3 Results

The data in figure 9a represent the amount of ^{14}C-bentazone which could not be removed by the washing solution after application to intact C. album leaves at 0930 or 2030 h. Total uptake after 7 days was approximately 70% regardless of treatment time although initial penetration of the herbicide occurred more rapidly if applied at 0930 h. (when mean LDR was 1.7 s.cm.$^{-1}$ and stomata relatively open) than at 2030 h. (when LDR was 15.6 s.cm.$^{-1}$ and stomata effectively closed). In fact, during the first 3 days after application uptake at 0930 h. was 100% greater than that following treatment at 2030 h. The majority of ^{14}C-bentazone which penetrated remained in the treated leaf if applied at 0930 h. (Fig. 9c) but was apparently translocated when applied at 2030 h. (Table 1.).

The incorporation of Actipron into the herbicide solution considerably enhanced uptake after treatment at 2030 h. (cf. Fig 9a and b). Indeed, similar amounts of ^{14}C-bentazone were found in the treated leaf regardless of application time in

FIGURE 9

Uptake of ^{14}C-bentazone after application to C.album leaves at different times of day

a) Total uptake of ^{14}C-bentazone.
b) Total uptake of ^{14}C-bentazone + 0·5%(v/v) Actipron.
c) ^{14}C-bentazone in treated leaf without Actipron.
d) ^{14}C-bentazone in treated leaf with Actipron.

Each point is the mean of four replicate plants. Bars representing standard deviation are shown where they exceed the symbol size. Open circles, applied 0930h; closed circles, applied 2030h.

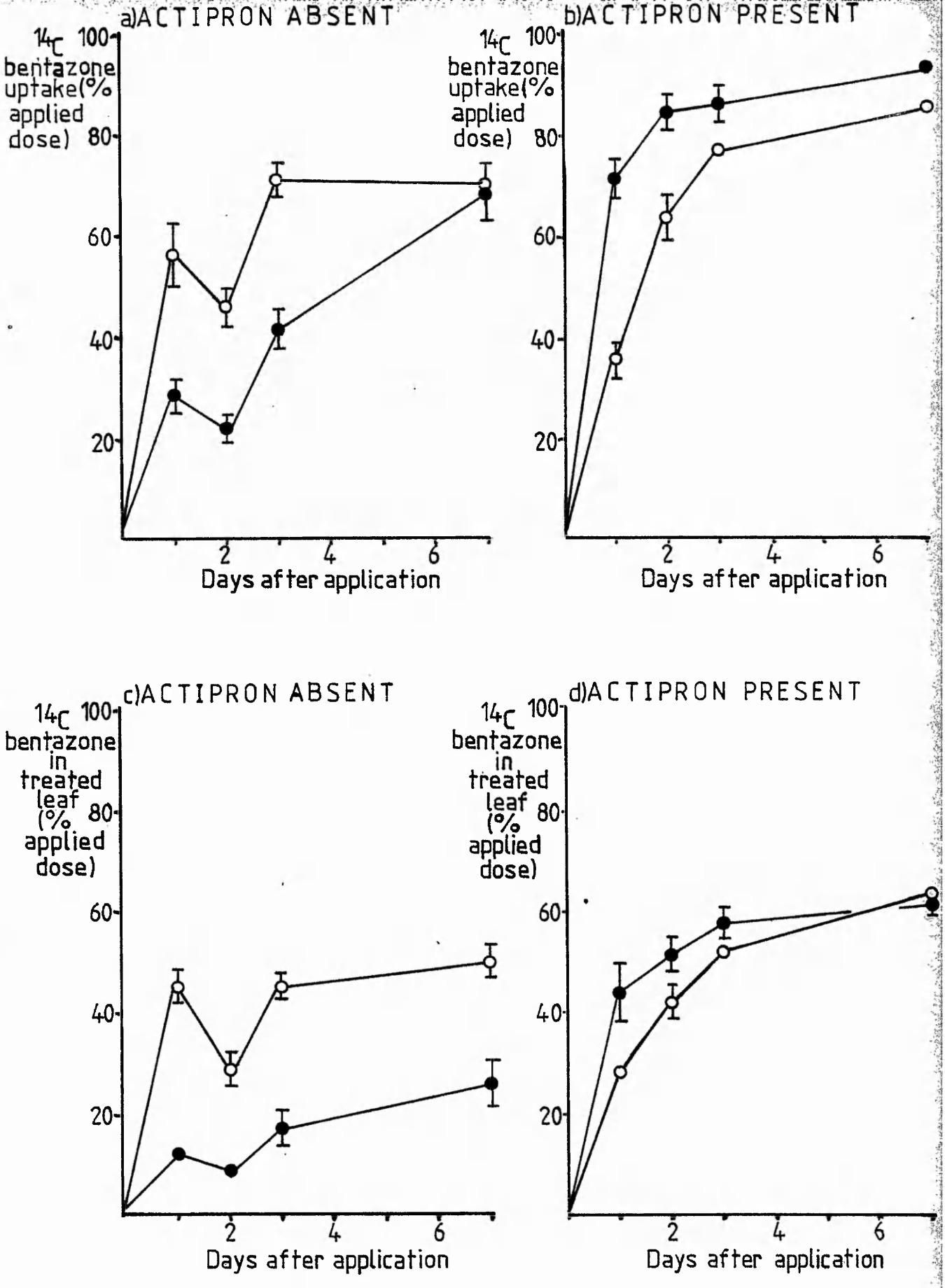

a)ACTIPRON ABSENT
b)ACTIPRON PRESENT
c)ACTIPRON ABSENT
d)ACTIPRON PRESENT
14C bentazone uptake(% applied dose)
14C bentazone in treated leaf (% applied dose)
100
80
60
40
20
2
4
6
Days after application

TABLE 1

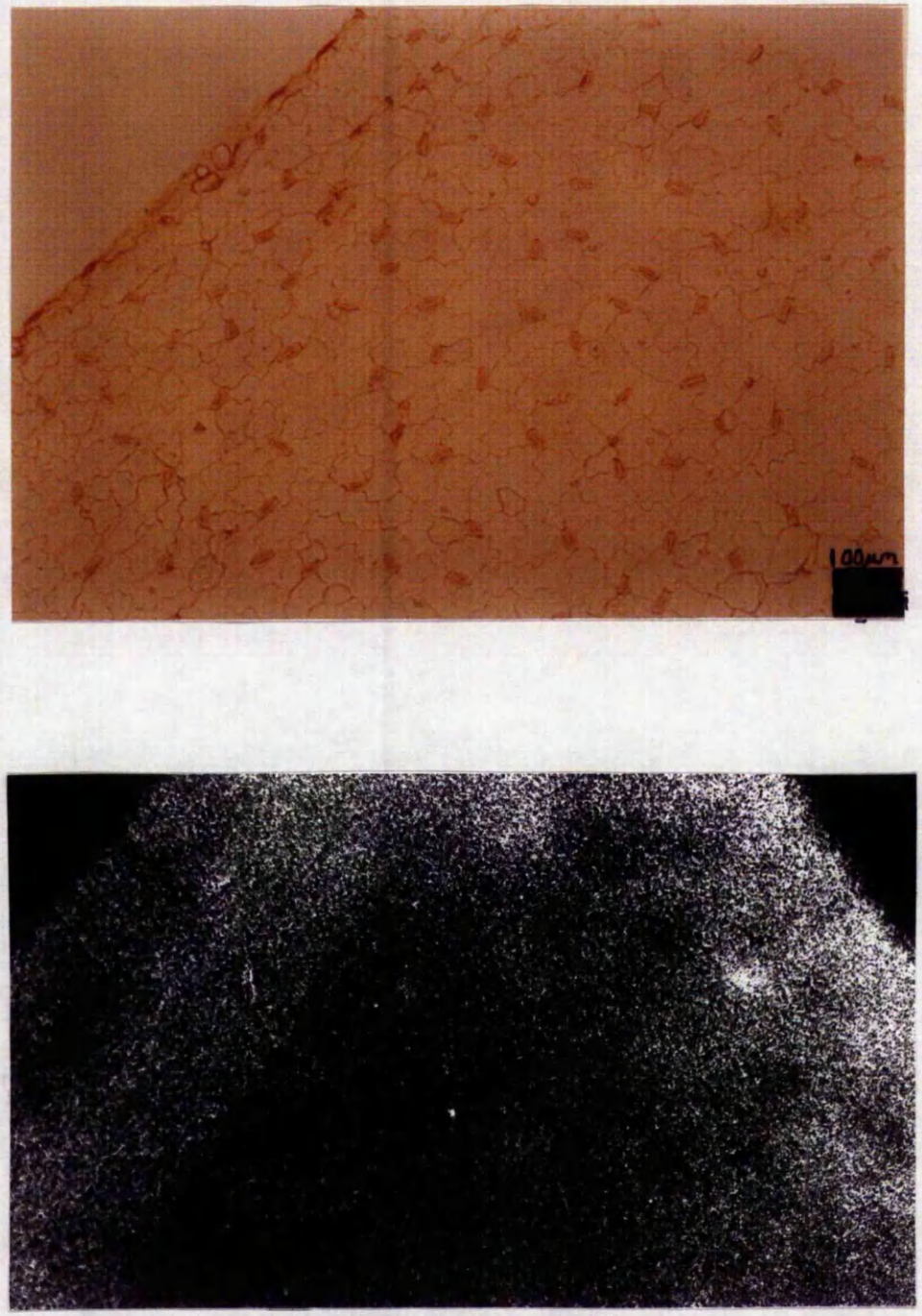
Translocation of ^{14}C-bentazone by C.album after application to leaves with open or closed stomata in the presence or absence of Actipron

The amount of ^{14}C-bentazone not recovered in either the washing solution or the treated leaf as a percentage of that recovered after zero time. Standard deviations are shown in brackets.

Treatment time(h)	Actipron	LDR ($s.cm^{-1}$)	Time after treatment(days)			
			1	2	3	7
0930	absent	1·7	10·2(16·7)	17·6(5·7)	26·2(8·2)	20·8(4·2)
0930	present	1·7	13·1(3·6)	21·2(6·2)	22·3(6·2)	21·8(4·8)
2030	absent	15·6	16·5(8·6)	12·8(8·2)	22·3(16·2)	41·8(14·4)
2030	present	15·6	27·0(7·9)	32·8(2·4)	28·8(1·9)	31·7(4·6)

PLATE 2

Photomicrograph of a C.album epidermal peel stained with safranin and the corresponding microautoradiograph obtained after floating on ^{14}C-bentazone solution (×100)



the presence of this oil (Fig. 9d) which had little influence on penetration at 0930 h. Furthermore, the total uptake in the presence of Actipron was greater after application when stomata were closed, rather than open, throughout the 7 days following treatment (Fig. 9b) primarily due to a consistently higher rate of apparent translocation at 2030 h. (Table 1.).

The microautoradiographs produced from C. album epidermes floated on ^{14}C-bentazone solution show that radioactivity was incorporated into all parts of the epidermis (Plate 2). Absorption was apparently uniform with no evidence of any coincidence between areas on the original peel and those on the impression made by ^{14}C-bentazone. Hence, under the conditions studied here, designed to cause stomatal closure, there was no indication of any preferential binding site for this herbicide on the epidermis of C. album.

3.4 Discussion

The data obtained in this investigation reveal that uptake of bentazone by intact C. album leaves is influenced by both the time of day of application and the inclusion of the oil adjuvant Actipron. Generally, the rate of penetration in the absence of oil was very slow under the conditions studied. Even when applied to actively photosynthesising plants with open stomata 30% of the herbicide could still be washed from the surface of the treated leaf after 7 days. This slow absorption may be a function of both the herbicide and the species under investigation. For example, in a similar experiment involving electron capture gas liquid chromatography to monitor 2, 4-D uptake by intact C. album plants, 28% of the applied dose could be washed from the leaves by chloroform after the same time period, compared with only 9% for Stellaria media (Hamilton, 1978). Growth conditions were not specified but the cause of differential uptake was considered to arise from variations between species in the relative proportions of polar components in the epicuticular waxes. Other workers have shown that penetration of ^{14}C-atrazine is 7 times greater into leaf sections of Zea mays than those of C. album within 24 hours and attributed their findings to slower

herbicide metabolism by the latter (Smith and Nalewaja, 1972). However, this hypothesis could not account for even less uptake by Setaria glauca since this species was shown to have metabolised 90% of the herbicide which had penetrated compared with only 70% by C. album. Moreover, the use of leaf slices submerged in herbicide solutions for uptake studies is open to criticism as herbicides may penetrate directly into the mesophyll tissue without first crossing the natural cuticular barriers. Nevertheless, the results of the present study showing relatively slow uptake of bentazone by C. album, together with the previous observations cited, may reflect a high resistance to herbicidal penetration in this species.

^{14}C-bentazone penetration appears to be extremely variable according to either the species to which it is applied or the washing method used for its recovery. For example, Zaunbrecher and Rogers (1973) found that this herbicide was rapidly absorbed by Glycine max and Xanthium pensylvanicum and that 95% of the applied dose remained in the treated leaf after 8 days. However, in a more detailed study, uptake by Amaranthus retroflexus was very slow in the absence of surfactants with 47, 30 and 28% being washed by water from the treated area after 1, 3 and 7 days respectively (Nalewaja and Adamczewski, 1977). In contrast, 80% uptake of ^{14}C-bentazone was found by these authors to occur within 24 hours in Brassica kaber whilst low initial penetration into G. max increased to 90% within 7 days. Other workers have shown that 75% of the applied bentazone is absorbed within 3 days as determined by ethanol washes of Helianthus annuus leaf discs punched from the treated area (Irons and Burnside, 1982). Hence, a variety of investigations have produced results which cannot be directly related since recovery of a herbicide will depend on its solubility in the washing medium. Indeed, certain solvents may penetrate plant tissues and dissolve herbicides inside the leaf thus giving misleading results. Consequently, little information is available regarding the penetration of bentazone relative to that of other herbicides since no recovery technique will be equally effective for all chemicals.

Stomata have been implicated as potential sites of preferential

uptake for herbicides in C. album (Currier, et al, 1964) and it has been proposed that the large wax-free ante-chamber surrounding the pore may facilitate bentazone entry (Taylor, et al, 1980). The 100% increase in ^{14}C-bentazone penetration in the first 3 days after application to leaves with open rather than closed stomata (Fig. 9a) may provide supporting evidence for this hypothesis. In addition, results obtained after treatment at 1830 h when mean LDR was 2.1 s.cm.$^{-1}$ showed penetration was intermediate between that found after 0930 h and 2030 h applications (data not shown). However, it is equally possible that the different environmental conditions during the first 8 hours after treatment may have influenced uptake and subsequent plant responses.

After incorporation of Actipron the amount of ^{14}C-bentazone found in the treated leaves was the same regardless of application time (Fig. 9d), although more total absorption was observed at 2030 h (Fig. 9b). Indeed, whilst penetration at 2030 h, when stomata were closed, was substantially improved by the incorporation of this oil, little effect was observed after application at 0930 h. Entry through the pore is less likely to occur when stomata are closed (Greene and Bukovac, 1974), and the reduced surface tension afforded by Actipron would probably have improved penetration when stomata were open (Schönherr and Bukovac, 1972) if the pore was of importance in the uptake of bentazone by C. album. It is therefore suggested that greater bentazone uptake by leaves with open stomata may have resulted from entry into the guard cells directly either via ectodesmata or across the wax-free cuticle of the more pronounced ante-chamber.

The enhanced uptake at 2030 h in the presence of Actipron suggests that the adjuvant acts by easing entry across the cuticle rather than through the pore. Oil additives have often been shown to improve absorption of bentazone (Nalewaja and Adamczewski, 1977) and other herbicides (e.g. Prendeville and Warren, 1975), and it has been proposed that the enhancement may result from better wetting of the leaf surface and increased lipophilicity of the herbicide-oil complex (Kirkwood, 1978). Such a theory,

however, does not explain the better penetration of ^{14}C-bentazone into C. album in the presence of Actipron at 2030 h. than at 0930 h. Changes in the leaf contour associated with stomatal movement may have contributed to this anomaly (Holly, 1976), but it is more probable that the reduced night temperature commencing 30 minutes after treatment combined with the humectant effect of Actipron may have sufficiently retarded drying of the herbicide solution to produce the observed improvement in uptake.

The data in Table 1, indicating that more ^{14}C-bentazone is translocated by C. album after treatment at 2030 h. than at 0930 h irrespective of Actipron, may provide supporting evidence that the route of penetration is dependent on the degree of stomatal opening during application. However, translocation was determined as the amount of label not recovered in either the treated leaf or washing solution and is therefore only assumed. Indeed, in one experiment where plants were sectioned and analysed for ^{14}C-bentazone, as described in this Chapter for treated leaves, only 91% of the total applied dose could be recovered after 7 days and less than 1% was found to be translocated (data not shown). This study was unrepresentative as light intensity was only 25 μE. $m^{-2}.s.^{-1}$ throughout, but it suggests that significant losses of radiolabel may be inherent in the experimental technique. Further examination of ^{14}C-bentazone translocation in C. album was not possible due to limitations on the supply of labelled herbicide.

The microautoradiographical studies (Plate 3) show that ^{14}C-bentazone was apparently taken up by all C. album epidermal cells and was not removed by the washing procedure indicating that the herbicide may have been bound to the tissue. The failure to detect any preferential binding sites for bentazone may provide evidence against stomatal involvement in its uptake although the method used here was probably inappropriate for penetration studies. Isolated abaxial epidermal peels were floated cuticle upwards on ^{14}C-bentazone solution creating rather artificial conditions for comparison with the agricultural context of spraying herbicide onto the adaxial leaf surface. Furthermore, the use of 15 μC; ^{14}C-bentazone, although less than that employed in L-(U ^{14}C)-malate studies in Commelina communis (Willmer and

Rutter, 1977), may have been excessive since permeability of the epidermal cells may be different for these two compounds. Malate is generally considered to be synthesised within the guard cells (Willmer, 1981) and is thus unlikely to be imported by these cells in large quantities. Hence, high C. album epidermal and guard cell permeability to bentazone may account for the inconclusive results obtained in the present study.

To summarise, bentazone penetration into intact C. album leaves was found to be a very slow process which occurred more rapidly after application at 0930 h than at 2030 h. With the addition of Actipron to the herbicide solution treated leaves absorbed similar quantities of ^{14}C-bentazone regardless of application time whilst translocation was apparently greater at 2030 h irrespective of the presence of this oil adjuvant. No direct evidence was obtained for involvement of the stomatal pore in uptake although it is suggested that the major route of bentazone penetration into this species may depend on the degree of stomatal opening during application and the surface tension of the herbicide solution.

CHAPTER 4

The effect of bentazone on stomatal movement in Chenopodium album epidermal peels

4.1 Introduction

Observations of *C. album* stomatal apertures in isolated epidermal peels have shown that under the conditions studied, bentazone invokes a rapid, light-dependent stomatal opening (Taylor, Cobb and Davies, 1980). However, all studies with epidermal peels should be treated with caution as detachment from adjacent mesophyll cells produces an artificial experimental system. Measurements taken immediately after peeling from the leaf often give false readings due to altered guard cell turgor relations caused by epidermal cell damage (Edwards and Meidner, 1979). Pre-incubation of peels in a suitable medium is therefore necessary before measurement of stomatal apertures. Care must also be taken in choice of bathing and mounting media for similar reasons. For example, the use of "Good"-buffers (Good, *et al*, 1966) in the bathing medium, as in the studies of Taylor, *et al*, (1980), is open to some criticism. These are imperfectly buffered against carbon dioxide and may affect guard cell metabolism if used over prolonged periods (Meidner, 1981).

However, epidermal peels have proved useful in elucidating many aspects of stomatal physiology and are considered an essential complement to such techniques as porometry (Meidner, 1981). For example, it is now well established that epidermal peel stomata can be induced to open in response to low intensity blue light or reduced intercellular carbon dioxide concentration, and that these responses are independent of each other (Mansfield, Travis and Jarvis 1981). Similarly, in many species potassium has been implicated as the major turgor-inducing cation accumulated during stomatal opening, (e.g. Willmer and Pallas 1973; Dayanandan and Kaufmann, 1975). Indeed, in *Vicia faba* it has been shown that when K^+ is actively taken up during stomatal opening H^+ ions are expelled into the solution with a consequent drop in pH

(Raschke and Humble, 1973).

Stomata can therefore be made to open in the presence of the above stimuli or close in their absence. The aim of this study is to investigate the effect of these stimuli on C. album epidermal peel stomata by following changes in stomatal aperture and bathing solution pH, in the presence or absence of bentazone.

4.2 Materials and methods

4.2.1. Isolation of Chenopodium album epidermal peels: For each experiment one healthy, fully expanded leaf of C. album, grown as described in section 2.2.1, was excised with a scalpel from the stem and placed in either daylight or darkness for 30 minutes with its petiole in distilled water. Up to 4 cm^2 of abaxial epidermal peels were then obtained with ease, using forceps, and floated cuticle upwards on 25 cm^3 of 10^{-4}M $CaCl_2$ in a petri dish and all visible mesophyll cell contamination removed. The peels were cut to a uniform size of approximately 0.25 cm^2, transferred to a further petri dish containing 25 cm^3 of the required pre-treatment solution, and incubated for 1 hour to cause stomatal opening or closure as required.

4.2.2 Measurement of epidermal peel area: After one hour of pre-treatment half the peels were removed for use in aperture studies, and the petri-dish containing the remaining peels was placed on a glass platform for area measurement. A lid covered with thin white paper, was placed on the dish and the peel images were projected onto the paper by illumination from below. This silhouette was traced, the shapes cut out and weighed on a chemical balance. Peel area was estimated by comparing the weight with that of a known area of paper. It is considered that this method may have given a slight overestimate of area, due to the small distance between peel and paper. However, this distance was constant throughout all measurements.

4.2.3 Control of experimental conditions: To gain a direct comparison with in vivo studies (Chapter 2), it was considered necessary that the effect of bentazone on C. album stomatal aperture and bathing solution pH change should be studied when

stomata were either open, closed, opening or closing. This was achieved by manipulation of conditions, during both the 1 hour pre-treatment and the 2 hour observation period.

Pre-treatment conditions:

(1) Stomatal opening. Clean epidermal peels were incubated on 25 cm^3 10^{-1}M KCl containing 10^{-4}M $CaCl_2$, in petri-dishes, under the conditions described in Figure 10 a, for 1 hour, Laboratory temperatures were $25 \pm 2^{o}C$, the atmosphere above the peels was maintained at 50 ppm CO_2 and illumination was with white light at a flux density of 50 $\mu E. m.^{-2}s.^{-1}$ (P.A.R.) supplied by an adjustable intensity angle-poise lamp.

(2) Stomatal closure. Incubation was, as shown in Figure 10 b, similar to that for stomatal opening, except the petri - dishes were kept in darkness. Air of approximately 500 ppm CO_2 was supplied and KCl was absent from the bathing medium. CO_2 concentration was accurately measured using an infra-red gas analysis circuit (described in detail in Chapter 5).

Treatment Conditions: During the 2 hour treatment period environmental conditions were either:-

(1) The same as pre-treatment so that changes could be monitored when stomata were induced to stay open or closed.

or:

(2) Reversed, so that measurements could be made when closed stomata were opening or when open stomata were closing.

Thus, the influence of the following bathing media was observed on stomata that were either open, closed, opening or closing:

(1) Control:

10^{-4}M Ca Cl_2

(2) With potassium:

10^{-4}M Ca Cl_2

10^{-1}M KCl

(3) With bentazone:

10^{-4}M Ca Cl_2

10^{-4}M Bentazone (95-99% technical grade).

(4) With bentazone and potassium:

10^{-4}M Ca Cl_2

FIGURE 10

Epidermal peel pre-treatment conditions

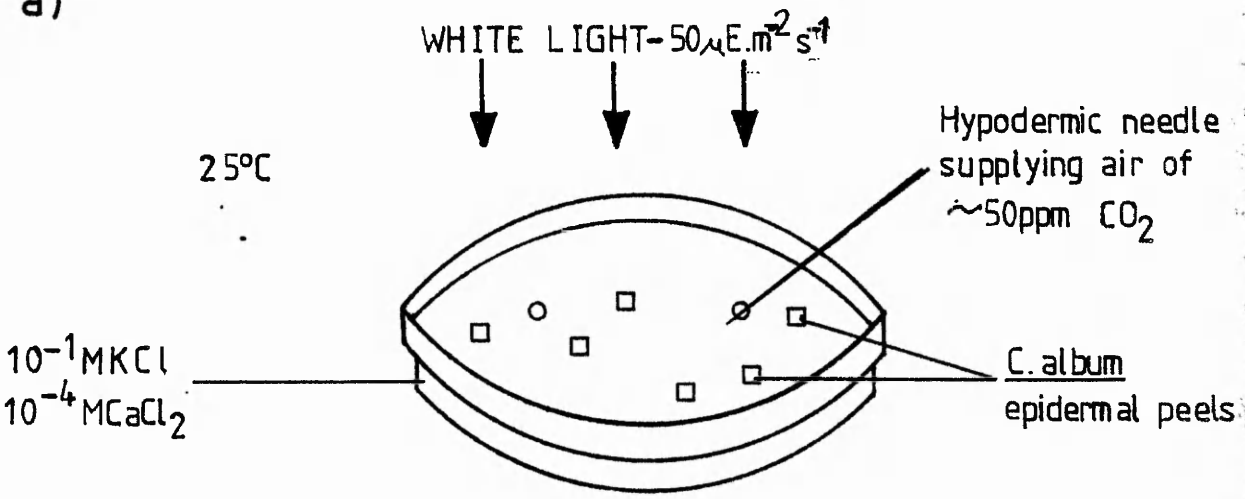
a) Opening conditions:

i.e. light, low $[CO_2]$, + KCl.

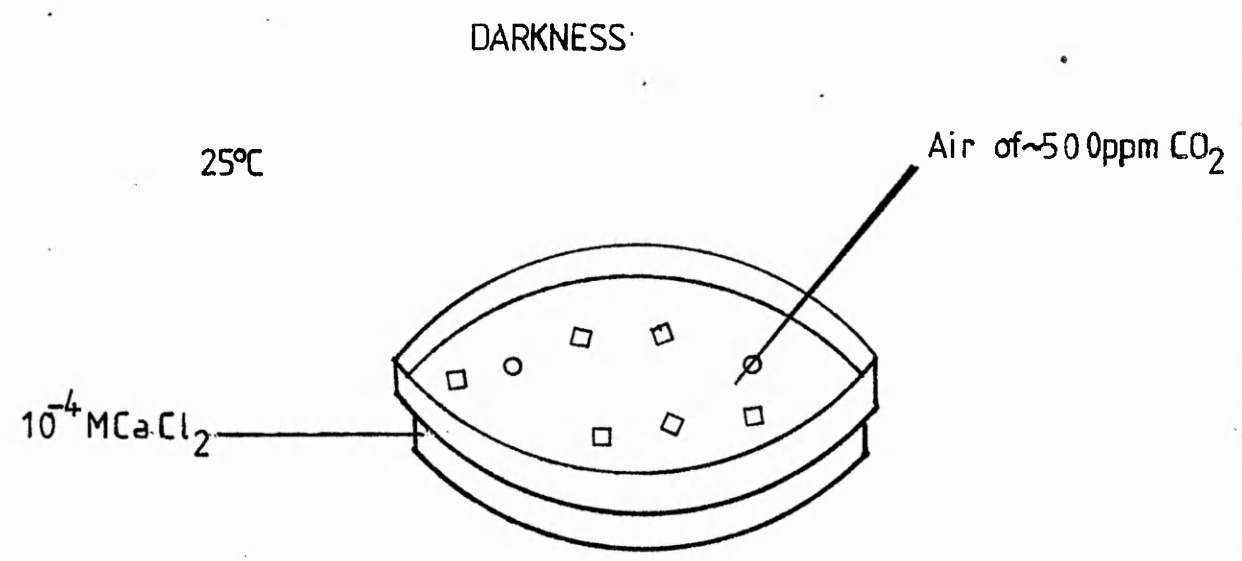
b) Closing conditions:

i.e. darkness, high $[CO_2]$, no KCl.

a)


WHITE LIGHT - 50 μE.m⁻²s⁻¹
25°C
Hypodermic needle supplying air of ~50ppm CO2
10⁻¹MKCl
10⁻⁴MCaCl2
C. album epidermal peels

b)


DARKNESS
25°C
Air of~500ppm CO2
10⁻⁴MCaCl2

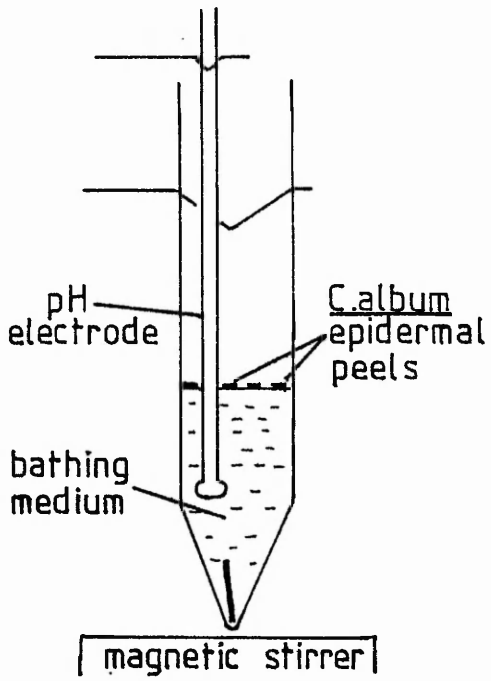
10^{-4}M Bentazone

10^{-1}M KCl

Bentazone was prepared as a 10^{-3}M stock solution in 5% (v/v) aq. ethanol, adjusted to pH 6.5 with a minimum quantity of 0.01M KOH and diluted as necessary.

Observations of pH and stomatal aperture change were made on 3 separate occasions for each of the 16 possible treatments.

4.2.4 Mesurement of pH change



After area measurement epidermal peels were floated on 3 cm^3 of the relevant bathing medium in a 10 cm^3 glass tapered centrifuge tube supported on a magnetic stirrer as shown im the diagram. Into the tube was placed a magnetic flea and an Activion miniature pH combination electrode. The environmental conditions were as described above and the pH allowed to equilibrate for 15 minutes. The solution was gently stirred and pH monitored every 15 minutes for 2 hours.

4.2.5 Measurement of stomatal aperture associated with pH change: A similar tube to that used for pH measurements was prepared alongside containing the remaining epidermal peels but no pH electrode. From this tube a peel was removed at times 0, 30, 60, 90 and 120 minutes, mounted in the bathing solution on a microscope slide, and the apertures of 15 stomata measured. Measurements were of the distance between the epicuticular lips (ECLD), as described by Taylor (1979), using the oil immersion lens of a Vickers light microscope fitted with a calibrated eyepiece micrometer. Each treatment was repeated on 3 separate occasions.

Staining with neutral red dye after 3 hour incubations showed that $>$ 95% of the guard cells remained healthy after treatment.

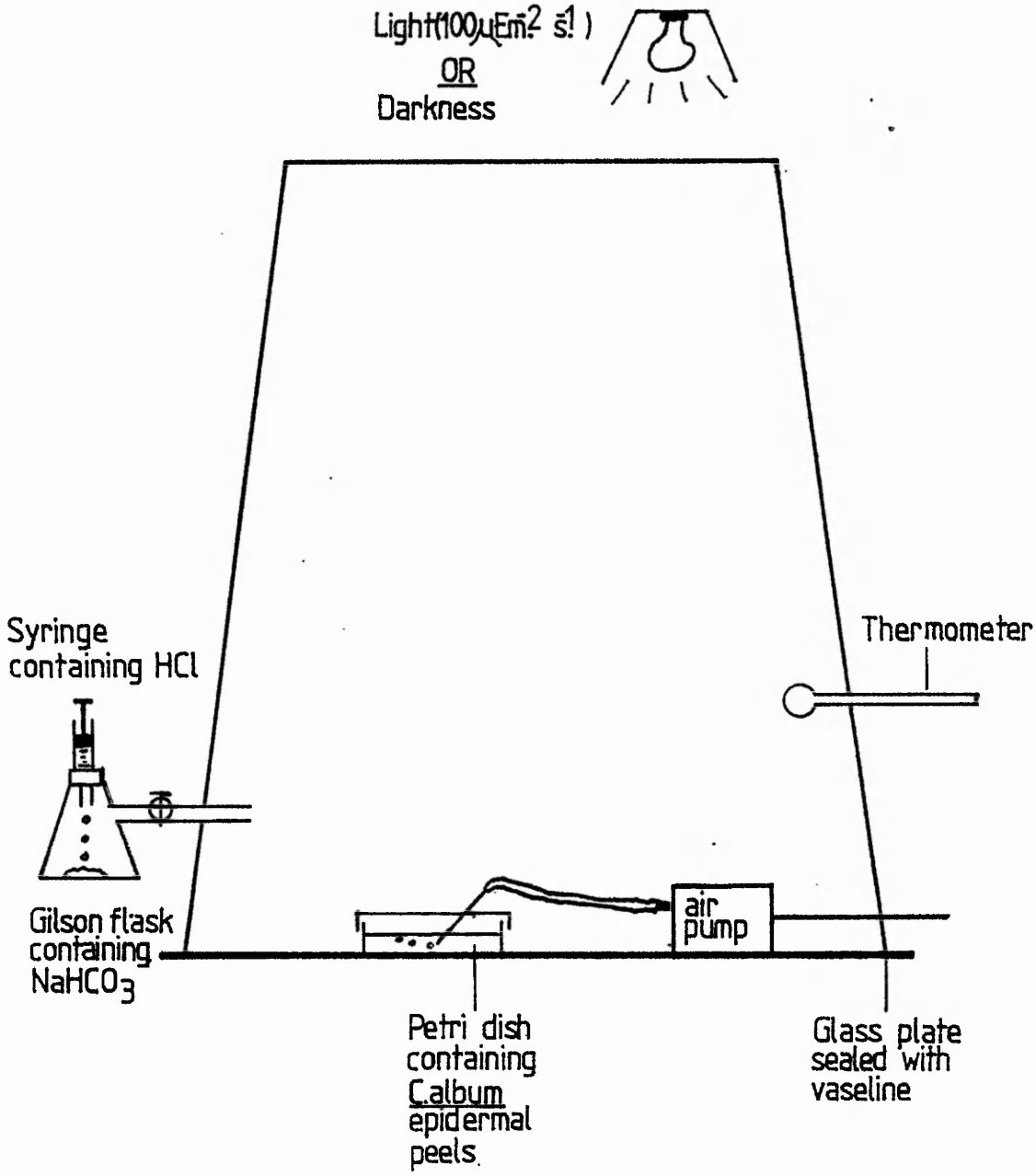
Observations on epidermal cells were obscured by large deposits of epicuticular wax.

4.2.6 The influence of bentazone on open or closed C. album stomata at different KCl concentrations: C. album epidermal peels were incubated to cause stomatal opening or closure (see section 4.2.3). After 1 hour the peels were removed and floated on 10^{-4}M Ca Cl_2 for 1 minute to rinse off excess KCl. Two peels were selected for aperture measurements and the rest transferred to 25 cm^3 of the relevant treatment solution for 2 hours in the same environmental conditions as during pretreatment. All treatment solutions contained 10^{-4}M Ca Cl_2 with bentazone at 0, 10^{-5} or 10^{-4}M and 0, 5, 10, 25, 50 or 100 x 10^{-3}M KCl at pH 6.5. Two further peels were removed for stomatal aperture measurement at the end of the treatment period. ECLD of 20 stomata from each peel were measured by taking a transect across the central area of the tissue ensuring that regions with any visible mesophyll contamination were avoided. The change in ECLD over 2 hours (ΔECLD) was then calculated for each treatment.

4.2.7 The influence of bentazone on CO_2 induced stomatal movement in C. album in light or darkness: C. album epidermal peels were prepared and pretreated for 1 hour to cause stomatal closure as in section 4.2.3. Two peels were removed and ECLD measured whilst the rest were transferred to a petri-dish containing 25 cm^3 of 10^{-4}M Ca Cl_2 with either 5 or 100 x 10^{-3}M KCl at pH 6.5. The dish was placed in a sealed chamber (volume 14 dm^3) into which CO_2 was introduced by evolution from $NaHCO_3$ in the presence of HCl to give experimental concentrations of 50, 500, 1000, 2500 or 5000 p.p.m. CO_2 as shown in figure 11. For the purpose of this study ambient CO_2 concentration was assumed to be 500 p.p.m. Thus, to produce an effective CO_2 concentration of, for example, 2500 p.p.m. 1M HCl was added drop-wise to sufficient $NaHCO_3$ to raise the ambient concentration by 2000 p.p.m. (i.e. 0.105g $NaHCO_3$) until no more gas was evolved. 50 p.p.m. CO_2 was achieved by passing air inside the isolated chamber through a soda-lime tower before bubbling into the bathing medium. A photon flux density of 100 μE. $m.^{-2}$ $s.^{-1}$ (P.A.R.) white light was provided and air within the chamber was bubbled through the bathing solution

FIGURE 11

Apparatus for the control of CO_2 concentration in C.album epidermal peel studies


Light(100μEm-2 s-1)
OR
Darkness
Syringe containing HCl
Thermometer
Gilson flask containing $NaHCO_3$
air pump
Petri dish containing C.album epidermal peels
Glass plate sealed with vaseline

for two hours after which ECLD of 20 stomata on each of two further peels were measured. △ ECLD was determined in the presence or absence of 10^{-4}M bentazone and the whole procedure repeated in darkness.

4.2.8 The influence of bentazone on ATP and ADP-induced stomatal movement in C. album: C. album epidermal peels, prepared and treated to cause stomatal closure as in section 4.2.3, were transferred to solutions containing 10^{-4}M $CaCl_2$ plus 5 or 100 x 10^{-3}M KCl and 10^{-3}M of either ATP or ADP at pH 6.5, and floated for 2 hours in the same environmental conditions. △ ECLD was determined as described, in the presence or absence of 10^{-4}M bentazone.

4.2.9 Histochemical staining of potassium in Chenopodium album guard cells: C. album epidermal peels, microscopically observed to have mainly open stomata, were floated on freshly prepared 40% (w/v) sodium cobaltinitrite in dilute acetic acid for 30 minutes at 0°C. Treated tissue was transferred to distilled water for 1 minute, to rinse off excess stain, and then to fresh distilled water for another minute. Next the peels were submerged in chloroform for 5 minutes to remove surface wax which interferes with microscopic observation of the stain (Dayanandan and Kaufmann, 1975). After a further 1 minute rinse in distilled water the peels were mounted on 10% ammonium sulphide in 5% (v/v) aqueous glycerine to precipitate black cobalt sulphide in areas of potassium deposits as $Na_3[Co(NO_2)_6] + 3K^+ \xrightarrow{\text{dilute acetic acid}} K_3[Co(NO_2)_6]\downarrow + 3Na^+$

$$Na_3[Co(NO_2)_6] + 3K^+ \xrightarrow{\text{dilute acetic acid}} K_3[Co(NO_2)_6]\downarrow + 3Na^+$$

$$K_3[Co(NO_2)_6] + 3(NH_4)_2S \longrightarrow CoS_3 + 3NH_4NO_2 + 3KNO_2$$

N.B. Precipitation of cobaltic sulphide will only occur in the alkaline conditions induced by ammonium sulphide.

Photomicrographs, of the prepared slides, were obtained using a Leitz Dialux E.B. microscope fitted with Ilford PANF black and white film.

4.3 Results

4.3.1 Changes in C. album ECLD under different environmental conditions in the presence or absence of bentazone: Table 2 shows the changes in pH cm^{-2} of epidermal peel which occurred in association with control experiments (i.e. no bentazone) causing stomata to be open, closed, opening or closing. The data shows no obvious relationship between change in C. album stomatal aperture and bathing solution pH. Of the 7 occasions where a fall in aperture was observed 4 were associated with a pH rise and 3 with a fall. Five treatments gave a rise in aperture and, of these, pH rose in all but one. Results in the presence of bentazone are not shown because of the lack of correlation in the control data for comparison.

Pretreatment of C. album epidermal peels in the closed conditions described gave stomata of mean ECLD between 6.1-7.3 µm. with open values between 9.7-11.1 µm. Figure 12 shows the changes in ECLD over 2 hours obtained with different chemical and environmental treatments. Closed stomata were induced to open in the presence of light at reduced CO_2 concentrations if 100 x 10^{-3}M KCl was present in the bathing medium (Fig. 12 b). Furthermore, this concentration of KCl produced an opening response even when conditions favoured remaining closed (Fig 12 a), and maintained stomata open regardless of environmental conditions (Fig. 12 c and d). When only $CaCl_2$ was available (i.e. control) closed stomata could not be induced to open (Fig. 12 a), whilst open stomata closed rapidly when conditions were conducive (Fig. 12 d).

In the absence of KCl, bentazone produced a rapid opening of closed stomata of similar magnitude in either light or darkness (Fig. 12 a and b). Indeed, in darkness the change in ECLD was appreciably greater than that induced by 100 x 10^{-3}M KCl, whilst in opening conditions it was only slightly less. However, the effect of bentazone alone on open stomata was to cause considerable reduction in aperture when conditions favoured remaining open (Fig. 12 c), and the herbicide did not maintain stomata open in closing conditions (Fig. 12 d).

When both KCl and bentazone were present, opening of closed stomata was not induced by any environmental treatment (Fig. 12 a and b).

TABLE 2

Changes in bathing medium pH(ΔpHcm.$^{-2}$epidermis) associated with stomatal movement in C.album epidermal peels

Changes, over 2 hours, of C.album mean ECLD, and bathing solution pHcm.$^{-2}$. Peels were pretreated for 1hour to cause stomatal opening or closure and measured for 2hours under the conditions described in section 4.2.3.

Treatment Conditions	Replicate	Tissue Area(cm.^{2})	Δ pHcm.$^{-2}$	ΔECLD (μm)
OPEN	1	0·47	+0·02	+1·64
	2	0·80	−0·19	+0·35
	3	0·66	−0·46	−0·51
CLOSED	1	1·36	+0·18	−1·64
	2	0·55	+0·15	−1·10
	3	0·87	−0·03	−1·10
OPENING	1	0·98	+0·21	+1·57
	2	0·51	+0·12	+3·92
	3	0·87	+0·24	+2·55
CLOSING	1	1·64	−0·01	−2·12
	2	0·55	+0·24	−1·85
	3	1·07	+0·03	−3·30

FIGURE 12

Changes in C.album ECLD under different conditions in the presence or absence of bentazone

Epidermal peels were pretreated in either opening or closing conditions. Each point is the mean of 15 stomatal apertures from 3 different peels measured at intervals up to 2 hours afterwards under the conditions described in 4.2.3. Bars indicate standard error of the mean (n=45). Open squares, 10^{-4} M $CaCl_2$, pH6·5; closed squares, 10^{-4} M $CaCl_2$ + 10^{-4} M bentazone, pH6·5; open circles, 10^{-4} M $CaCl_2$ + 10^{-4} M KCl, pH6·5; closed circles, 10^{-4} M $CaCl_2$ + 10^{-1} M KCl + 10^{-4} M bentazone.

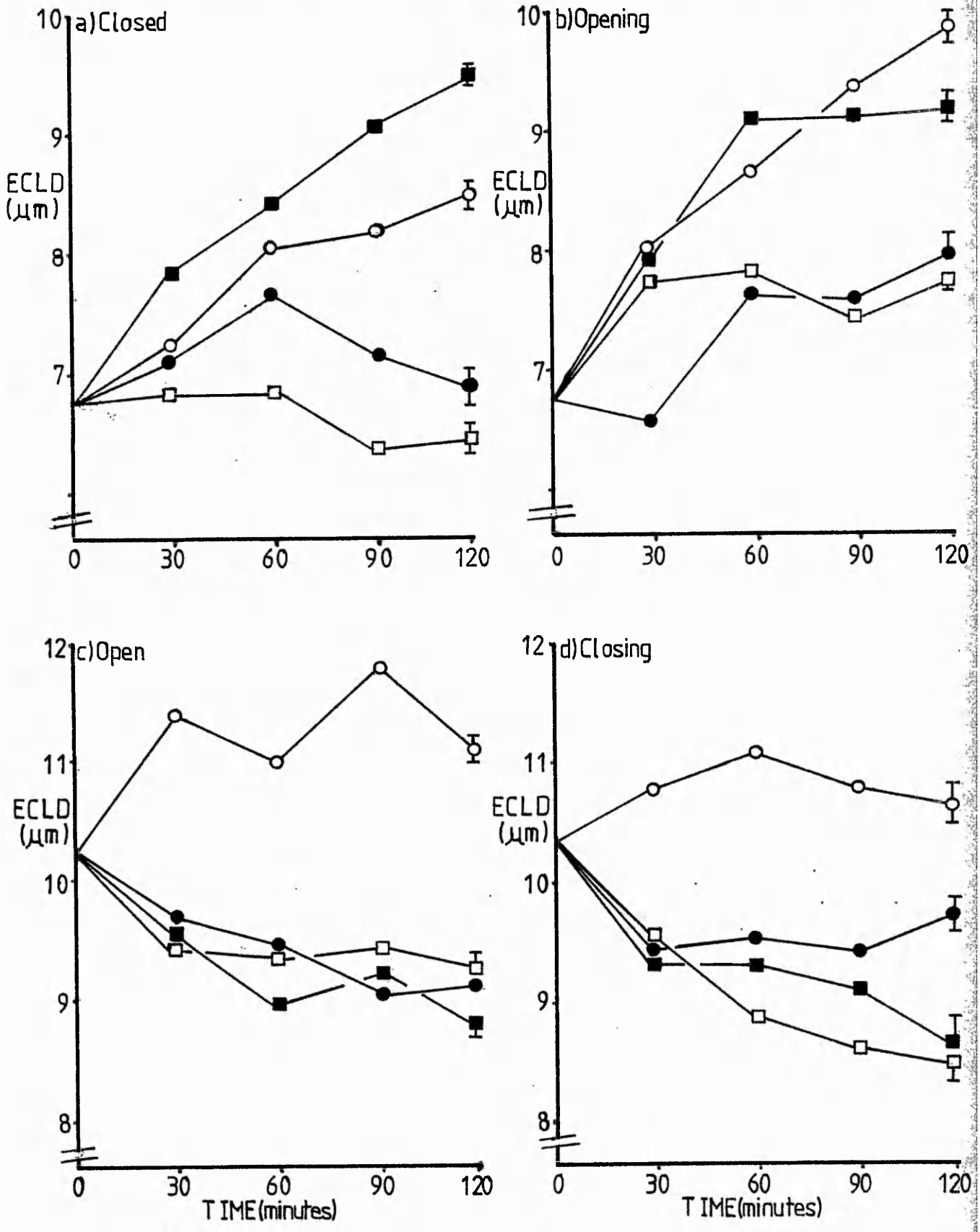

a) Closed
b) Opening
c) Open
d) Closing
ECLD (µm)
10
9
8
7
12
11
0
30
60
90
120
TIME (minutes)

FIGURE 13

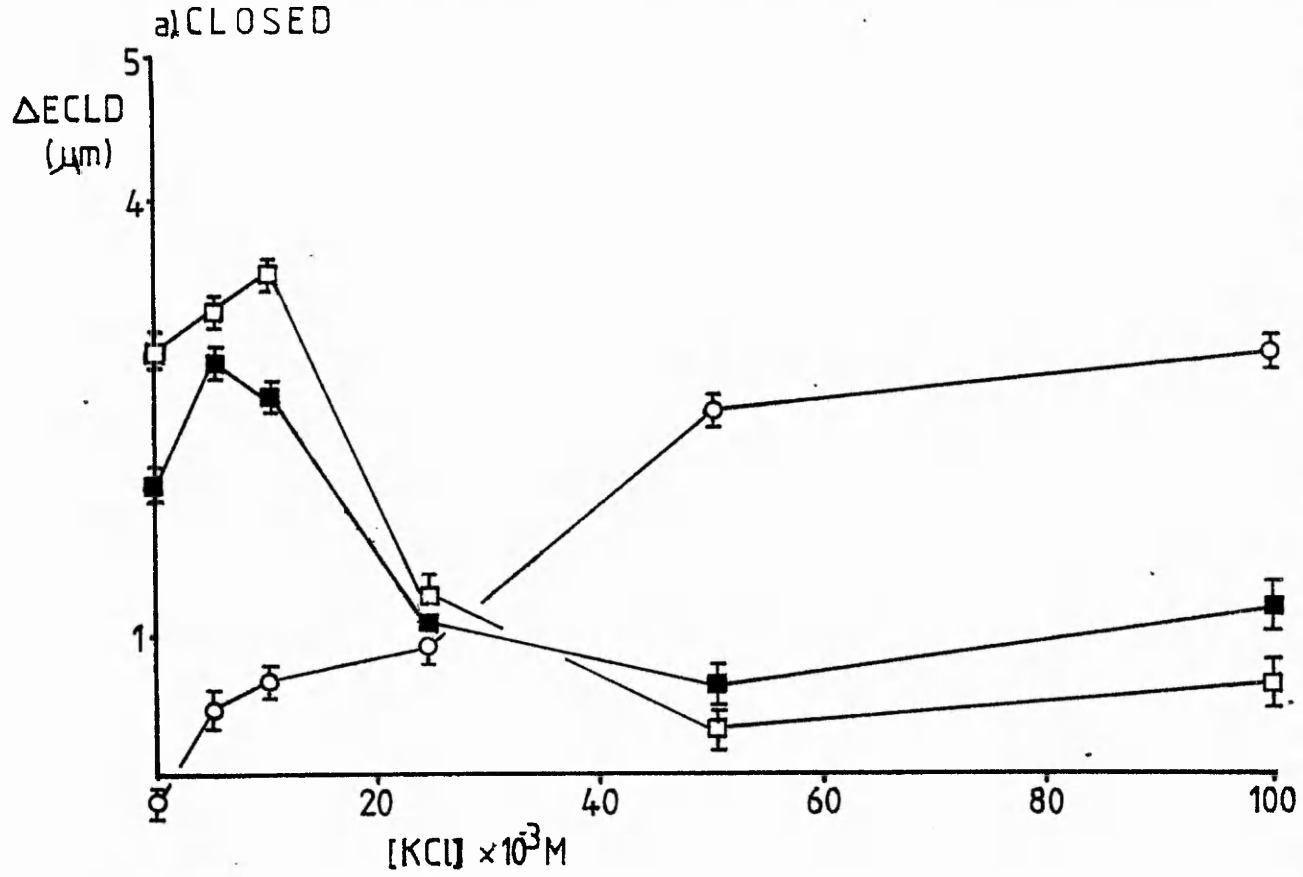
The influence of bentazone on C.album stomatal aperture at different KCl concentration

The change in ECLD during 2 hours incubation of C.album epidermal peels on solutions of different KCl concentrations in the presence or absence of bentazone after pretreatment to cause stomatal opening or closure. Each point is the mean of measurements on 2 epidermal peels taken before and after treatment in the same conditions.

a)Closing conditions, i.e.darkness + 500ppm CO_2.

b)Opening conditions, i.e.light + 50ppm CO_2.

Bars representing standard error of the mean(n=40) are shown where they exceed the symbol size. Open circles, 10^{-4}M $CaCl_2$, pH6·5; closed squares, 10^{-4}M$CaCl_2$+ 10^{-5}M bentazone, pH6·5; open squares, 10^{-4}M$CaCl_2$+ 10^{-4}M bentazone, pH6·5.


a) CLOSED
ΔECLD
(μm)
5
4
1
20
40
60
80
100
[KCl] ×10⁻³M

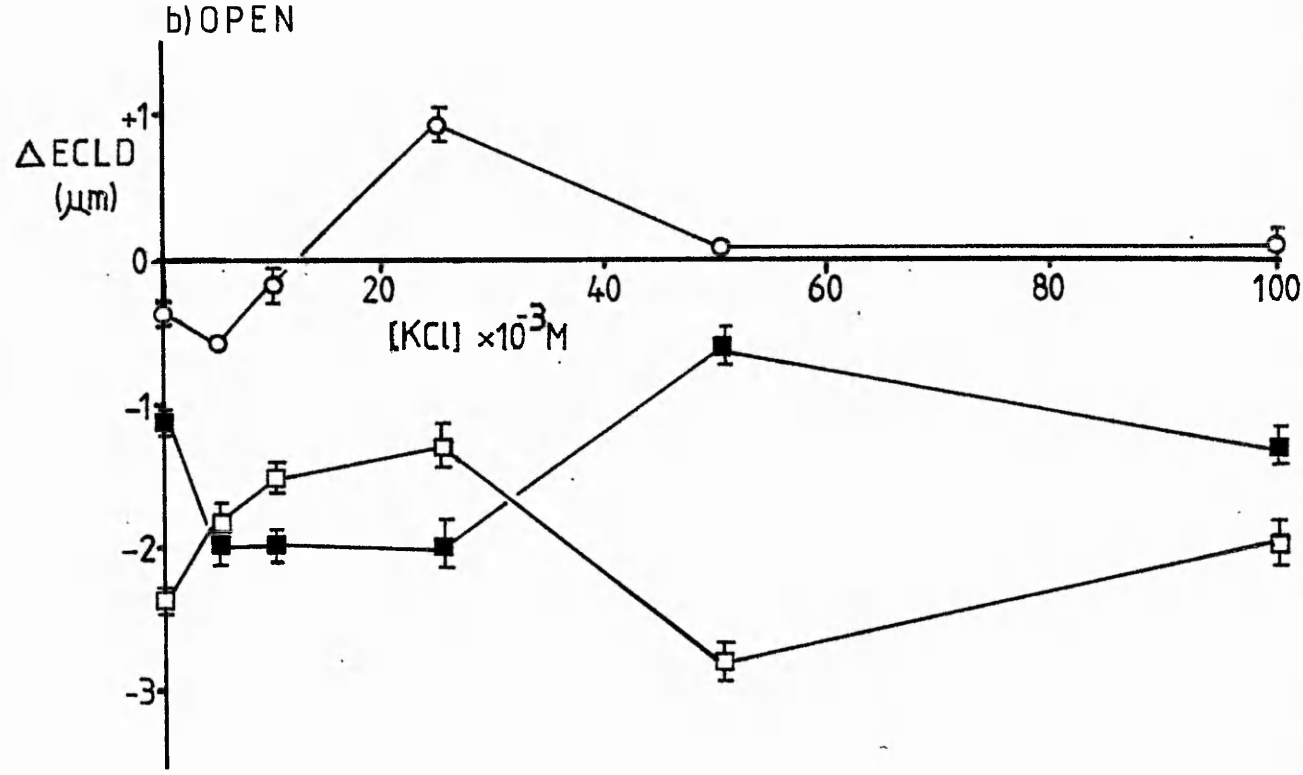

b) OPEN
ΔECLD
(μm)
+1
0
-1
-2
-3
20
40
60
80
100
[KCl] ×10⁻³M

FIGURE 14

The influence of CO_2 on C.album stomatal movement in the presence or absence of bentazone

The changes in ECLD of C.album epidermal peel stomata during 2 hours at different CO_2 concentration in light or darkness and the presence or absence of 10^{-4}M bentazone. All peels were pretreated for 1 hour to cause stomatal closure.

a) White light, 5×10^{-3}MKCl, 10^{-4}M$CaCl_2$, pH6·5.

b) White light, 100×10^{-3}MKCl, 10^{-4}M$CaCl_2$, pH6·5.

c) Darkness, 5×10^{-3}MKCl, 10^{-4}M$CaCl_2$, pH6·5.

d) Darkness, 100×10^{-3}MKCl, 10^{-4}M$CaCl_2$, pH6·5.

Open symbols, no bentazone; closed symbols, with bentazone. Bars represent standard error of the mean(n=40).

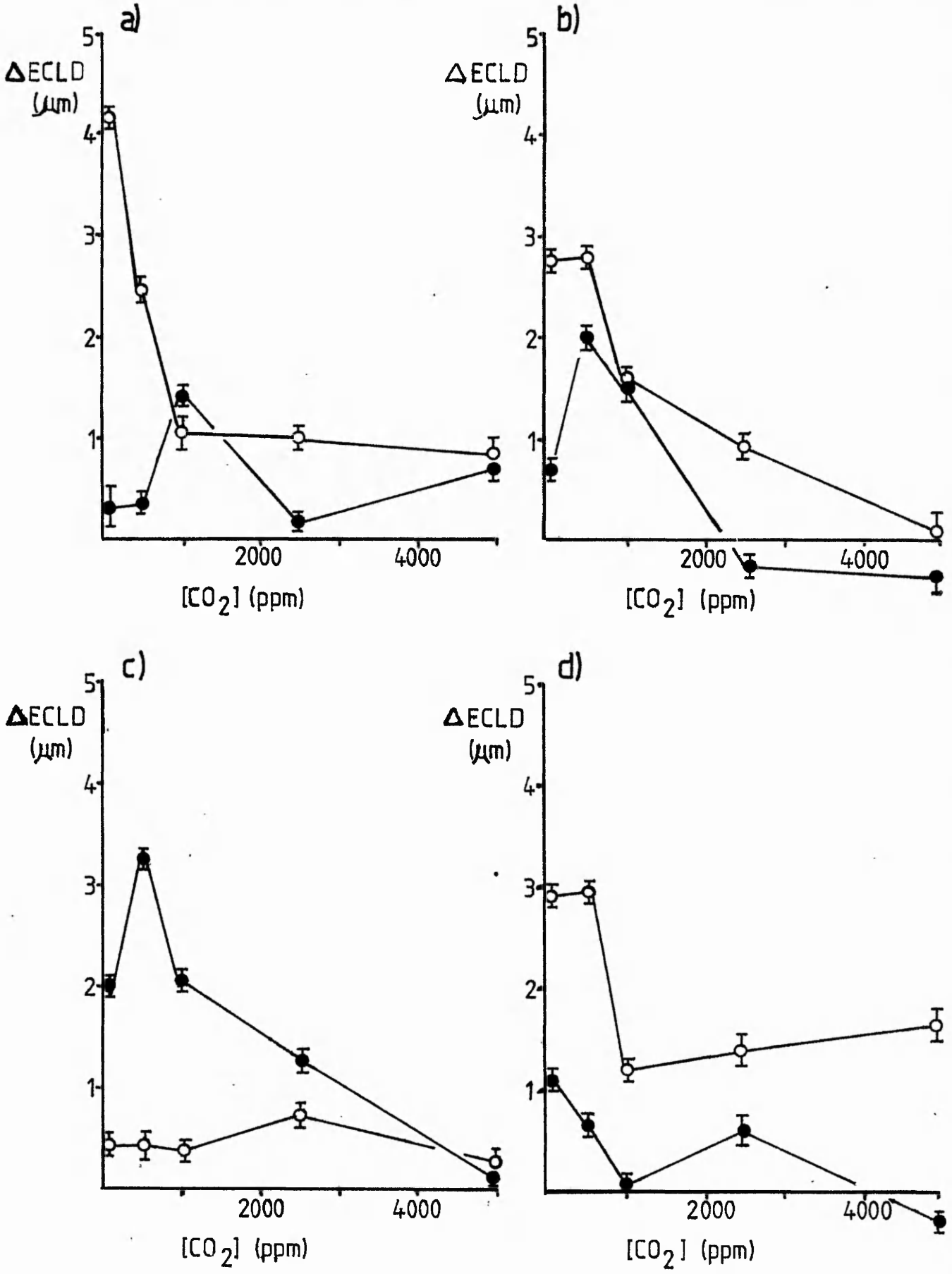

a)
b)
c)
d)
ΔECLD (μm)
[CO2] (ppm)
1
2
3
4
5
2000
4000

FIGURE 15

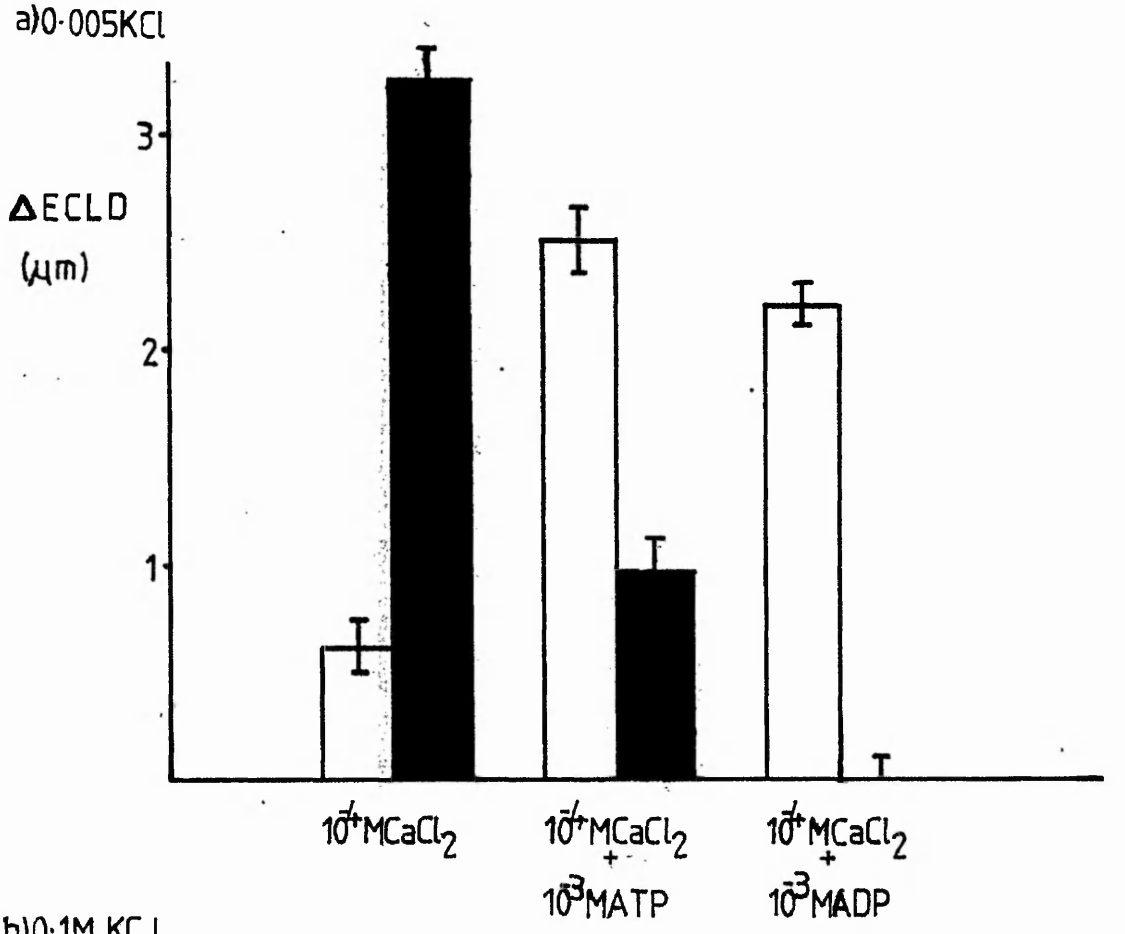
The influence of bentazone on ATP- and ADP-induced stomatal movement in C.album epidermal peels

The change in ECLD during 2 hours in darkness and 500ppm CO_2 as influenced by ADP or ATP in the presence or absence of bentazone. All peels were pretreated for 1 hour to cause stomatal closure.

a) 5×10^{-3} MKCl

b) 100×10^{-3} MKCl

Open histogram area, control; closed histogram area, 10^{-4} M bentazone. Bars represent standard error of the mean (n=40).


a)0·005KCl
ΔECLD
(μm)
3
2
1
10^{-4}MCaCl$_2$
10^{-4}MCaCl$_2$ + 10^{-3}MATP
10^{-4}MCaCl$_2$ + 10^{-3}MADP

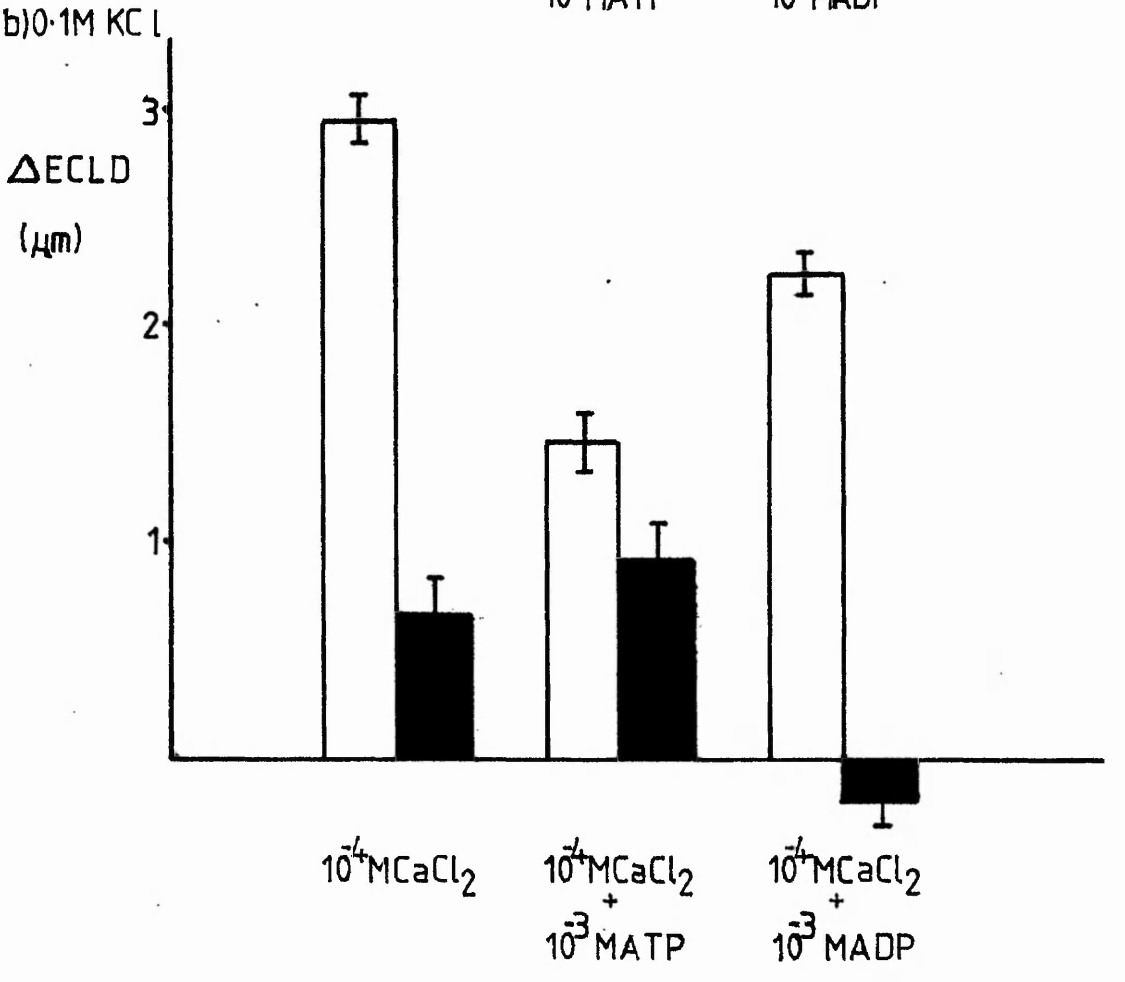
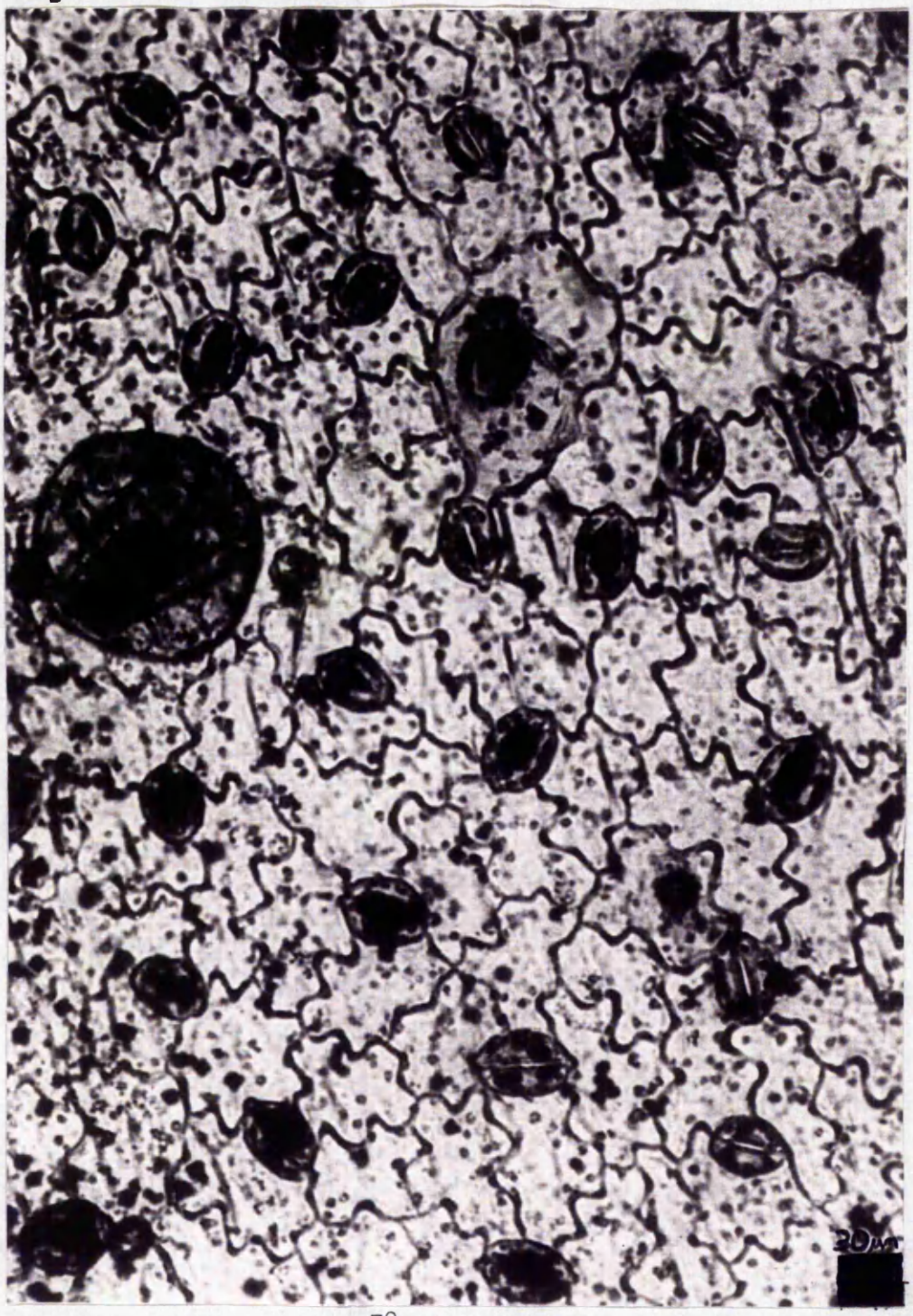

b)0·1M KCl
ΔECLD
(μm)
3
2
1
10^{-4}MCaCl$_2$
10^{-4}MCaCl$_2$ + 10^{-3}MATP
10^{-4}MCaCl$_2$ + 10^{-3}MADP

PLATE 3

Histochemical stain of C.album epidermal peel showing localisation of potassium by CoS precipitation

Dark areas represent regions of high potassium concentration. Stomata were open prior to staining. Magnification × 480.



In fact, ECLD values were similar to those obtained in the absence of either. With open stomata the combination caused considerable closure relative to KCl (Fig. 12 c and d) which was of the same order as that induced by bentazone alone. In all cases, the presence of KCl plus bentazone appeared to be antagonistic to the effect of KCl alone.

4.3.2 The influence of bentazone on open or closed epidermal peel stomata at different KCl concentrations: Figure 13 a shows the change in ECLD of closed C. album epidermal peel stomata during 2 hours in darkness at 500 p.p.m. CO_2 i.e. closing conditions maintained. Incorporation of KCl produced a gradual increase in ECLD at concentrations up to 25 x 10^{-3}M and invoked significant stomatal opening when present at 50 or 100 x 10^{-3}M. Moreover, inclusion of bentazone in this system resulted in an appreciable increase in ECLD even in the absence of KCl. Further stomatal opening occurred with the addition of KCl, reaching an optimum at 5 or 10 x 10^{-3}M dependent on the bentazone concentration, but this response was negated by subsequent increases in KCl concentration. For example, when the herbicide was present at 10^{-4}M, ΔECLD was only 0.7 µm compared with 3.0 µm for the control at 100 x 10^{-3}M KCl, whilst at 10 x 10^{-3}M KCl the corresponding changes were 3.5 and 0.6 µm respectively. Both the herbicide-induced opening of C. album stomata and its inhibition by higher [KCl] occurred at all bentazone concentrations studied (data for 10^{-6}M not shown), but became more pronounced at 10^{-4}M possibly suggesting direct interference with stomatal control of potassium movement. However, Limeweaver-Burke plots constructed from this data did not reveal any typical disruption of enzyme-type kinetics due to competitive or non-competitive binding.

When C. album epidermal peels, pretreated with 100 x 10^{-3}M KCl in light and reduced CO_2 concentration to initiate opening, were incubated for 2 hours in the same opening conditions (Fig. 13 b), little change in ECLD was observed at any [KCl]. However, the effect of bentazone was to cause partial stomatal closure in all cases, but with no distinctive trend regarding the concentration of either KCl or the herbicide.

4.3.3 The influence of CO_2 concentration on C. album stomatal opening in the presence or absence of bentazone and light: The changes in ECLD of closed C. album epidermal peel stomata at different CO_2 concentrations and 5 or 100 x 10^{-3}M KCl in either light or darkness are shown in figure 14. With the lower [KCl], light caused considerable stomatal opening at 50 p.p.m. CO_2 which was appreciably reduced by increasing CO_2 concentration (Fig. 14 a) whilst little or no opening occurred at any [CO_2] in darkness (Fig. 14 c). When 100 x 10^{-3}M KCl was present in the bathing solution, significant opening occurred at 50 and 500 p.p.m. CO_2 independently of light, although the inhibition of stomatal opening by high [CO_2] was not as complete in darkness (Fig. 14 b and d). C. album stomata were therefore shown to react to changes in ambient [CO_2] with the magnitude of the response being dependent on the presence of sufficient light and the KCl concentration of the surrounding medium.

At 5 x 10^{-3}M KCl, bentazone inhibited the light-induced increase in C. album ECLD at all CO_2 concentrations (Fig. 14 a) but in darkness invoked considerable stomatal opening which was optimal at 500 p.p.m. CO_2 but completely negated by 5000 p.p.m. CO_2 (Fig. 14 c). Furthermore, stomatal opening induced by 100 x 10^{-3}M KCl was significantly inhibited by bentazone in darkness at all CO_2 concentrations (Fig. 14 d) and at 50 p.p.m. CO_2 in light (Fig. 14 b). However, light-induced stomatal opening was observed at 100 x 10^{-3}M KCl in the presence of the herbicide. This response, which was of lower magnitude than that produced by 100 x 10^{-3}M KCl alone, was also inhibited by high CO_2 concentrations with an apparent optimum at 500 p.p.m. CO_2. Thus, the general effect of bentazone was to interact with C. album stomatal responses to CO_2 resulting in differential changes in ECLD which were influenced by both the presence or absence of light and the amount of KCl in the bathing medium.

4.3.4 The influence of bentazone on ATP and ADP induced stomatal movement in C. album epidermal peels: The histograms in figure 15 represent the response of closed C. album epidermal peel stomata to the inclusion of 10^{-3}M ATP or ADP in the bathing solutions, in the presence of 5 or 100 x 10^{-3}M KCl, when environmental conditions

favoured stomata remaining closed. The previously observed failure of stomata to open when only 5 x 10^{-3}M KCl was available was overcome by the addition of 10^{-3}M ATP or ADP, to produce increases in ECLD slightly less than those effected by bentazone under the same conditions (Fig. 15 a). However, the response to ATP was reduced by 62% and that to ADP completely negated when the herbicide was present. Thus, C. album stomatal opening induced by ADP or ATP at low KCl concentration was inhibited by bentazone.

When 100 x 10^{-3}M KCl was present the large increase in control ECLD was reduced by 33 and 52% in the presence of 10^{-3}M ADP and ATP respectively (Fig. 15 b). Furthermore, the observed inhibition of stomatal opening by bentazone under these conditions was relatively unaffected by the presence of ATP but was more pronounced with ADP in the bathing medium. Hence, whilst ATP was a more effective inhibitor of C. album dark stomatal opening induced by 100 x 10^{-3}M KCl only ADP totally prevented opening when used in combination with bentazone.

4.3.5 Histochemical staining of C. album epidermes to show areas of potassium localisation: Plate 3 is a typical photomicrograph of a C. album epidermal peel obtained after treatment to cause stomatal opening, and stained with sodium cobaltinitrite and ammonium sulphide to reveal black areas of cobaltic sulphide corresponding to localisation of potassium density in the tissue. Accumulation of this ion in the guard cells relative to the epidermal cells is clearly shown. Some of the hair-like "bladder cells" were also found to contain potassium but it was not possible to determine if these were intact.

4.4 Discussion

The experiments conducted in this study on C. album epidermal peels have provided considerable evidence that stomata of this species show characteristic reactions to the environmental stimuli of light and CO_2 if provided with a suitable supply of potassium ions. Darkness and 500 p.p.m. CO_2 were found to maintain stomatal closure if potassium was absent from the incubation medium (see Fig. 12) in a similar manner to that found in other species

(e.g. Travis and Mansfield, 1979 b). Furthermore, wide stomatal opening was observed in light at 50 p.p.m. CO_2 when only 5 x 10^{-3}M KCl was available (Fig. 14) and incorporation of 100 x 10^{-3}M KCl caused opening regardless of environmental conditions (Fig. 12), as observed by other workers with Vicia faba (Raschke and Humble, 1973). This ability of high KCl concentrations to override stomatal environmental responses is well documented and thought to result from enhancement of potassium uptake by guard cells or inhibition of its leakage to the surrounding cells or media (Mansfield, Travis and Jarvis, 1981). In the present study, however, pre-incubation in the absence of KCl under the closing conditions described is likely to yield tissue containing little or no potassium ions (Raschke, 1979). In such circumstances, inhibition of potassium leakage alone could not result in stomatal opening and thus the former hypothesis, i.e. enhancement of K^+ uptake, is considered more valid.

The observation that significant opening of closed C. album stomata in response to a range of KCl concentrations, occurred only above 25 x 10^{-3}M in closing conditions (Fig. 13 a) may provide supporting evidence for potassium stimulated opening. Indeed, under favourable conditions, only small amounts of KCl were required to maintain stomata open after pre-incubation on 100 x 10^{-3}M KCl, and little further opening could be induced by high potassium concentrations (Fig. 13 b), indicating a possible role for light and CO_2 as mediators of ion leakage or efflux.

Stomatal ion transport is commonly thought to be controlled by membrane-bound proton pumps with monovalent cations providing the electrical balance (Zeiger, Bloom and Hepler, 1978; Moody and Zeiger, 1978). However, Macrobbie (1981) has proposed that since all plant cells are capable of taking up ions to maintain turgor, the unusual property of guard cells is their ability to discharge metal ions causing loss of turgor and that stomatal aperture may be determined by the relative activities of the uptake and efflux processes. Furthermore, whilst both Na^+ and K^+ ions will cause stomatal opening in Commelina communis subsequent ABA-induced closure has been shown to occur only when KCl was the original osmoticum (Jarvis and Mansfield, 1980), providing strong evidence

for the existence of a potassium specific efflux mechanism in guard cells. It is therefore proposed that the opening response to high KCl concentration under adverse environmental conditions may be due to saturation of potential binding sites on the guard cell membrane causing a shift in the equilibrium of potassium transport in favour of uptake.

The response of closed C. album stomata to varied CO_2 concentrations in either light or darkness clearly shows that these two environmental parameters initiate ion movement independently (Fig. 14). In the absence of light CO_2-depletion only caused stomatal opening if excess KCl was available, whilst in its presence maximal opening occurred at 50 p.p.m. CO_2 with only 5×10^{-3}M KCl. Increased CO_2 concentration gradually inhibited this pronounced response inferring that light and CO_2 may be antagonistic in their effects on stomata. Similar independence of stomatal reactions to light and CO_2 has been demonstrated for other species (Mansfield, Travis and Jarvis, 1981), but the inhibition by high CO_2 concentrations of KCl-induced stomatal opening in vitro, as found in the present study, has not been previously reported. Other workers have shown that whole plant stomatal resistance is proportional to CO_2 concentration up to 4000 p.p.m. in most species investigated (Heath and Russell, 1954; Gaastra, 1959; Pallas, 1965), but studies of this type on epidermal peel stomata are scant and often limited to CO_2 concentrations close to ambient (e.g. Travis and Mansfield, 1979 a). However, inhibition of KCl-induced epidermal peel stomatal opening by treatment solutions containing more than 25 μm bicarbonate has been demonstrated for Commelina benghalensis (Mrinalini, Latha, Raghavendra and Das, 1982). Moreover, recent studies with protoplasts of Vicia faba have indicated that guard cell dark respiration is 175 μ mol. O_2 $mg.^{-1}$. Chl. $h.^{-1}$ compared with only 6 μ mol. O_2 $mg.^{-1}$. Chl. h^{-1}. for mesophyll protoplasts under the same conditions (Shimazaki, Gotow and Kondo, 1982). Indeed, guard cells have abundant mitochondria (Raschke, 1979), and, clearly, intracellular CO_2 concentration may rise appreciably, particularly in the absence of photosynthetic activity. Under such circumstances CO_2 may act by inhibiting the processes responsible for potassium uptake or

promoting its efflux with similar consequences.

It has been proposed that the most probable sites of CO_2 action to inhibit stomatal opening are either directly on the proton pumps which maintain high potassium gradients between the vacuole, cytoplasm and surrounding cells, or on the process including PEP carboxylation which produces malate to ionically balance incoming potassium ions (Jarvis and Morison, 1981). However, chloride uptake, sufficient to balance up to half the accumulated potassium, has been demonstrated for epidermal peel stomata of other species floated on KCl (Pallaghy and Fischer, 1974; Raschke and Schnabl, 1978) and thus inhibition by CO_2 of malate formation alone could not completely halt stomatal opening as found in the present study at 5000 p.p.m. (Fig. 14 b). The results of this investigation therefore suggest that the role of CO_2 in reducing stomatal opening may be due to direct action on the proton pumps which regulate potassium transport although other effects on guard cell metabolism cannot be excluded.

Incorporation of 10^{-3}M ATP or ADP resulted in appreciably enhanced opening of closed C. album epidermal peel stomata in the presence of 5 x 10^{-3}M KCl (Fig. 13 a). In contrast, stomatal opening induced by 100 x 10^{-3}M KCl was considerably reduced by ATP and partially inhibited by ADP (Fig. 13 b). However, information regarding the effect of nucleotides on stomatal movement is conflicting. Several studies have demonstrated stomatal opening in response to exogenous ADP or ATP (e.g. Fujino, 1967; Das and Raghavendra, 1974) whilst others have found no significant change (e.g. Fischer, 1967).

Disagreement about the effect of ATP may arise from the ability of this chemical to act as an ion chelator (Luttge, Schöch and Ball, 1974) since such a property may interfere with stomatal cation transport. Indeed, the very different influence on C. album stomata of exogenous ATP and ADP observed in the present study at either 5 or 100 x 10^{-3}M KCl may reflect activity of this kind. However, the bulk of evidence suggests that stomatal ion movements occur via active processes (e.g. Pallaghy and Fischer, 1974) and that the ATP required to drive the proton pumps considered

to be responsible for this transport may be derived from oxidative and/or photophosphorylation (Raghavendra, 1981). Thus, whilst the results of the present investigation of C. album may support the involvement of high energy nucleotides in dark stomatal opening of this species and show differential responses dependent on KCl availability which may indicate two distinct transport systems, they do not provide any further information about the mechanism of these processes.

The failure to correlate change in stomatal aperture with bathing solution pH (Table 2) as observed by Raschke and Humble (1973), may be purely a technical problem. It is not possible to obtain such a large area of epidermis from one leaf of C. album as that used by these authors with Vicia faba. This would not only reduce possible proton extrusion but also the ability of the peels to buffer against pH variation due to changing carbon dioxide concentration. However, large accumulations of potassium in open C. album stomata, shown in Plate 3, are clearly similar to those found in other species (Willmer and Pallas, 1973; Dayanandan and Kaufmann, 1975) and exchange with protons is not the only proposed mechanism of potassium uptake by guard cells. Potassium and chloride ions may enter simultaneously as in Zea mays (Raschke and Fellows, 1971), which would presumably give little or no pH change. Chloride is detected in the absence of reducing agents by precipitation with silver nitrate (Raschke and Fellows, 1971) but such investigations with C. album epidermes were inconclusive due to the white precipitate being obscured by large deposits of epicuticular wax in this species.

A further complication to pH studies may have been caused by the presence of "bladder cells" on the epidermis of C. album. These trichomes, typical of halophytes, have a remarkable affinity for salts of alkali metals (Osmond, et al, 1969). During salt uptake, H^+ ions are passively excreted into solutions surrounding leaf slices (Pallaghy and Luttge, 1970). However, the light-induced membrane potential responsible for the affinity is lost when the epidermis is peeled from the mesophyll (Luttge and

Pallaghy, 1969) due to rupture of the connecting stalk cell. "Bladder cells" may have influenced pH in the above experiments as some are shown to contain K^+ ions (Plate 3), but it is not possible to say which, if any, of these were functional.

When considering the influence of bentazone on C. album stomatal movements the prevailing environmental conditions are of vital importance since a major target of this herbicide is the inhibition of chloroplast electron transport (Pfister, et al, 1975; Suwanketnikom, et al, 1982). Guard cells of this species contain appreciable numbers of chloroplasts and hence may be susceptible via this mechanism. Consequently, several of the experiments detailed in this study have been conducted in both light and darkness to account for the contribution of photosynthetic electron transport, and its inhibition, to the observed responses. In addition, the majority of experiments were performed on C. album epidermal peels pretreated to cause stomatal closure and yield tissue virtually devoid of solutes (Raschke, 1979) thus ensuring that any subsequent opening response was the result of accumulation from the bathing medium.

The data obtained in the presence of bentazone confirm that this herbicide causes wide opening of C. album epidermal peel stomata as reported by Taylor, et al, (1980). The response does not appear to be light-dependent as suggested by these authors but, in the absence of KCl, occurred regardless of environmental conditions (Fig. 12). However, suitable pre-treatment of epidermes to determine stomatal condition at the start of an experiment has been found in the present study to be vital for subsequent investigation of responses to the factors involved in their movement. It is therefore considered that ambiguity may result from the lack of pre-incubation combined with little control of CO_2 concentration in the earlier study. Furthermore, investigations in this laboratory have since shown that solutions of Hepes buffer adjusted to pH 6.5 by the addition of alkali as used by the above workers may contain up to 15×10^{-3}M of monovalent cations. This is within the region where bentazone-induced dark stomatal opening may be inhibited by KCl (Fig. 13 a) and thus may have obscured responses. Such variability in ion concentration

was not found in the present study where buffer was absent, but small amounts of potassium were present in all solutions due to the type of glassware used for storage and the addition of a few drops of 0.01M KOH to adjust the pH of bentazone stock solutions.

The general response of C. album stomata to bentazone is shown by figure 12 to depend on the degree of opening prior to its addition rather than the environmental conditions afterwards. Thus bentazone was found to cause opening of closed stomata (Fig. 12 a and b), closure of open stomata (Fig. 12 c and d), and to inhibit KCl-induced stomatal opening (Fig. 12 a and b) but did not interfere with the normal closing response in the absence of KCl (Fig. 12 d). Moreover, bentazone-stimulated stomatal opening in darkness was shown to be dependent on the amount of KCl in the incubation medium (Fig. 13 a) whilst closure of open stomata in light at 50 p.p.m. CO_2 was induced at all KCl concentrations studied (Fig. 13 b). In addition, the herbicide was found to interfere with stomatal CO_2 responses of this species in either light or darkness (Fig. 14), via a mechanism which was also influenced by exogenous KCl concentration, and to inhibit ATP-and ADP-induced changes in stomatal aperture. No previous investigators have studied the influence of metabolic inhibitors at different KCl concentrations and comparisons are therefore difficult. However, it is known that the effect of the natural inhibitory plant hormone ABA is significantly influenced by potassium ion availability (Wilson, Ogunkanmi and Mansfield, 1978; Weyers and Hillman, 1979).

The effect of other non-cyclic photosynthetic electron transport inhibitors such as CMU, which causes stomatal closure in detached leaves (Allaway and Mansfield, 1967), and DCMU, which inhibits KCl-induced stomatal opening in epidermal peels of C. communis in light (Willmer and Mansfield, 1970), are usually reversed if the experimental system is flushed with CO_2-free air. This is clearly not the case with bentazone since at 50 p.p.m. CO_2 inhibition of C. album stomatal opening in light was almost total (Fig. 14 b). Moreover, whilst both DNP and salicylaldoxime were found by the latter authors to inhibit stomatal opening in

the light regardless of CO_2 concentration, none of the chemicals investigated had any effect in darkness. Although, in the present study, bentazone was also shown to inhibit stomatal opening in light at comparable potassium concentrations (Fig. 14 b), the response was more pronounced in darkness (Fig. 14 d). This data and that showing marked stimulation of dark stomatal opening at 5×10^{-3}M KCl (Fig. 14 c) infer that bentazone influences guard cell metabolism by some mechanism other than simply inhibiting photosynthetic electron transport.

Several ionophores, ie. compounds which facilitate transmembranal ion fluxes, have been found to interfere with stomatal movement by altering guard cell potassium transport. For example, nigericin and valinomycin were reported to cause loss of potassium from guard and subsidiary cells resulting in irreversible stomatal closure (Arntzen, Haigh and Bobick, 1973), whilst the synthetic polyether benzo-18-crown-6, which strongly binds potassium ions, has been shown to reversibly inhibit stomatal opening in C. communis epidermal peels in a way which is apparently antagonistic to ABA (Richardson, et al, 1979). Some uncouplers of oxidative phosphorylation are also known to produce ionophoric responses in animal systems but little evidence exists regarding the influence of bentazone on this process in plant cells. However, this herbicide has been shown to cause an initial slight increase followed ultimately by strong inhibition of dark respiration in both microbial populations (Marsh, Wingfield, Davies and Grossbard, 1978) and intact leaves of Cirsium arvense (Penner, 1975), which often indicates uncoupling of oxidative phosphorylation and/or inhibition of mitochondrial electron transport. Preliminary investigations in this laboratory have shown that bentazone is unable to counteract ABA-invoked stomatal closure (data not shown) but the possibility that the herbicide acts as an ionophore cannot be excluded since the observed responses are significantly affected by KCl concentration in the bathing medium.

The fungal toxin fusicoccin stimulates potassium uptake into plant cells by increasing proton extrusion (Marré, 1977) and as a result causes stomatal opening in epidermal peels (Raschke, 1977). However, stomata normally open wider under air

containing reduced CO_2 concentrations, with a corresponding increase in guard cell malate levels, but this response is reversed in C. communis by fusicoccin (Travis and Mansfield, 1979 a). Thus, a larger increase in both guard cell malate and stomatal aperture was observed at 350 than at 0 p.p.m. CO_2 in the presence of this toxin. Very low concentrations of potassium were used in these experiments and the magnitude of reversal was much greater in light than in darkness. Under similar conditions bentazone has also caused reversal of C. album stomatal CO_2 responses with the effect being considerably more pronounced in darkness (Fig. 14 c) than in light (Fig. 14 a) within the range 50 - 500 p.p.m. CO_2. Further increases in CO_2 concentration inhibited bentazone-stimulated opening in darkness but the response was only completely negated at 5000 p.p.m. whilst in light bentazone caused a shift in the apparent CO_2 concentration required for maximal stomatal opening to 1000 p.p.m. Indeed, the general suggestion from this data is that there is an optimum CO_2 concentration for stomatal opening, and supporting evidence in the literature indicates that closed stomata of some species often open more readily in air containing 100 - 200 p.p.m. CO_2 than in CO_2-free air (Raschke, 1977). Travis and Mansfield (1979 a) suggest that CO_2 exerts its major effect on guard cells by controlling the proton electrogenic pump or one of the other factors which regulate net uptake of K^+, and that fusicoccin causes the H^+ pump to operate in a manner which can no longer be controlled by CO_2. However, more recently, epidermal peel stomata of Commelina benghalensis have been shown to respond to increases of bicarbonate in the incubation medium up to 6 μM by opening more widely (Mrinalini, et al, 1982). Further increases in HCO_3^- reduced the degree of opening whilst the inclusion of fusicoccin raised the optimum concentration for stomatal opening to 25 μ M HCO_3^- above which toxin-stimulated opening was also appreciably reduced. The observation in the present study that bentazone induces stomatal responses in darkness which are apparently the same as those caused by fusicoccin in light, may not only infer a similar mode of action for these two chemicals but may also indicate the existence of separate proton pumps for uptake and efflux of potassium. Furthermore, inhibition

of the response to both compounds by high concentrations of CO_2 or HCO_3^- may provide support for the hypothesis outlined earlier in this Chapter that CO_2 controls guard cell potassium transport by direct action on the proton pumps responsible for its movement.

As discussed previously, the influence of ATP or ADP on C. album epidermal peel stomata pre-incubated on 10^{-4}M $CaCl_2$ to cause stomatal closure was dependent on the concentration of potassium in the surrounding medium. Previous workers with these nucleotides have used freshly peeled epidermes (Fujino, 1967; Raghavendra and Das, 1972) which probably contained appreciable levels of potassium ions in the subsidiary cells even when stomata were closed. Consequently, background information regarding the effects of inhibitors on energy-dependent stomatal movement is scant and not directly comparable to data obtained in the present study. Any interpretation of the observed responses is therefore speculative although effects on photosynthesis can be safely excluded as all experiments were conducted in darkness.

C. album stomatal opening induced by bentazone at low KCl concentration was effectively reversed by the inclusion of ATP or ADP with the latter being more effective (Fig. 15 a). Conversely, neither nucleotide restored the ability of the stomata to open widely in the presence of 100 x 10^{-3}M KCl which was inhibited by the herbicide (Fig. 15 b). Since bentazone interferes with dark respiration (Penner, 1975) it may effectively inhibit the majority of energy supply under these conditions, where little substrate would be available for cytoplasmic energy production via glycolysis as guard cell starch levels are high when stomata are closed (Allaway, 1981). Thus it could be predicted that the effect of bentazone might be reversed by the incorporation of ATP but not by ADP if the herbicide acts on stomata in this way. However, this is clearly not the case and similarities between stomatal responses to bentazone and fusicoccin suggest that the mechanism may involve interaction with proton pumps associated with guard cell potassium transport. Both the influx and efflux processes are likely to involve such pumps and their action may depend on the supply of ADP and ATP being regulated by environmental constraints. It is therefore considered that bentazone

may act in some way to modify the ability of the nucleotides to bind to the ATPase either by direct competition or by altering the conformation of the binding site. Support for this hypothesis is provided by the observation that, in contrast to the control response, the change in aperture due to ATP in the presence of bentazone was unaffected by KCl concentration, as was the change due to ADP. Indeed, except in the case of ATP and 100 x 10^{-3}M KCl the normal stomatal response to exogenous nucleotides was considerably inhibited by bentazone perhaps inferring altered binding kinetics at their sites of action rather than herbicidal inhibition of energy production.

In conclusion, the experiments reported here have shown that C. album epidermal peel stomatal responses to both environmental and chemical stimuli are in accordance with those reported for other species. Furthermore, the effect of 0-5000 p.p.m. CO_2 on changes in stomatal aperture indicates that high concentrations of CO_2 will completely inhibit stomatal opening in this species in light or darkness regardless of potassium ion availability. It is suggested that CO_2 may control guard cell potassium transport directly via action on proton pumps. The herbicide bentazone has been shown to exert a profound influence on the ability of stomata to respond in vitro to various stimuli possibly by disturbing the internal CO_2 "sensing" mechanism with consequent disruption of guard cell energetics. It is proposed that the herbicide may inhibit binding of ATP and ADP to their active sites and that there may be a similar target point for bentazone and the fungal toxin fusicoccin both of which interfere with guard cell responses to CO_2.

CHAPTER 5

Construction and use of an infra-red gas analysis system to determine the response of Chenopodium album photosynthesis and transpiration to applications of bentazone

5.1 Introduction

Intact whole leaf photosynthesis in higher plants is influenced by a variety of environmental constraints. It follows, therefore, that for accurate measurement of photosynthesis, these factors must be under strict control. The relevant environmental parameters and their control in an infra-red gas analysis circuit were first described in detail by Gaastra (1959). This author showed that a well designed assimilation chamber could be used to monitor leaf CO_2 uptake with an infra-red gas analyser(IRGA), provided that effective control of light intensity, temperature, humidity and CO_2 concentration was achieved. Similarly, transpiration may also be studied if a suitable hygrometer is incorporated into the circuit (e.g. Singh, Turner and Rawson, 1982). Indeed, IRGA methods have been refined and adapted by other workers in a wide variety of investigations including, for example, leaf age effects (Yamaguchi and Friend, 1979), stomatal responses to humidity (Farquhar, Schulze and Küppers, 1980) and the influence of herbicides (West, Muzik and Witters, 1976).

Bentazone efficacy in C. album has been shown to depend on environmental conditions immediately before and after application (Taylor, 1979). Hence, the purpose of the present investigation is to characterise the photosynthetic responses of this species to variation in temperature, light intensity and humidity by the construction of an IRGA system which may then be used to monitor photosynthesis and transpiration in response to bentazone application.

5.2 Materials and methods

5.2.1 Design of the experimental IRGA system: Figure 16 a shows the air circuit used in this study. The tubing was mainly 5 mm diameter polyester, but some 6 mm diameter silicone rubber was

FIGURE 16

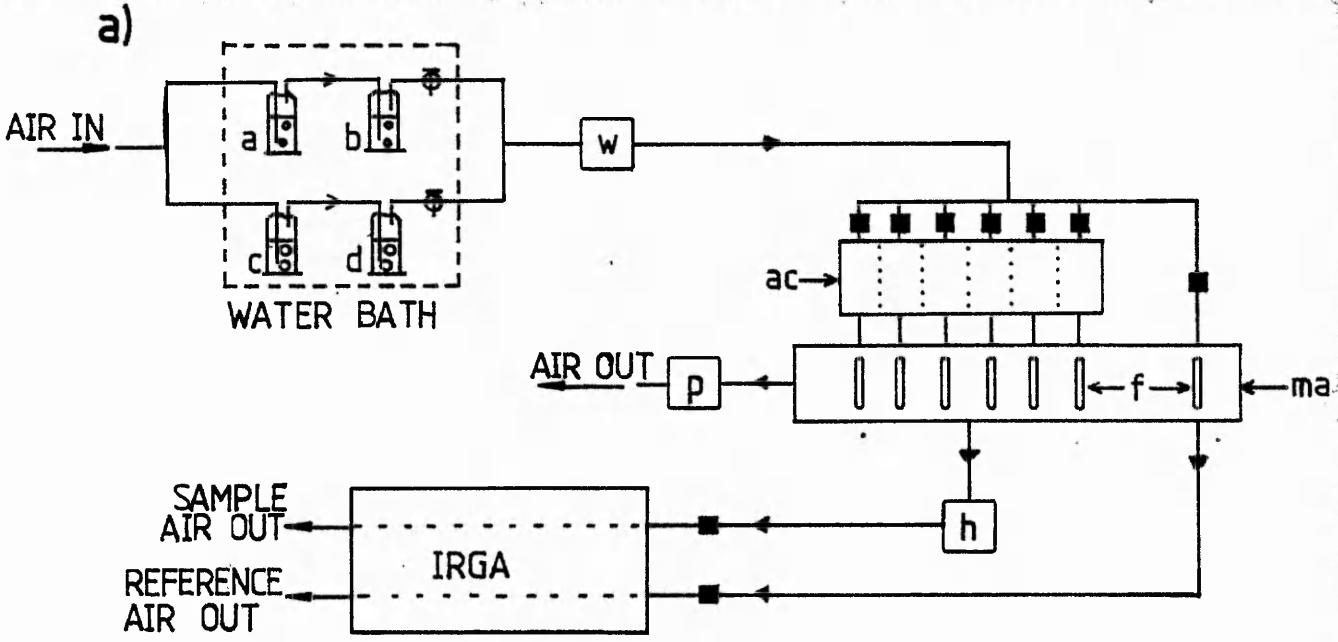
a) The air circuit

a,b	:	Dreschel bottles containing distilled water
c,d	:	Dreschel bottles containing glycerol
f	:	flow meters
h	:	humicap sensor and thermocouple
p	:	by-pass pump
Φ	:	adjustable valve
■	:	air filter
→—	:	direction of air flow
w	:	water trap
ac	:	assimilation chamber
ma	:	6-point sequential sampler

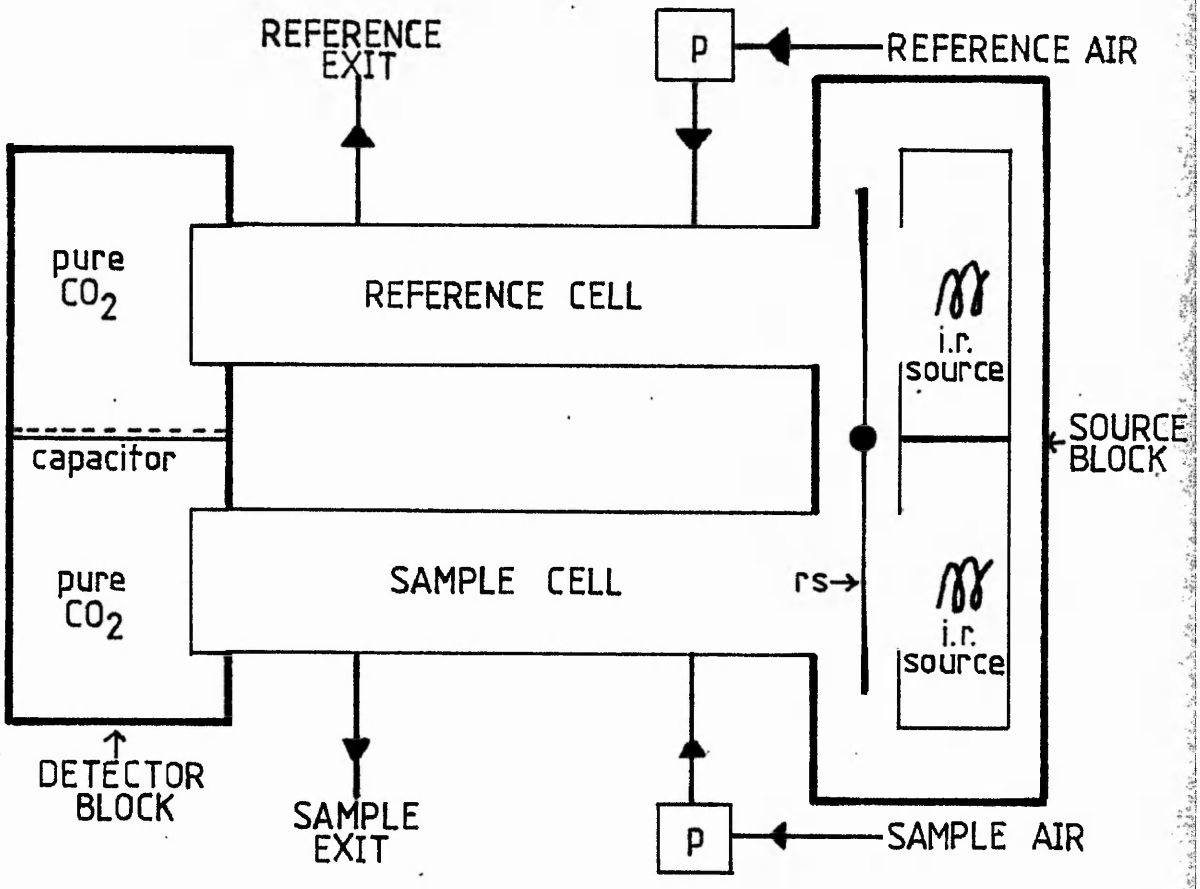
b) Internal organisation of the GP IRGA 120

p	:	internal air pumps
rs	:	rotating shutter

a)


AIR IN
a
b
c
d
WATER BATH
w
ac
AIR OUT
p
f
ma
h
SAMPLE AIR OUT
REFERENCE AIR OUT
IRGA

b)


REFERENCE EXIT
p
REFERENCE AIR
pure CO_2
REFERENCE CELL
i.r. source
capacitor
SOURCE BLOCK
pure CO_2
SAMPLE CELL
rs
i.r. source
DETECTOR BLOCK
SAMPLE EXIT
p
SAMPLE AIR

used for connections. Both these materials are impermeable to CO_2. Great care was taken to avoid leaks in the system as these caused large fluctuations in the IRGA readout and considerable loss of accuracy.

Air entering the circuit passed initially through a series of Dreschel bottles in a constant temperature water bath and the flow rate through a and b or c and d was regulated to give the required ambient humidity as monitored by the humicap sensor h (Vaisala: HMP 13 ST). Humidified air was drawn via the water trap (W) into the assimilation chambers which were sequentially selected by an auto-sampler (ADC: WA161) for measurement by an IRGA (Sieger: GP IRGA 120) of CO_2 concentration relative to a reference sample.

5.2.2 IRGA - theory and calibration: Figure 16 b shows the internal organisation of the IRGA used in this study. Two infra-red beams of equal energy are interrupted by a rotating shutter which allows them to pass intermittently but simultaneously through a sample cell assembly and a parallel reference cell into the detector. The detector consists of two sealed absorption chambers separated by a thin metal diaphragm, which, with an adjacent perforated plate, forms an electrical capacitor. The two chambers are filled with CO_2 so that energy characteristic of CO_2 is selectively absorbed. The instrumemt, therefore, monitors CO_2 concentration by measuring the relative absorption of the two cells. If the reference cell is filled with CO_2-free air and normal air passed into the sample cell the CO_2 present in this cell absorbs some of the energy, to which the detector is sensitised. This results in an imbalance of energy and deflection of the detector diaphragm changing the capacitance. This change is measured electrically and the output connected to a recorder. Thus, if the gas in the sample cell has a known CO_2 concentration then the recorder may be calibrated.

Similarly, the reference cell may be filled with ambient air, and used to monitor changes in the analysis cell CO_2 concentration away from ambient.

5.2.3 Control of factors influencing C. album photosynthesis:

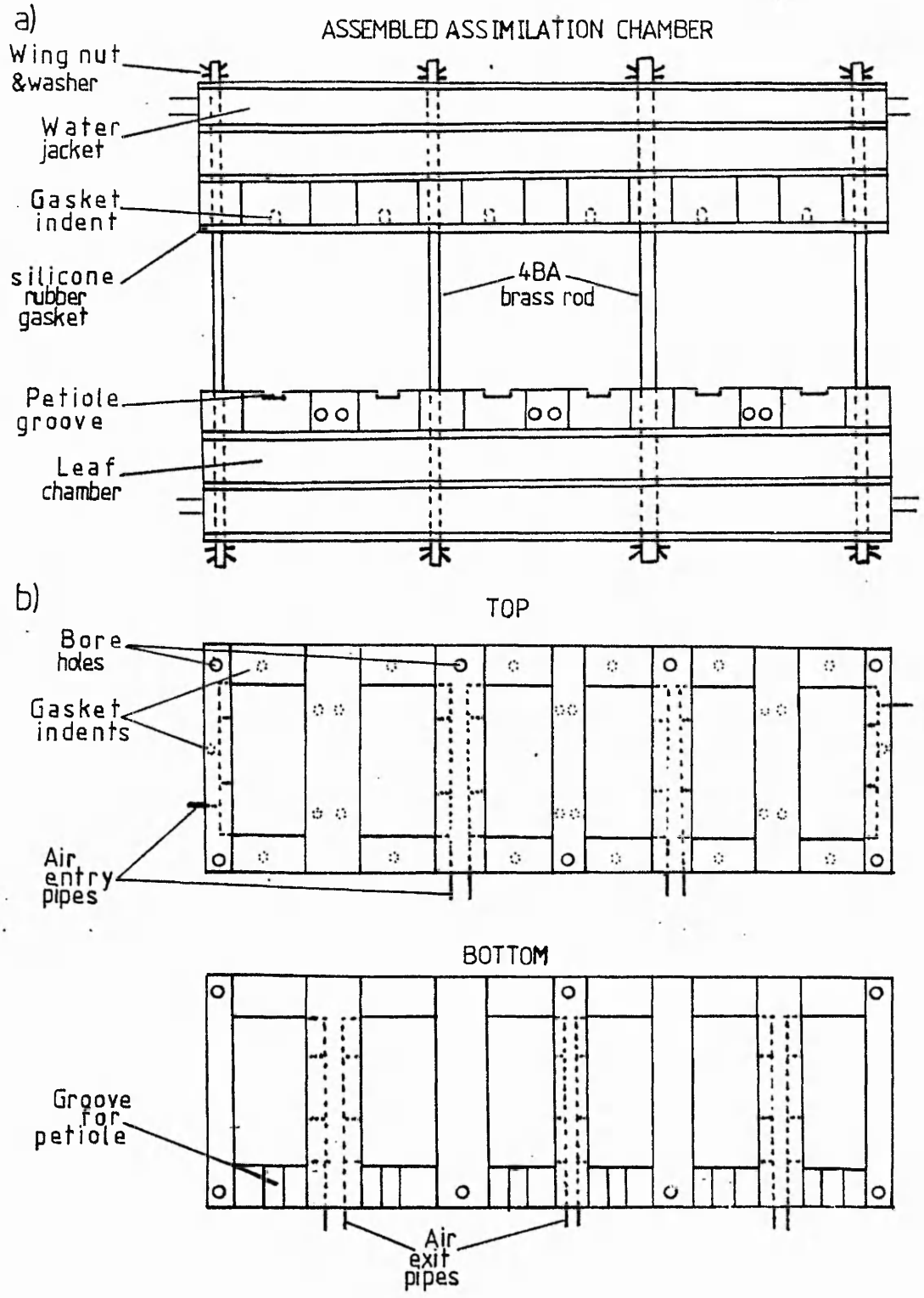
1. The assimilation chamber. The leaf assimilation chamber

FIGURE 17

Diagram to show details of assimilation chamber design

a) side view

b) plan


a)
ASSEMBLED ASSIMILATION CHAMBER
Wing nut &washer
Water jacket
Gasket indent
silicone rubber gasket
4BA brass rod
Petiole groove
Leaf chamber
b)
TOP
Bore holes
Gasket indents
Air entry pipes
BOTTOM
Groove for petiole
Air exit pipes

was specially constructed by the author, to hold 5 C. album leaves with one empty chamber (i.e. control) from perspex according to tne specification outlined in figure 17. It was intended to fulfil the following criteria:-

a) The intact leaf attached to the plant via its petiole could be positioned in the centre of the chamber.

b) The chamber could be made airtight once the leaves were in position by means of a silicone rubber gasket. The leaf entry holes were secured with non-phytotoxic sealing putty.

c) A regular distribution of air could be passed over the cross-section of the chamber. This was achieved by having 4 small inlet vents and similar outlet vents so that air passed across the width of the leaf.

d) The chambers were the minimum size to accommodate a large C. album leaf so that minimal dead space was available for the accumulation of air pockets.

e) The 6 equal-sized chambers were maintained at the same temperature by means of a double water-jacket fed by a constant temperature water circulator (Tecam C400).

2. Photon Flux density. Controlled irradiation was by a Camrex Solarcolour LG HPS/U sodium fluorescent tube (400w). With this light source it was possible to obtain specific light intensities within the range 0-1500 $\mu E.m.^{-2}$ $s.^{-1}$ (P.A.R.) with a good degree of uniformity ($\pm$ 5%) over the length and width of the assimilation chamber. The distance between the light source and the chamber required for individual light intensities was calibrated and checked weekly with a quantum sensor (LI-170, Crump Scientific Products). Above 1500 $\mu E.m.^{-2}$ $s.^{-1}$ variation in light intensity between chambers was somewhat higher ($\pm$ 10%). This was due to the close proximity of the tube element to the chamber and light intensities in this region were not studied.

As considerable heat was emitted by the lamp it was necessary to provide a shield through which light, but not heat, could pass. This was constructed as a perspex tray with a 3 cm deep continuously circulating stream of cold water. Using this device, placed between the light and the assimilation chamber, it was found that whilst temperatures above the reservoir reached 35-40°C, those below it were unchanged.

3. Air temperature. Air entering the gas circuit was first passed through Dreschel bottles, containing water or glycerol, in a constant temperature water bath. The assimilation chambers were surrounded by a water-jacket linked to a constant temperature water circulator. Both water systems were kept at the same temperature. This method was found to be accurate at temperatures in the range 15-35°C using the thermocouple in probe h (Fig. 16).

4. Relative humidity. Air entering the gas circuit was split into two streams (see Fig. 16). One stream was passed through glycerol to dry the air whilst the other was bubbled through water at the same temperature to humidify. The flow of humid and dry air was then regulated to give air of the required humidity as monitored by the Humicap sensor in probe h. Relative humidity was monitored continually during experiments and was not allowed to vary by more than 5%. However, at 80% R.H. condensation sometimes formed inside the assimilation chambers indicating an occasional increase on the probe value.

5. Carbon dioxide concentration. The city centre location of the laboratory meant that ambient CO_2 levels varied considerably and the use of air cylinders was considered impractical and costly. However, it was found that provided readings were taken between 0930-1630 h, ambient levels of CO_2 would vary by less than 5 p.p.m. throughout the experimental period. Concentration varied with the prevailing weather conditions but generally fell within the range 340-370 p.p.m CO_2 as measured by the IRGA.

6. Flow rate. Air flow was controlled by the external pump p

(Fig. 16) and the internal pumps of the IRGA and maintained at 1dm^3. $min.^{-1}$ through each leaf chamber by means of the automatic sampler. Reference air flow was 0.4 $dm.^3$ $min.^{-1}$ and very little fluctuation was observed during normal operation.

5.2.4. Measurement of leaf area: Measurement of C. album leaf area was similar to that described for epidermal peels in section 4.2.2. The height of a glass topped platform was adjusted so that the relevant leaf was resting flat on the surface. Light was shone through the glass and a piece of white paper placed over the leaf. The leaf silhouette was accurately traced and the replica cut out and weighed on a chemical balance. Leaf area was obtained by comparison with the known weight per unit area of the paper.

5.2.5. Calculation of photosynthesis: The equation used for calculation of photosynthesis was as follows:

$$P = \frac{Q.\ (c_i - c_o)}{A.V.}$$ (After Gaastra, 1959).

Where P = the rate of photosynthesis (Moles CO_2 $dm.^{-2}$ $s.^{-1}$),
Q = the rate of air supply (dm^3. $s.^{-1}$),
c_i = the CO_2 content of the inlet air ($dm.^3$ $dm.^{-3}$),
c_o = the CO_2 content of the outlet air ($dm.^3$ $dm.^{-3}$),
A = the total leaf area ($dm.^2$), and
V = the molar volume of CO_2 ($dm.^3$ $Mole^{-1}$).

Thus, under constant environmental conditions, accurate measurement of leaf area, flow rate, and carbon dioxide concentration of air entering and leaving the assimilation chamber allowed photosynthesis and dark respiration to be calculated.

5.2.6. Calculation of transpiration: C. album intact leaf transpiration was determined by monitoring increases in R.H. of air passed through leaf chambers compared to the empty control chamber using the humicap sensor h (Fig. 16 a). Relative humidity, in the range 40-100%, is directly proportional to absolute humidity (Long, 1982). Thus, accurate measurement of leaf area and flow-rate

allows calculation of transpiration as follows:

$$T = \frac{Q.\ \Delta RH}{A.M} \quad X \quad C$$

Where T = the rate of transpiration (Moles H_2O $dm.^{-2}$ $s.^{-1}$),
Q = the rate of air supply ($dm.^3$ $s.^{-1}$),
ΔRH = the increase in R.H. due to transpiration,
A = the total leaf area ($dm.^2$),
M = the relative molar mass H_2O ($g.\ mole^{-1}$), and
C = the humidity constant = 1.72×10^{-4} $g.H_2O$ $dm.^{-3}$ dry air (i.e. 1% R.H. ≡ 1.72×10^{-4}g. H_2O $dm.^{-3}$ dry air @ 20°C).

5.2.7. Measurement of Chenopodium album photosynthesis under different environmental conditions: Five 4-6 week old C. album plants grown as described in section 2.2.1., were taken to the laboratory each day from the growth cabinet. The nearest fully expanded leaf to the apex of each plant was placed inside a leaf assimilation chamber, via its petiole, and the chamber sealed. Air flowing over the leaves at 1 $dm.^3$ $min.^{-1}$ was adjusted to the required temperature and relative humidity and the plants were allowed to equilibrate for 30 minutes at a light intensity of 125 µE. $m.^{-2}$ $s.^{-1}$. The acclimatised leaves were sequentially analysed for CO_2 uptake, each being studied for 3 x 1 minute periods in 18 minutes. Light intensity was then increased to 250 µE. $m.^{-2}$ $s.^{-1}$ and the leaves equilibrated for a further 15 minutes, after which time CO_2 uptake was measured under the new conditions. This procedure was repeated at light intensities of 500, 750, 1000, 1250, 1500 and finally 0 µE.$m.^{-2}$ $s.^{-1}$.

The relationship between leaf CO_2 uptake and light intensity was studied at 40, 60, and 80% RH over a temperature range of 15-35°C.

5.2.8. The influence of bentazone on C. album photosynthesis and transpiration in the presence or absence of Actipron: C. album plants, grown as described in section 2.2.1. were taken to the laboratory each day and sprayed at 0930 h with either bentazone or an equal volume of water (to act as controls) in a fume cupboard

using a Shandon chromatography sprayer. Immediately after treatment the youngest fully-expanded leaf of each plant was installed in the assimilation chamber receiving 1 $dm.^3$ of air per minute at 20°C, 60% R.H. and illuminated with 750 $\mu E.\ m.^{-2}\ s.^{-1}$ (P.A.R.). Transpiration and net photosynthesis were measured continuously under these conditions for 325 minutes after herbicide application. The effect of bentazone was studied at 1/10, ½ or field-rate concentration (3 $dm.^3$ BAS 3517H in 280 $dm.^3$ water $ha.^{-1}$) using two different plants on three separate occasions i.e. six plants per treatment. A control plant, sprayed with water only was monitored alongside each treated plant and the data used to determine the relationship between intact leaf photosynthesis and transpiration of C. album under the conditions described. The response to half field-rate bentazone was further monitored for herbicide solutions containing 0.5 or 5% (v/v) aqueous Actipron when controls were sprayed with adjuvant solution only. Transpiration and net photosynthesis of all treated plants were calculated as percentages of the relevant control values.

5.3 Results

Typical gas exchange data for intact leaves of C. album obtained at 60% R.H. over a range of temperatures and photon flux densities is shown in figure 18. Net photosynthesis in this species is clearly shown to have achieved light saturation at approximately 500-750 $\mu E.\ m.^{-2}\ s.^{-1}$. Furthermore, whilst temperature influenced the rate of photosynthesis it did not significantly alter the light saturation point above which net assimilation of CO_2 remained relatively constant. Results at 40 and 80% R.H. showed essentially the same trend although the maxima obtained were slightly lower at both these extremes of the humidity range (data not shown).

The photosynthetic response of C. album to temperature varied according to the prevailing photon flux density. For example, at 125 $\mu E.\ m.^{-2}\ s.^{-1}$ maximum photosynthesis occurred at 30°C and for 250 and 500 $\mu E.\ m.^{-2}\ s.^{-1}$ at 25°C. Moreover, above saturating light intensity this species was able to sustain high

FIGURE 18

Calbum intact leaf photosynthesis in different environmental conditions

The variation in net photosynthesis at 60%R.H. obtained over a range of photon flux densities from 0-1500$\mu E.m^{-2}.s^{-1}$ at 5 different temperatures between 15 and 35°C. Each point represents the mean of measurements taken on 5 different plants. Bar indicates maximum standard error of the mean.

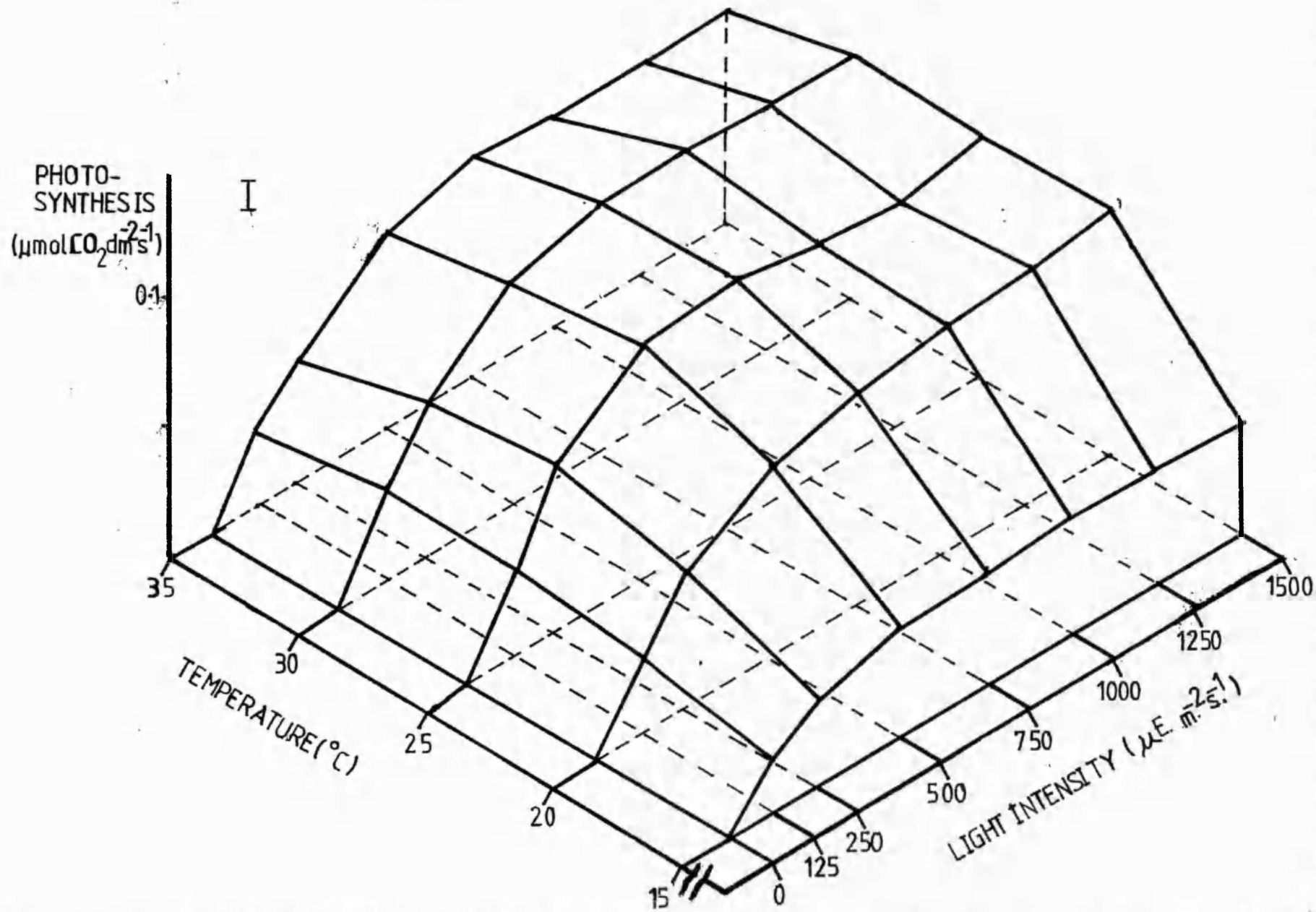


PHOTO-
SYNTHESIS
(μmol CO2 dm-2 s-1)
I
0·1
35
30
25
20
15
TEMPERATURE (°C)
0
125
250
500
750
1000
1250
1500
LIGHT INTENSITY (μE. m-2 s-1)

FIGURE 19

The relationship between CO_2 assimilation and transpiration in intact leaves of C.album

The measured values for net photosynthesis and transpiration from intact leaves of C.album at 20°C, 750 $\mu E.m^{-2}.s^{-1}$ and 60%R.H.

$r=0{\cdot}843 (n=18)$ i.e. significant at $p=0{\cdot}001$

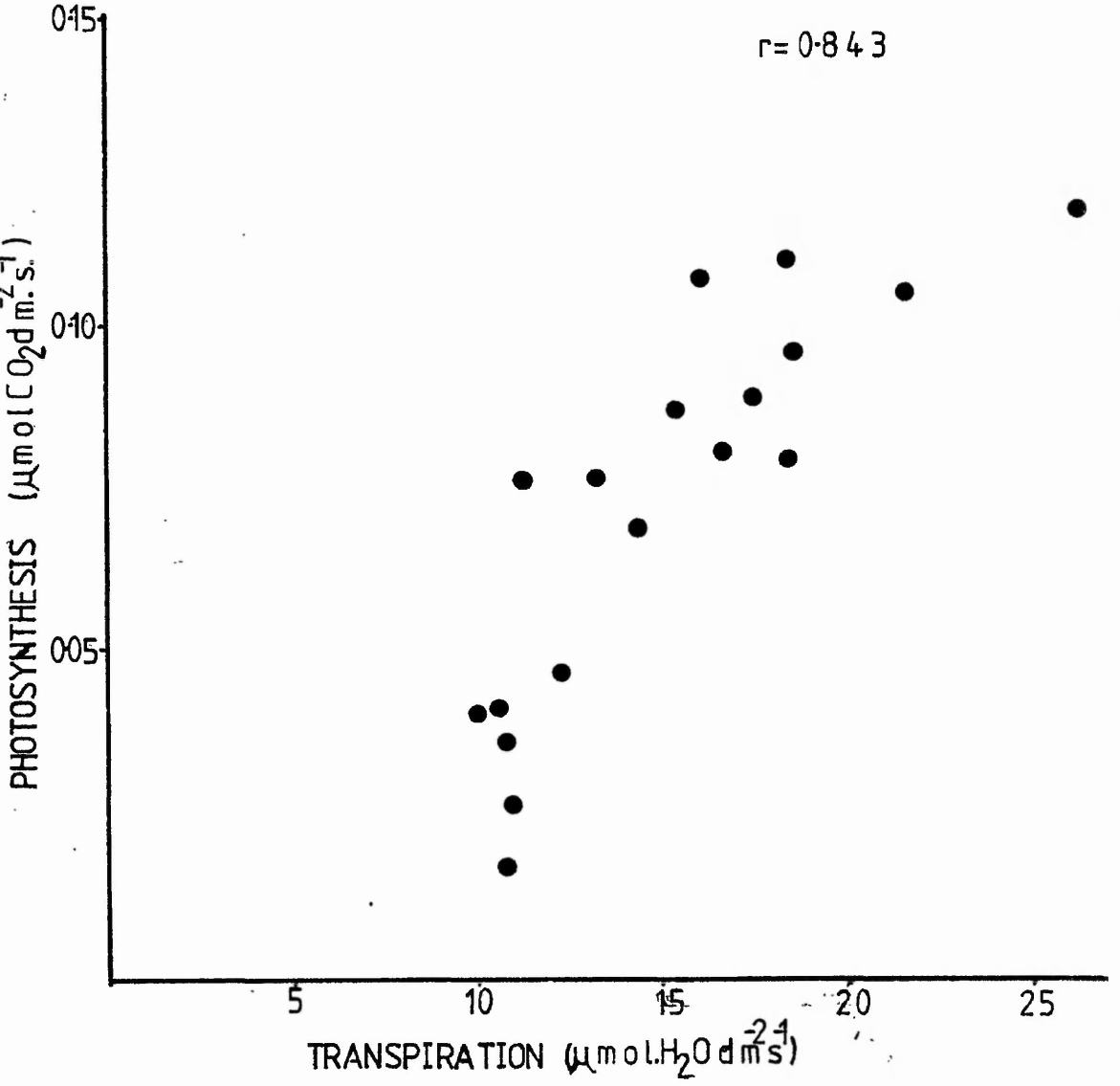
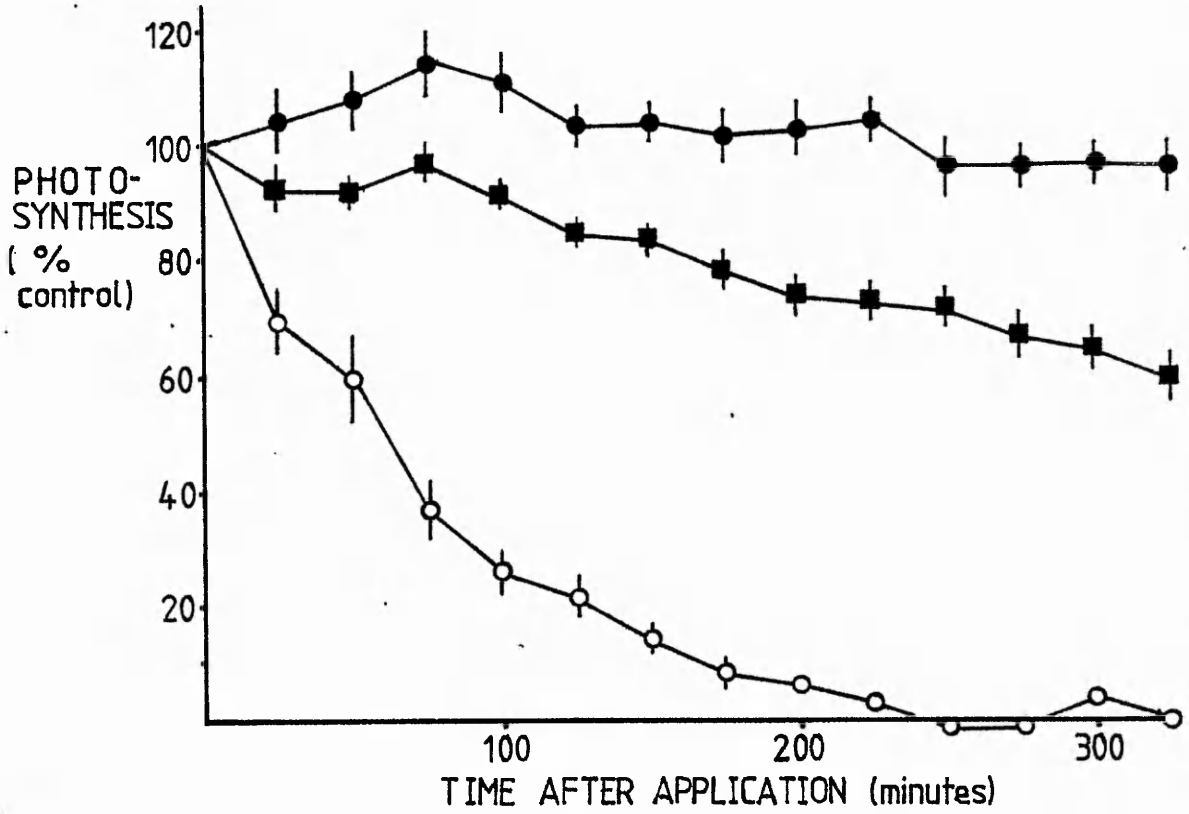

0·15
0·10
005
r= 0·843
PHOTOSYNTHESIS (μmol CO_2 $dm^{-2}.s^{-1}$)
5
10
15
20
25
TRANSPIRATION (μmol.H_2O $dm^{-2}s^{-1}$)

FIGURE 20

The influence of bentazone at different rates on intact leaf photosynthesis and transpiration in C.album

The change in net photosynthesis and transpiration during the first 325 minutes after bentazone application. Closed circles, 1/10 field-rate bentazone; closed squares, 1/2 field-rate bentazone; open circles, field-rate bentazone. Bars representing standard error of the mean(n=6) are shown where they exceed the symbol size.


120
100
80
60
40
20
PHOTO-
SYNTHESIS
(%
control)
100
200
300
TIME AFTER APPLICATION (minutes)

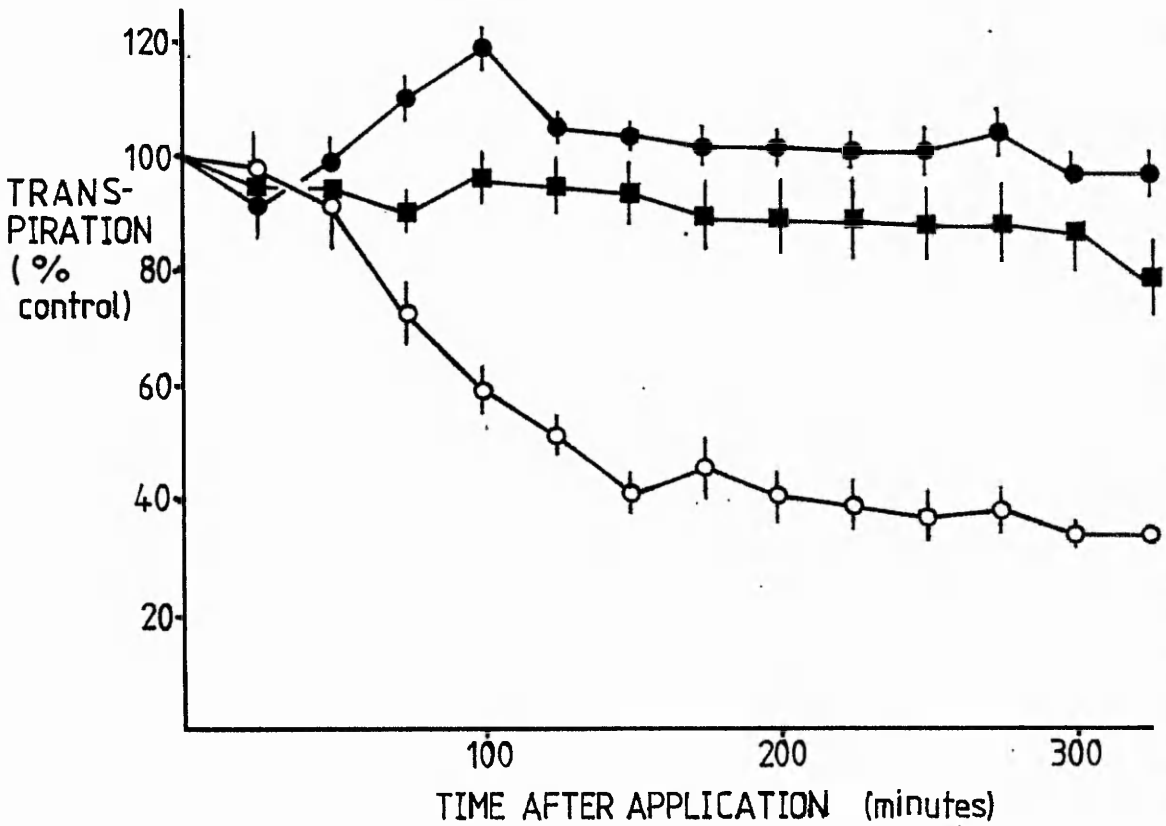
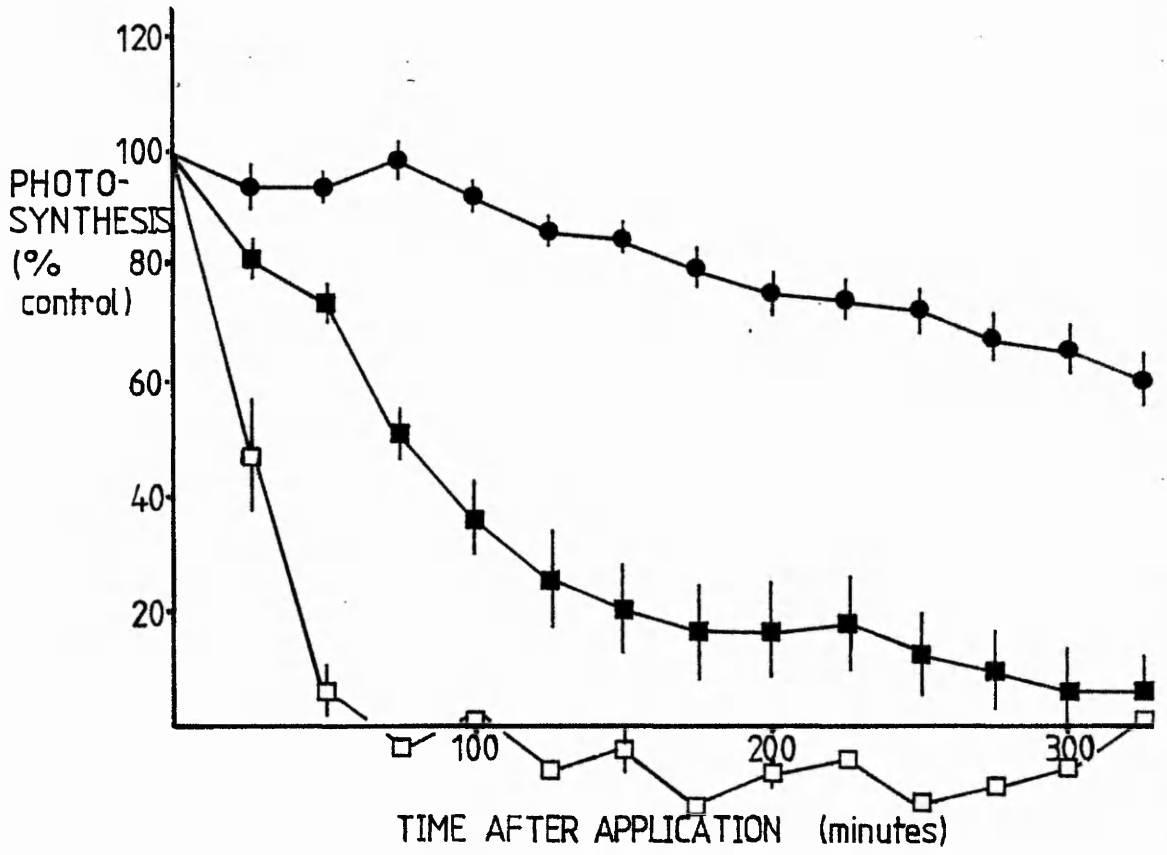

120
100
80
60
40
20
TRANS-
PIRATION
(%
control)
100
200
300
TIME AFTER APPLICATION (minutes)

FIGURE 21

The influence of Actipron on inhibition of C.album intact leaf photosynthesis and transpiration by bentazone

The change in net photosynthesis and transpiration during the first 325 minutes after application of bentazone and Actipron mixtures. Closed circles, half field-rate bentazone; closed squares, half field-rate bentazone including 0·5%(v/v) aq. Actipron; open squares, half field-rate bentazone including 5%(v/v) aq. Actipron. Bars representing standard error of the mean are shown where they exceed the symbol size(n=6)


120
100
80
60
40
20
PHOTO-
SYNTHESIS
(% control)
100
200
300
TIME AFTER APPLICATION (minutes)

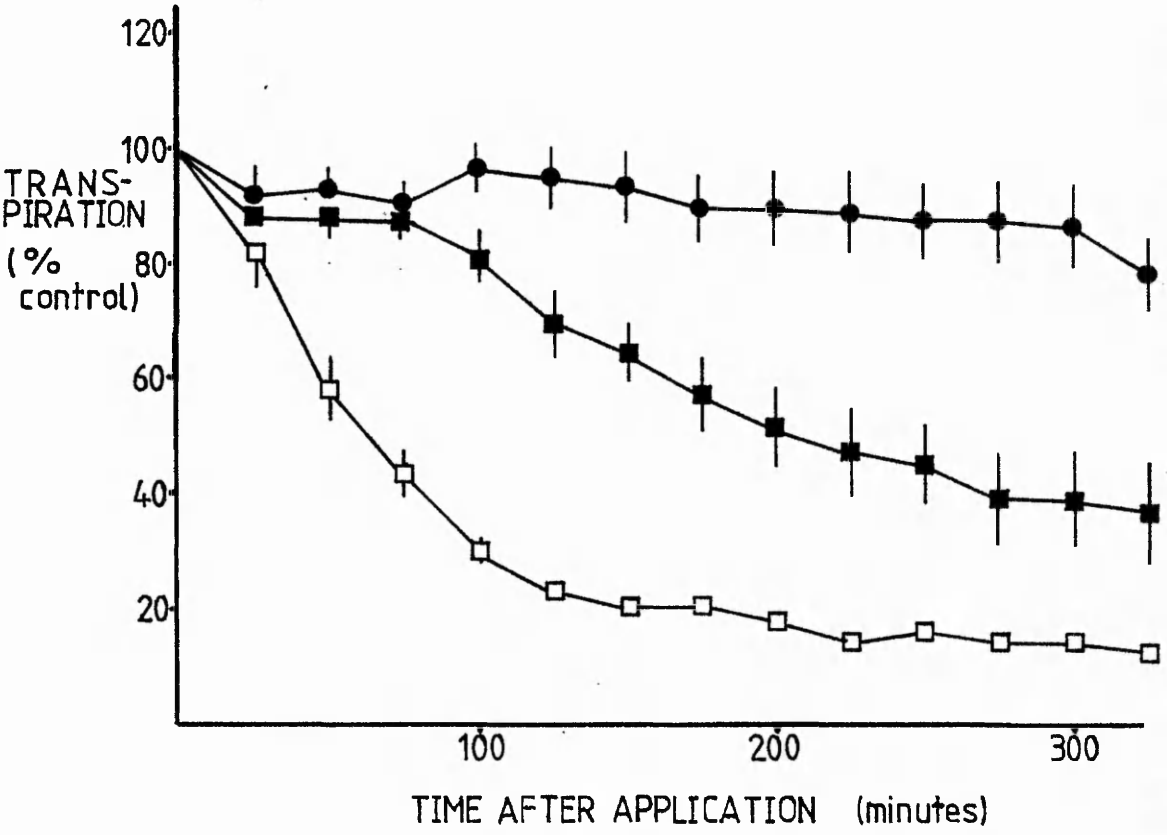
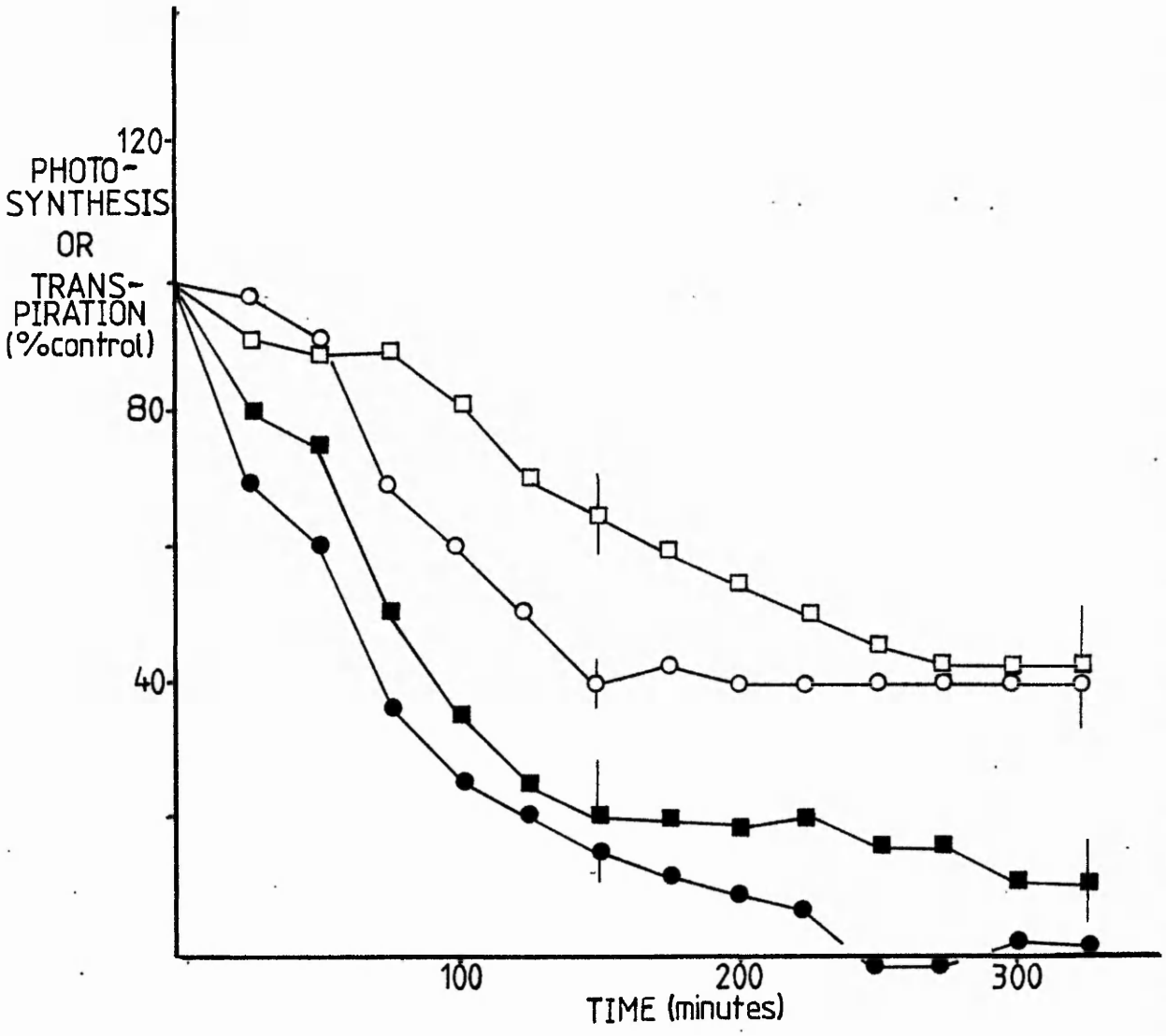

120
100
80
60
40
20
TRANS-
PIRATION
(% control)
100
200
300
TIME AFTER APPLICATION (minutes)

FIGURE 22

The influence of bentazone on *C.album* intact leaf CO_2 assimilation and transpiration as affected by Actipron

The change in net photosynthesis and transpiration of plants treated with field-rate bentazone (circles) or half field-rate plus 0·5%(v/v)aq.Actipron (squares). Open symbols, transpiration; closed symbols, photosynthesis. Bars indicate standard error of the mean(n=6).


120
PHOTO-
SYNTHESIS
OR
TRANS-
PIRATION
(%control)
80
40
100
200
300
TIME (minutes)

levels of CO_2 assimilation over a range of temperatures from 20-30°C. In all cases a reduction in photosynthesis occurred at 35°C to give an apparently optimal response. Thus, optimum environmental conditions for photosynthesis of C. album grown as described, were found to occur at temperatures between 20-30°C and photon flux densities above 500 μE. $m.^{-2}$ $s.^{-1}$. Maximum CO_2 assimilation, observed over a considerable range of the conditions studied was shown to be 0.10 - 0.12 μ moles CO_2 $dm.^{-2}$ $s.^{-1}$. Furthermore, the combined dark respiration values associated with this study, shown in Table 3, indicate that photosynthetic CO_2 assimilation in this species at ambient temperatures is a factor of approximately 10 times greater than respiratory CO_2 output. CO_2 compensation points at light saturation were determined regularly during the investigation and found to be in the range 40-80 p.p.m. CO_2 dependent upon temperature.

The observed relationship between C. album intact leaf transpiration and net photosynthesis presented in figure 19 indicates a strong correlation between these two processes in this species. However, the mean rates of CO_2 assimilation achieved in this study, by plants which were transferred directly to 750 μE. $m.^{-2}$ $s.^{-1}$, 60% R.H. and 20°C after spraying with water, were only 0.08 μ moles CO_2 $dm.^{-2}$ $s.^{-1}$ i.e. approximately $\frac{3}{4}$ of the values observed by those which were introduced gradually to the same environmental conditions. The associated transpiration values were generally in the range 10-20 μ moles H_2O $dm.^{-2}$ $s.^{-1}$ beyond which no appreciable increase in photosynthesis was observed. Thus, to take account of these observed reductions in photosynthesis and any other possible fluctuations in gas exchange due to the rapid change of environment, data for herbicide studies have been expressed as percentages of the relevant control values.

Fig. 20 shows the response of intact leaf net photosynthesis and transpiration in C. album to 1/10, $\frac{1}{2}$ or field-rate bentazone upon transfer to 750 μE. $m.^{-2}$ $s.^{-1}$, 60% R.H. and 20°C. Photosynthesis was completely inhibited by field-rate bentazone in 250 minutes whilst transpiration was reduced to levels typical of leaves maintained in darkness within only 150 minutes. However, only 40% reduction in CO_2 assimilation and 20% in transpiration

TABLE 3

The relationship between dark respiration and ambient temperature for intact leaves of C.album

CO_2 output of intact C.album leaves in darkness at different temperatures. Figures in brackets indicate standard error of the mean (n=15) for data at 40, 60 and 80%R.H. combined.

TEMPERATURE (°C)	RESPIRATION (moles CO_2 $dm.^{-2}s.^{-1}$)
15	$8{\cdot}1(2{\cdot}7) \times 10^{-9}$
20	$11{\cdot}5(3{\cdot}5) \times 10^{-9}$
25	$15{\cdot}2(0{\cdot}8) \times 10^{-9}$
30	$18{\cdot}0(4{\cdot}6) \times 10^{-9}$
35	$18{\cdot}9(2{\cdot}2) \times 10^{-9}$

cf. figure 18 showing maximum photosynthesis of 0·10-0·12umoles CO_2 $dm.^{-2}s.^{-1}$

TABLE 4

The influence of Actipron on C.album photosynthesis and transpiration

C.album intact leaf photosynthesis and transpiration 5 hours after application of Actipron solutions ($280 dm^{-3} ha^{-1}$) at the concentrations indicated followed by the standard error of the mean (n=5).

Actipron concentration (%v/v)	Net photosynthesis ($\mu mol.CO_2 dm^{-2} s^{-1}$)	Transpiration ($\mu mol H_2O dm^{-2} s^{-1}$)
0	0·07 ± 0·009	8·8 ± 0·46
0·5	0·05 ± 0·003	7·1 ± 0·50
5·0	0·04 ± 0·006	7·1 ± 0·60

were induced by ½ field-rate bentazone during the whole 325 minutes of observation. Moreover, in response to 1/10 field-rate C. album transpiration was initially stimulated by 20% during the first 100 minutes after treatment with a corresponding small increase in photosynthesis. Indeed, although subsequent rates of both processes returned to control values no inhibition of either was observed during the experimental period.

The data in figure 21 shows that the performance of ½ field-rate bentazone as an inhibitor of net photosynthesis and transpiration in C. album may be appreciably enhanced by the incorporation of Actipron into the herbicide solution. However, with the inclusion of the oil adjuvant, changes in CO_2 assimilation and transpiration no longer occurred in parallel. For example, at 0.5% (v/v) aq. Actipron photosynthetic inhibition by half field-rate bentazone followed a similar pattern to that observed with the herbicide alone at field-rate, whilst the respective times required to reduce transpiration to dark levels were 275 and 150 minutes (see Fig. 22). With 5% (v/v) aq. Actipron, rapid total inhibition of net photosynthesis was induced by half field-rate bentazone within 75 minutes by which time transpiration was reduced to rates normally attained in darkness. Subsequent further reductions in transpiration to less than 20% of control values, inferring complete stomatal closure, were associated with abnormally high respiratory rates indicated by CO_2 output.

The influence on C. album photosynthesis and transpiration of Actipron alone is shown in Table 4. Whilst transpiration may have been marginally influenced by this adjuvant it is important to note that appreciable depression of net photosynthesis was caused by both concentrations studied even in the absence of bentazone.

5.4. Discussion

As a result of the need to test the newly constructed IRGA circuit considerable information regarding the photosynthetic competence of C. album has been accumulated in this study. When grown under the controlled conditions described net CO_2 assimilation in this species has been shown to saturate at a photon flux density of 500-750 $\mu E.\ m.^{-2}\ S.^{-1}$. In combination with the observed

compensation points of 40-80 p.p.m. CO_2 this strongly suggests that photosynthesis in C. album follows a typical C_3 carbon reduction pathway. Furthermore, the maximum photosynthetic rates of 0.10-0.12 μ mol. CO_2 $dm.^{-2}$. $s.^{-1}$ at light saturation are comparable to those obtained by other workers using IRGA systems with leaf discs of this species (Günther, Baumann, Klos, and Balfanz , 1979) whilst the light-response curves are equivalent to those found in other C_3 weed species (Holt, Stemler and Radosevich, 1981).

The ability of C. album to maintain high levels of net photosynthesis over a wide range of temperatures at photon flux densities above 500 μE. $m.^{-2}$ $s.^{-1}$ probably contributes to the success of this species as a weed in arable situations. Midsummer daylight has been measured during the present study and found to be in the range 1500-2000 μE. $m.^{-2}$ $s.^{-1}$. Thus, the reported response of C. album to light regimes typically found under canopy cover of producing rapid stem extension (Morgan and Smith, 1978; Child, et al, 1981) combined with a capacity for high photosynthetic rates, even when grown under relative shade, would enable the plant to increase production appreciably once a crop stand was outgrown. Furthermore, the considerable temperature range for maximum photosynthesis in C. album will confer further ecological advantage in terms of acclimation. The observed interaction between temperature and light intensity as determinants of assimilation rates is typical in that the temperature curve was more pronounced at higher light intensities (Ludlow and Wilson, 1971) and although the plants were grown at 20°C the optimum temperatures for photosynthesis were higher, particularly at low light intensities. This phenomenon occurs in many species and may arise from a compromise between the need to optimise photosynthesis and the extra energy expenditure of increased rate-limiting enzyme production (Berry, and Bjorkmann, 1980).

The relationship between net photosynthesis and transpiration in C. album presented in figure 19 clearly demonstrates the large expenditure of water vapour required for CO_2 fixation by plants. Water use efficiency i.e. the molar ratio of H_2O lost in relation to CO_2 fixed may vary, for example, in Xanthium strumarium between

156-336 depending on stomatal conductance (Raschke, 1979). Thus, the maximum values of approximately 230 obtained for C. album in the present study (Fig. 19) suggest a relatively high degree of stomatal control of the ratio in this species under the conditions described. However, the possibility of slightly higher ratios associated with the greater photosynthetic rates obtained in the environmental study (Fig. 18) cannot be excluded.

The effect of field-rate bentazone (i.e. 1.4 kg. ha.$^{-1}$ a.i.) on net photosynthesis in C. album as shown in figure 20 was to cause 100% inhibition within 250 minutes at 20°C, 60% R.H. and 750 µE. m.$^{-2}$ s.$^{-1}$ with no subsequent recovery. This response is in close agreement with several other studies into the influence of this herbicide on susceptible species. For example, applications of 2 kg. ha.$^{-1}$ bentazone to Sinapis alba have been shown to irreversibly inhibit CO_2 assimilation in 120 minutes (Retzlaff and Hamm , 1976) whilst 69% inhibition was demonstrated within 180 minutes of application to Cirsium arvense at 2.24 kg. ha.$^{-1}$ (Penner, 1975). Furthermore, Mine and Matsunaka (1975) observed 100% inhibition of photosynthesis in Cyperus serotinus 240 minutes after foliar application of approximately 5 x 10^{-3} g. bentazone per plant. Thus, the results of the above studies indicate that irreversible inhibition of photosynthesis in susceptible species by comparable concentrations of bentazone occurs between 2 and 5 hours after foliar application.

In contrast, although 2 kg. ha.$^{-1}$ bentazone was found in a similar investigation to inhibit photosynthesis of Gallium aparine in 4⅓ hours, CO_2 assimilation in three other species, including Xanthium pensylvanicum, was inhibited in less than 1 hour (Dannigkeit, 1977 b). Evidence supporting rapid inhibition by this herbicide in X. pensylvanicum suggests that a 50% reduction of photosynthesis occurs within 5 minutes of application at 1 kg. ha.$^{-1}$ (Potter, 1977) and that 100% inhibition may occur in the same species in 180 minutes after foliar treatment with only 0.1 kg. ha.$^{-1}$ (Potter and Wergin, 1975). In comparison, no inhibition of C. album CO_2 assimilation was observed in the present study during the 325 minutes immediately after treatment with 1/10 field-rate (0.14 kg. ha.$^{-1}$) bentazone.

Variable susceptibility was attributed by Dannigkeit (1977 b) to differential uptake and metabolism of the herbicide, since after 72 hours X. pensylvanicum contained 30% of the applied bentazone in an unmetabolised form, whilst only 10% was detected in G. aparine. However, whilst the effect of bentazone on photosynthesis may vary according to the species involved, the nature of the leaf surface and the degree of formulation to improve uptake, it seems unlikely that penetration of sufficient bentazone to completely inhibit photosynthesis could have occurred within 1 hour as claimed by this author. Indeed, doubt must be expressed as to the validity of this study since the observed rates of photosynthesis were so low that even in species tolerant to bentazone maximum CO_2 uptake in light was exceeded by dark CO_2 output (cf. photosynthesis: respiration was 10:1 for C. album in the present study). Furthermore, in the studies of Potter and Wergin (1975; 1977) bentazone plus 0.1% nonoxynol was applied by brush or syringe and the data is therefore not directly comparable with other studies. In addition, the herbicidal application rates were calculated by these authors on a leaf area basis and thus would be appreciably higher if allowance was made for the bentazone which, under normal circumstances, would not have come into contact with the plants after spray application.

Inhibition of C. album photosynthesis became progressively less as the applied concentration of bentazone was reduced (Fig. 20). Thus, field-rate bentazone completely inhibited photosynthesis in 250 minutes whilst ½ field-rate caused only 40% inhibition during the entire experimental period. However, when plants were treated with only 1/10 field-rate bentazone an initial 15% stimulation of CO_2 assimilation was observed relative to the controls and, although the rate returned to normal after 125 minutes, there was no subsequent inhibition. Furthermore, in all cases a change in net photosynthesis was associated with a concurrent and parallel change in transpiration.

A similar study into the effect of atrazine on two Amaranthus retroflexus biotypes has also revealed simultaneous falls in both photosynthesis and transpiration although calculations of leaf diffusive resistance suggested that the major obstruction to CO_2 movement was at the mesophyll rather than the

stomatal level (West, et al, 1976). Such determinations of leaf diffusive resistance are dependent on continuous monitoring of leaf temperature (Gaastra, 1959) which was not possible in the present investigation. Moreover, the calculation assumes zero chloroplast CO_2 concentration which may not apply to studies of herbicides known to inhibit photosynthesis. Indeed, the large increase in mesophyll resistance considered by West, et al, (1976) to be primarily responsible for reduced CO_2 uptake did not occur until 4 hours after atrazine application, by which time photosynthesis and transpiration were already reduced by more than 60 and 40% respectively. Thus, it is suggested that stomatal closure indicated by reduced transpiration in both the results of the above workers and in the present study may significantly inhibit CO_2 assimilation independently of action at the chloroplast level, particularly during the first few hours after treatment when little herbicide may have penetrated into the mesophyll.

At least one other study has demonstrated that field concentrations of bentazone not only inhibit photosynthesis but also transpiration in a susceptible species, although the two processes were investigated separately (Penner, 1975). Indeed, this author observed significantly increased transpiration in two varieties of resistant Glycine max in the 24 hours following treatment with bentazone, although during the equivalent time photosynthesis fell by 21%. This herbicide is therefore clearly able to influence transpiration and hence stomatal movement independently of photosynthesis in resistant species. It is, however, not certain if the changes in C. album transpiration in the present study resulted in the concurrent changes in CO_2 assimilation or vice versa. Not only would increased internal CO_2 concentration due to cessation of chloroplastic photosynthesis cause stomatal closure but, under the conditions described, herbicide-induced stomatal closure would inhibit both transpiration and photosynthesis as discussed earlier. However, C.album stomatal opening in response to 1/10 field-rate bentazone implied by increases in transpiration and CO_2 assimilation of treated plants is unlikely to have resulted from herbicide stimulation of photosynthesis. Thus, the ability of bentazone to modify stomatal apertures in this species (see Chapter 4)

may, in some circumstances, contribute significantly to the mechanism of action of this herbicide.

The observation that bentazone induced inhibition of C. album photosynthesis and transpiration is appreciably enhanced by the inclusion of Actipron, above its critical micelle concentration (Taylor, 1979), probably explains reports of more effective weed control by the herbicide when this adjuvant is used (King, 1976; King and Handley, 1976). When incorporated into the treatment solution at 0.5% (v/v) aq. Actipron effectively doubled the photosynthetic inhibition caused by bentazone (Fig. 21). Indeed, at 5% (v/v) aq. Actipron, inhibition of photosynthesis was so rapid that CO_2 assimilation was only 50% of control values 25 minutes after treatment. Moreover, unlike the response in the absence of the adjuvant, inhibition of photosynthesis occurred at a much faster rate than the corresponding reduction in transpiration (Fig. 22). It is therefore suggested that improved bentazone penetration afforded by Actipron (see Chapter 3) may have resulted in disruption of mesophyll CO_2 assimilation in advance of any stomatal response. This in turn would have increased internal CO_2 concentration and, in combination with the effects of bentazone alone, caused the subsequent almost complete stomatal closure. However, Actipron at both concentrations was also found to inhibit C. album photosynthesis in the absence of herbicide but had relatively little effect on transpiration (Table 4). Similar observations of photosynthetic inhibition by mineral oil additives have been reported elsewhere (Gudin, Syratt and Boize, 1976) and thus it is difficult to separate the influence of the oil and the herbicide from possible interactions between them which may have caused the observed responses.

To summarise, the results of this study demonstrate the remarkable acclimation capacity of the C_3 weed C. album. Furthermore, under the experimental conditions described the herbicide bentazone has been found to inhibit CO_2 assimilation in this species via a mechanism which may involve stomatal closure. However, when used in combination with the oil adjuvant Actipron photosynthesis is rapidly inhibited independently of stomatal responses possibly as a result of enhanced cuticular penetration of the herbicide although bentazone/Actipron interactions may have contributed to the improved phytotoxicity.

CHAPTER 6

FINAL DISCUSSION

6.1 An assessment of the techniques used in this study

Every effort has been made in this series of investigations to design experiments which were relevant to each other in order to yield information which was directly comparable. Thus, growth conditions in controlled environment cabinets were constant throughout and herbicide formulation BAS 3517H was used at the manufacturer's recommended field-rate, or fractions thereof, for all studies into whole plants which in turn were derived from a single seed batch. In addition, individual plants which displayed atypical growth morphology, usually identified by reduced apical dominance, were discarded along with those showing evidence of nutrient deficiency or viral infection. Moreover, all observations were made on the youngest fully-expanded leaf of plants which had suffered no water stress prior to treatment and hence were physiologically active.

In spite of these precautions it was not possible to completely standardise all procedures. For example, in the LDR studies (Chapter 2) plants were treated using a pressurised Binks-Bullows gravity sprayer, whilst in photosynthesis experiments (Chapter 5) bentazone was applied with a Shandon chromatography sprayer. It is possible that these different application techniques may have produced variations in droplet size, evaporation rate and hence spreading of the herbicide with unknown consequences on absorption and activity. Moreover, neither method produced droplets as large as those used in the ^{14}C-bentazone uptake studies. Such inconsistences may have influenced the rate of development of herbicide responses, but it is considered unlikely that the general trends observed would have been substantially altered by employing different methods to which there was no alternative at the time. Since the completion of these investigations a specially designed Mardrive controlled application pot-sprayer has become available in this laboratory and it is recommended that this be employed in future studies of

this type to overcome any possible ambiguity.

In view of the pronounced circadian rhythm of C. album LDR it was also considered desirable that the time of day for treatments and observation should be standardised and that readings during the midday stomatal closure period should be avoided. Thus, bentazone was usually applied at 0930 h in all whole plant studies, and measurements of ECLD in epidermal peels were confined to the early morning and the mid-afternoon. However, whilst the LDR and bentazone uptake experiments may be directly comparable, since they involved relatively little time away from the growth cabinet, the procedure for photosynthesis/transpiration studies required the plants to be transferred to the laboratory. Consequently, mean LDR of such plants had risen from less than 1 s. cm.$^{-1}$ to 6.3 s. cm.$^{-1}$ by the time bentazone was applied, suggesting that the physiological condition of the treated plants was not representative for time of day investigations. Indeed, the complexities involved in transporting, spraying and installing the plants in the assimilation chamber, then producing the desired environment quickly enough to obtain values for CO_2 assimilation and transpiration within 30 minutes of herbicide application, imposed severe limitations on this technique for the study of short-term time of day responses. Furthermore, these problems may account for the slightly lower rates of photosynthesis in herbicide studies although photo-inhibition due to the sudden transfer from the growth regime to experimental light intensities of 750 μE. m.$^{-2}$ s.$^{-1}$ may also have contributed. Nevertheless, the procedure was highly satisfactory for monitoring responses of C. album photosynthesis and transpiration to different concentrations and formulations of bentazone, since the initial rates observed for both processes in treated and control plants were very similar.

Another problem in short-term studies of bentazone application time and the dependence of the subsequent response on C. album LDR was posed by the controlled environment. Thus, whilst the data in Chapter 3 apparently shows that ^{14}C-bentazone penetration is significantly greater in this species when applied to leaves with open rather than closed stomata, the possibility that this results from some environmental difference during the early post-treatment period

cannot be excluded. Unfortunately, this problem was unavoidable unless plants were subjected to highly artificial conditions. For example, a regime of continuous light could have been imposed after treatment but this would not have accounted for possible rhythmic interactions. Alternatively, treatment at 0930 h of leaves with stomata either open or induced to close by one of a variety of techniques may have been a more satisfactory approach to determine the involvement of these structures in bentazone absorption by C. album independently of time of day. However, stomata respond quickly to new environments and artificially-induced closure may therefore have been only temporary or, indeed, too permanent depending on the method used.

The studies on C. album LDR in relation to bentazone application (Chapter 2) may have been a more realistic reflection of any time of day response, since drastic disruption of the leaf environment was avoided by utilising the natural circadian rhythm of stomatal movement , leaving sufficient time before the end of the light regime to nullify any environmental interactions. Hence, although the evidence in Chapter 3 is not strong enough to prove that bentazone penetration into C. album was better at 0930 h because stomata were open, the LDR study does indicate that the herbicide is more effective if applied when stomata are widely open than partially closed. Both long and short-term investigations showed a more rapid increase in C. album LDR after treatment at 0930 h than 1230 h which correlated with damage data. Moreover, whilst final fresh and dry weights were unavoidably determined after 11 and 14 days for the 0930 h and 1230 h bentazone applications respectively, and hence may not be comparable, the earlier data shows that 0930 h treatments had already suffered significantly more damage within 7 days. However, improved control at 0930 h may have resulted from interference by bentazone with some unknown fluctuating physiological process coincidental with the circadian rhythm in LDR.

The use of porometry to determine changes in C. album LDR has been found in this investigation to be particularly suitable for growth cabinet studies. If the precautions outlined in Chapter 2 were observed then remarkably consistent results were very quickly obtained. A minor problem was presented when both

treated and untreated plants were monitored in the same study as it was found that the stick-on foam rubber seals used to modify the sensor aperture retained some herbicide, but this was easily remedied by using separate seals for each treatment. In addition, excessive disturbance of the controlled environment, as in the short-term herbicide studies tended to disrupt the normally observed circadian rhythm so that midday closure occurred earlier, later or not at all depending on when observations commenced. Thus, a major improvement in the procedure for short-term studies would be the incorporation of "arm-holes" in the growth-cabinet door to totally avoid any disturbance of the environmental conditions.

The porometer was also used satisfactorily to determine stomatal condition in several other laboratory experiments but strong reservations must be expressed regarding its field-use. Although described by the manufacturers as a "field porometer" it was found that very slight changes in temperature necessitated recalibration and that in relatively minor winds the instrument gave large fluctuations. Hence, it was only possible to obtain consistent readings under field conditions if the whole area surrounding the porometer was completely shielded from all wind by a large frame covered in transparent polythene.

Measurement of uptake and translocation of radiolabelled herbicides by liquid scintillation counting is a very commonly used technique. However, its use in the present study has highlighted some major drawbacks of which the author was previously unaware. Firstly, inexplicable losses of ^{14}C-bentazone were observed, both from stock and from scintillation vials containing the "applied dose" as a control, which were sometimes higher than 10%. Consequently, it was necessary to calculate uptake as a percentage of the applied dose recovered after zero time to allow for this discrepancy. Although it is not known why such losses might have occurred it is suggested that they may explain the apparently high translocation rates at 2030 h in the absence of evidence that label was present in other plant parts. Indeed, if the difference between the applied dose and the amount recovered is due to high volatility, thermal breakdown or photodegradation

of bentazone, as seems possible (Nilles and Zabik, 1975), then the higher translocation calculated for 2030 h than for 0930 h applications of this herbicide may simply reflect lower uptake and a greater loss of label to the atmosphere.

A further source of error in bentazone penetration studies may have been the recovery technique. This herbicide is reported to be soluble in water at up to 0.5 g. $dm.^{-3}$ (Fedtke, 1982) but it has been found necessary in practice during preparation of 10^{-3}M stock solutions (i.e. only 0.24 g. $dm.^{-3}$) to initially dissolve technical grade bentazone in absolute ethanol before dilution with distilled water to give the desired concentration. By this procedure the herbicide was soluble in 5% (v/v) aq. ethanol but it was not possible to dissolve the same amount directly into this solvent mixture. It is therefore doubtful if the 0.5% (v/v) Actipron in 5% (v/v) aq. ethanol used to recover ^{14}C-bentazone in uptake experiments would have removed all the label from the leaf surface. Consequently, it is probable that absorption of this herbicide by C. album leaves was even less than that determined. Moreover, if a large portion of the label not recovered was lost by decomposition and the remainder relatively insoluble in the washing solution, then the total found in the treated leaf of only 26.1% 7 days after application of ^{14}C-bentazone alone at 2030 h may include a significant quantity of herbicide from the leaf surface. Thus, it is possible that under certain conditions C. album leaves are almost impermeable to unformulated bentazone.

The efficiency of detection of ^{14}C-labelled herbicides in plant sections by liquid scintillation counting is highly dependent on accurate allowance being made for "quench" due to the presence of either treatment solutions or undigested vegetable matter. Few problems were posed by the latter provided that suitably small sections were used whilst the influence of the former were assumed to be constant throughout. However, the solubilisation technique produced samples for counting which varied from clear and colourless to bright yellow due to inefficient bleaching by H_2O_2. It is therefore possible that counting efficiency in the presence of chloroform, used to correct the observed data, may not

accurately reflect "quenching" due to plant pigment debris. Hence, the determination of ^{14}C-bentazone present in treated leaves may represent a slight underestimate which may also have contributed to the low recovery although it is likely that similar reductions would have occurred in the controls.

As discussed in Chapter 3 the procedure for microautoradiographic studies which showed "blanket" uptake by all epidermal cells may be unrepresentative as an investigation of bentazone penetration into C. album leaves to determine the existence of preferential uptake sites. Indeed, it is not possible to determine from the observed response whether the herbicide was absorbed into all cells or simply adsorbed onto the surface of the tissue. Facilities were not available for treatment of intact whole leaves with ^{14}C-labelled herbicides by spray application which may have yielded valuable data, but it is considered that more suitable in vitro approaches may also have successfully pinpointed any preferential uptake sites (e.g. Kirkwood, McKay and Livingstone, 1982). However, details of such techniques were not known and, consequently, the present study employed a method designed to complement both the herbicide penetration and epidermal peel investigations. Indeed, the observation that the herbicide remained bound to the epidermis after the washing procedure may suggest a high affinity of bentazone for this tissue which, in itself, may explain the relatively slow penetration into intact C. album leaves.

Whilst the use of epidermal peels as an experimental system may be open to further criticism, since stomatal movement may be dependent on transport of essential metabolites from the adjacent mesophyll tissue (e.g. Mansfield, Wellburn and Moreira, 1978), it was generally found that stomata in suitably pretreated epidermes responded characteristically to environmental stimuli provided they were obtained from healthy non-stressed leaves. Furthermore, remarkably similar responses were obtained in replicate experiments, although consistency of the data was further enhanced by using peels from a single leaf for each treatment and calculating ΔECLD by taking measurements before and after the 2 hour incubation period. This may reflect the narrow range of stomatal movement in this species (i.e. approximately 5 μm from fully closed to fully open) since

relatively minor differences between leaves would significantly increase the degree of error. However, it is also considered that measurements of stomatal movement, such as ΔECLD, may more accurately depict the response to external stimuli than the commonly-used procedure of simply recording apertures at the end of a treatment without reference to a starting point.

Many other workers in stomatal physiology have incubated epidermal peels on solutions containing biological buffers. This practice has been criticised as such compounds may interfere with certain plant metabolic activities and because they are relatively ineffective at maintaining pH as mentioned in Chapter 4. In addition, many of these chemicals are "zwitterionic" salts of alkali metals and often require the use of stong bases to adjust their pH to a desired range, rendering them particularly unsuitable for studies on a process which may be strongly dependent on the available supply of monovalent cations. Thus, the exclusion of buffers in the present study is considered to be justified. Moreover, only very small pH changes were observed during incubations of C. album epidermes on low volumes of several different treatment solutions under a variety of experimental conditions without buffer, suggesting that pH fluctuations may be negligible when larger volumes are used. However, localised pH changes may occur due to proton build-up or depletion at the surface of epidermes undergoing changes in stomatal aperture which may go undetected and/or result in feed-back inhibition of stomatal movement (C. M. Willmer, personal communication), although this is unlikely to have happened in the present study where peels were vigorously stirred by a continuous flow of air bubbling through the incubation medium.

A further influence on pH of treatment solutions may have been the external CO_2 concentration since this gas may dissolve in water to form a weak acid. Whilst this was probably unimportant for most of the experiments, when only 0-500 p.p.m. CO_2 was studied, it may have been responsible for pH changes when used in concentrations as high as 5000 p.p.m. Although no procedure was devised to measure pH under these conditions, the observed data does not indicate that stomatal apertures were influenced by any pH reduction.

Indeed, whilst the predicted response to reduced pH is stomatal opening due to epidermal cell disruption, almost total inhibition of opening was observed in the presence of these very high CO_2 concentrations. Furthermore, the inclusion of 10^{-4}M $CaCl_2$ in all treatments may have contributed to pH regulation in an experimental system devoid of buffers. The use of Ca^{2+} in epidermal peel studies has been strongly recommended since it may help retain integrity and K^+ specificity of the guard cell membrane Hsiao, 1976), although it is apparently inhibitory to stomatal movement in some species (Raschke, 1979). Hence, although there is no evidence that Ca^{2+} is required for stomatal movement in C. album epidermal peels it has not been found in the present study to be inhibitory and is doubtless present in vivo in the intercellular spaces. Moreover, observations on guard cell ATP ase activity suggest that Ca^{2+} may activate an ATPase isoenzyme associated with stomatal closure in Commelina benghalensis (Raghavendra, Rao and Das, 1976) and thus may be an important co-factor in guard cell cation transport.

The procedure used to regulate CO_2 concentration in these experiments may have had questionable accuracy since it relies on an assumed ambient CO_2 concentration and a completely airtight chamber surrounding the incubation vessels. Laboratory CO_2 concentration was regularly determined at approximately 500 p.p.m. CO_2 using the IRGA circuit described in Chapter 5, and whilst slight deviations may have occurred, they would only represent a small error in the range studied. Furthermore, the sealed chamber used in this investigation was found to give steady values after filling with IRGA calibration gas ($\sim$ 350 p.p.m.) for a period of more than 5 hours although it was not possible to reduce the CO_2 concentration of the air within this vessel to zero by continuous circulation through soda-lime. IRGA measurements under such conditions gave CO_2 concentrations in the range 30-50 p.p.m., perhaps reflecting the efficiency of the absorber which was invariably spent after 2 hours use. It is therefore considered that satisfactory control of CO_2 concentration was achieved but it is not, however, suggested that the imposed atmosphere represented an absolute indication of the intercellular CO_2 concentration, merely that there would be a reasonable degree of proportionality

between the two values.

Routine measurements of ambient CO_2 concentrations using the IRGA circuit were thus found to be an invaluable complement to epidermal peel experiments. However, technical problems associated with the regulation and monitoring of R.H. imposed limitations on IRGA investigations into C. album photosynthesis and transpiration. Several methods were evaluated for humidity control including, for example, passage of air through saturated salt solutions and the mixing of humid air with air dried by $CaCl_2$ or glycerol. Saturated solutions were found to slowly form salt deposits in the tubing and block air flow within the circuit whilst $CaCl_2$ was quickly exhausted and difficult to recharge since it solidified in the drying columns. Glycerol was unable to dry air below 50% R.H. for extended periods but was satisfactory for humidities above this level, eventually absorbing sufficient water to give the required R.H. without supplementation. Consequently, this method was found to be both consistent and economical for lengthy use in the ambient humidity range, provided regular checks were made on the relative flow of dried and humidified air.

The combined humicap sensor and thermocouple obtained from Vaisala U.K. was found to limit the scope of these investigations in several ways. Whilst temperature measurement was found to be fast and accurate the response to a new humidity level by this probe was very sluggish requiring up to 5 minutes to adjust depending on the magnitude of the change. Consequently, the instrument was unsatisfactory for monitoring rapid changes in transpiration. In addition, although the humicap sensor was relatively stable for short-term studies it was prone to drift in the long-term and required daily recalibration. Similar criticisms regarding the speed and accuracy of responses by these probes have been expressed elsewhere (see Tibbitts and Kozlowski, 1979). However, the freshly-calibrated probe was found to respond consistently to a new humidity if 5 minutes equilibration was permitted between readings, thus allowing accurate determination of transpiration. Whilst such measurements of transpiration have proved useful to the present study a more relevant parameter would

have been stomatal diffusive resistance which excludes cuticular water loss. The calculation of the diffusive resistance of stomata to water vapour or CO_2 requires continuous measurement of leaf temperature by incorporation of a thermocouple in the assimilation chamber. Moreover, in order to improve accuracy the circuit should ideally include a water vapour IRGA to give more rapid monitoring of humidity change due to stomatal factors.

In conclusion, this study into the nature of bentazone action on C. album leaf physiology has used a variety of methods which, despite the limitations outlined above, have been found to be particularly valuable in herbicide studies. Specifically, porometry may be used to monitor phytotoxicity prior to the onset of visible symptoms, ^{14}C-herbicide penetration studies may be used to determine optimum application times and IRGA techniques provide a rapid herbicidal screen without recourse to expensive field trials, whilst epidermal peels may eventually prove to be an important in vitro system for detecting transpirational activity of herbicides independent of effects on photosynthesis. However, results obtained from plants reared in growth cabinets may not accurately reflect the field responses. Consequently, whilst confidence may be placed in the procedures described, care is necessary when extrapolating data from growth cabinet studies.

6.2 The mode of action of bentazone in C. album-a general interpretation

The variety of techniques used in this study have provided considerable information regarding the sequence of events leading to C. album injury following bentazone application. Although some of these procedures are subject to limitations, as outlined above, there are no major contradictions in the observed data. Thus, unformulated bentazone was found to penetrate only slowly into intact leaves of this species. However, within a relatively short time after foliar treatment with field-rate bentazone plants exhibited a sharp rise in LDR, indicative of stomatal closure, and subsequent phytotoxicity was correlated with LDR at application time. IRGA studies confirmed that C. album transpiration was rapidly decreased by the herbicide at this concentration and that CO_2 assimilation was inhibited proportionally.

Furthermore, isolated epidermal peel investigations indicated that open stomata closed rapidly in response to bentazone via a mechanism which was not related to increased intercellular CO_2 concentration due to photosynthetic inhibition. Consequently, it is proposed that under normal circumstances slow penetration of bentazone may result in a major portion of the applied dose remaining on the leaf surface, particularly during the first few hours following treatment. Moreover, it is suggested that stomatal closure, induced by bentazone at the leaf surface may lead to inhibition of C. album CO_2 assimilation independently of action at the chloroplast level. Hence, plant damage may result from either gradual inhibition of photosynthetic electron transport associated with slow movement of bentazone across the cuticle to the mesophyll, continued stomatal obstruction to CO_2 uptake or a combination of these effects.

The suggestion that bentazone may inhibit CO_2 assimilation in C. album via a mechanism which involves stomatal closure is a relatively new concept. Indeed, stomatal closure has long been considered as a secondary effect of herbicides due to CO_2 build-up caused by inhibition of photosynthesis (e.g. Smith and Buchholz, 1962). In contrast, more recent investigations have shown, for example, that glyphosate may inhibit net photosynthesis in Phaseolus vulgaris as a direct result of reducing stomatal conductance (Shaner and Lyon, 1979). In the case of bentazone the available evidence suggests that effects on stomata may be a major contributing factor in the mechanism of this herbicide. For example, although bentazone is xylem-mobile after application to Cyperus serotinus under flooded-field conditions, 90% inhibition of CO_2 fixation required 2 days compared to only 4 hours after foliar treatments (Mine and Matsunaka, 1975). Consequently, these authors concluded that "a rapid contact effect" probably plays an important role after foliar application. Furthermore, 6-hydroxy bentazone, the major metabolite of this herbicide in resistant plants, is non-phytotoxic when applied as a root treatment to bentazone-susceptible Sagittaria pygmaca (Mine, Miyakado and Matsunaka, 1975) and has no effect on electron transport in the Hill reaction (Retzlaff and Hamm, 1979). However, after foliar application this derivative caused 70% inhibition of

CO_2 assimilation by susceptible Sinapis alba within the time required by active bentazone to totally halt photosynthesis in the same species (Retzlaff and Hamm, 1976). Complete recovery was eventually observed in plants treated with this metabolite although CO_2 assimilation was still inhibited by 50% 12 hours after treatment. It is therefore inferred that the majority of photosynthetic inhibition caused by bentazone may initially result from stomatal closure, hence limiting CO_2 availability, and that the ability to invoke this response may be retained by the 6-hydroxy derivative although this metabolite is not ultimately phytotoxic.

The relative contribution of the proposed stomatal activity will depend to a large extent on the ability of bentazone to penetrate the cuticle into the mesophyll, which in turn will be determined by the species concerned. Thus, in species with comparatively thin cuticular barriers where absorption may be quite rapid, the herbicide may quickly interfere with chloroplast activity rendering stomatal responses unimportant. However, bentazone is a poor inhibitor of photosynthetic electron transport, requiring some 12 times higher concentrations to inhibit the Hill reaction by 50% than, for example, simazine which is effective at 3.8×10^{-6}M in Spinacea oleracea chloroplasts (Mine and Matsunaka, 1975). Consequently, if uptake through the cuticle is poor as a result of wax composition, quantity or the nature of the leaf surface then effects of bentazone on stomata may assume a wider significance. This may be the case with C. album which has an abundance of hydrophobic epicuticular wax that is likely to present a substantial barrier to the relatively polar bentazone molecule. Indeed, very slow penetration was observed in the present study particularly if stomata were closed when the herbicide was applied. Moreover, the observation that bentazone caused a more rapid increase in LDR, which correlated with plant damage (Chapter 2), following treatment when LDR was low and stomata wide open, rather than partially closed at midday, infers a significant contribution of the stomatal response in this species and may provide an explanation for reports of reduced control in hot dry weather (King, 1976). Under such conditions plants often suffer water stress which results in ABA-induced stomatal closure and although bentazone may in some circumstances cause opening of closed stomata it has been

found during the present study to be unable to reverse this hormonal effect in epidermal peels. Hence, the reduced susceptibility of C. album to bentazone when stomata are only partially closed may develop into a form of resistance to this herbicide when these structures are tightly closed due to water stress.

Tolerance of C. album to bentazone in hot dry conditions was overcome by the incorporation of Actipron into the herbicide mixture. It was assumed that this oil adjuvant afforded better spread which enhanced herbicide penetration and that reduced susceptibility of this weed had been caused by increased epicuticular wax deposition (King and Handley, 1976). This explanation of the observed tolerance was found to be unlikely since quantity and composition of epicuticular wax in this species could not be related to the prevailing environmental conditions (Taylor, 1979), and stomata were proposed as potential uptake sites due to the presence of large apparently wax-free antechambers which may be inaccessible during water stress (Taylor, et al, 1980). Improved uptake of ^{14}C-bentazone by C. album with open rather than closed stomata has been observed in the present study although further investigations are necessary to exclude environmental influences. However, Actipron has undoubtedly been shown to enhance uptake of this herbicide by leaves with closed stomata suggesting that this oil may improve cuticular rather than stomatal penetration. Furthermore, in contrast to the normally observed simultaneous reduction in both photosynthesis and transpiration of intact C. album leaves in response to bentazone alone, the herbicide inhibited CO_2 assimilation well in advance of transpiration when Actipron was present, inferring more rapid penetration to the mesophyll chloroplasts. Indeed, improved control of C. album by bentazone in the presence of this adjuvant was accompanied by increased damage to Phaseolus vulgaris (King and Handley, 1976) which may reflect the enhanced uptake, suggesting that tolerance of certain crops to bentazone may depend on the normally slow penetration, although the injury may have been caused by Actipron itself.

The enhancement of bentazone activity by Actipron observed in Chapter 5 suggests that the efficiency of this herbicide may be manipulated by changing the formulation. Indeed, the rate of inhibition of C.album photosynthesis by $\frac{1}{2}$ field-rate bentazone,

which was usually very slow, was almost equivalent to that produced by field-rate when 0.5% (v/v) aq. Actipron was included and was more rapid if the adjuvant was used at 5%. Incorporation of this oil at levels as high as 5% is unsatisfactory since it is in itself phytotoxic and may lead to crop damage, but it is apparent that Actipron and other surfactant additives may be usefully employed at levels which are above the c.m.c. but sufficiently low to have negligible effects on the crop. In this way herbicide dosage could be reduced significantly with the consequent benefits of lower costs, improved environment and the possibility of increased yields due to less herbicidal damage to the crop, particularly if such measures were employed during dry weather when the inclusion of a surfactant may be desirable for weed control.

Another interesting feature of bentazone action is highlighted by the response of C. album to sub-lethal doses of this herbicide. When only 1/10 field-rate bentazone was applied transpiration was initially stimulated by 20% relative to the control values with a corresponding small increase in CO_2 assimilation. This response is not considered to be an artefact as LDR readings taken prior to treatment indicate that the stomata were relatively closed as discussed earlier, and bentazone has been found to also cause opening of closed epidermal peel stomata of this species. Although both photosynthesis and transpiration returned to control rates within 2 hours no subsequent inhibition was observed suggesting that the effects of this herbicide in vitro may also occur in the intact plant. Indeed, 1/10 field-rate bentazone represents a herbicide solution concentration of 2×10^{-3}M which is five times greater than the optimum for bentazone-induced stomatal opening in C. album epidermal peels but within the region where opening is observed (Taylor, et al, 1980). However, field-rate solutions are within the concentration range where negligible opening occurs. Consequently, the differential response in IRGA studies may simply reflect a previously observed dependence on bentazone concentration. Hence, it may be possible to use bentazone not only as a herbicide but also as an agricultural "tool" to modify transpiration since it has been shown that the stomatal response in epidermal peels may be controlled by manipulation of the

various chemical and environmental stimuli. However, it is not suggested that bentazone at sub-lethal doses may be used to increase productivity, merely that different concentrations could be employed to adjust the degree of stomatal opening in a resistant crop stand. Obviously, the range of uses for such a property is diverse since stomata are mediators of a variety of biotic and non-biotic agricultural processes which are often beyond human control.

Bentazone has already been reported to alter the activity of several different types of pesticide when used in combinations (Sprankle, et al, 1973; 1975; Woldetatios and Harvey, 1977; Campbell and Penner, 1982) and it is possible that the effects on stomata observed in the present study may be responsible for some of these interactions. However, whilst no details of LDR are available from these investigations, it is likely that some form of interaction will occur whenever two chemicals with differing modes of action are applied together. Indeed, bentazone is sold commercially as a mixture with phenoxyacetic acid herbicides, several of which have also been shown to influence stomatal movement. Thus, the effect of bentazone on stomata may be of importance when the herbicide is used in tank-mixtures, although it would be necessary to study the responses in detail before such a practice could be recommended on a wider scale. A more useful application of bentazone may be as a means of inducing stomata to open prior to application of chemicals which penetrate only very slowly since, although there is little evidence concerning the open pore as an uptake route under field conditions, penetration has generally been found in the laboratory to be greater into leaves with more open stomata (Greene and Bukovac, 1974).

The potential of an ability to control stomata is perhaps most obvious in the context of water use efficiency. Thus, the field-worker may wish to conserve the available moisture, for example, to maintain the level of the underlying water-table in forestry or in the agricultural environment to avoid excessive water use in case of drought. Whilst bentazone itself may only be employed in such applications when its herbicidal spectrum was suitable it is possible that the 6-hydroxy derivative may have a more general-purpose use as an anti-transpirant. There is wide scope for such

compounds particularly in regions where large scale irrigation is necessary. In addition, there may be further applications of the stomatal opening response to sub-lethal doses of bentazone in cases where water-logging has occurred due to excessive rainfall or poor drainage. Treatment with low doses may stimulate transpiration to alleviate the problem although it is possible that bentazone may be ineffective under these conditions since the symptoms of flooding are often similar to those caused by other forms of water stress.

The preceding discussion has outlined how the observed influence of bentazone on C. album stomata may contribute to the complete mode of action of this herbicide and suggested ways in which these properties may be useful in the agricultural context. However, it is probable that other species may show differential reactions to bentazone since it is a selective herbicide. Moreover, it is considered that other herbicides may have similar properties which have gone undetected, as in the case of bentazone, because effects on transpiration are not usually investigated in the normal course of screening compounds for herbicidal activity. Indeed, it is important to remember that herbicides often have several target sites which may contribute to phytotoxicity to different degrees depending on the relative physiological activity of the treated plant. Consequently, the desirability of further investigations into the effect on stomatal physiology and transpiration of bentazone and other pesticides cannot be emphasised too strongly.

6.3 The effect of bentazone on C. album stomata - a mechanistic approach

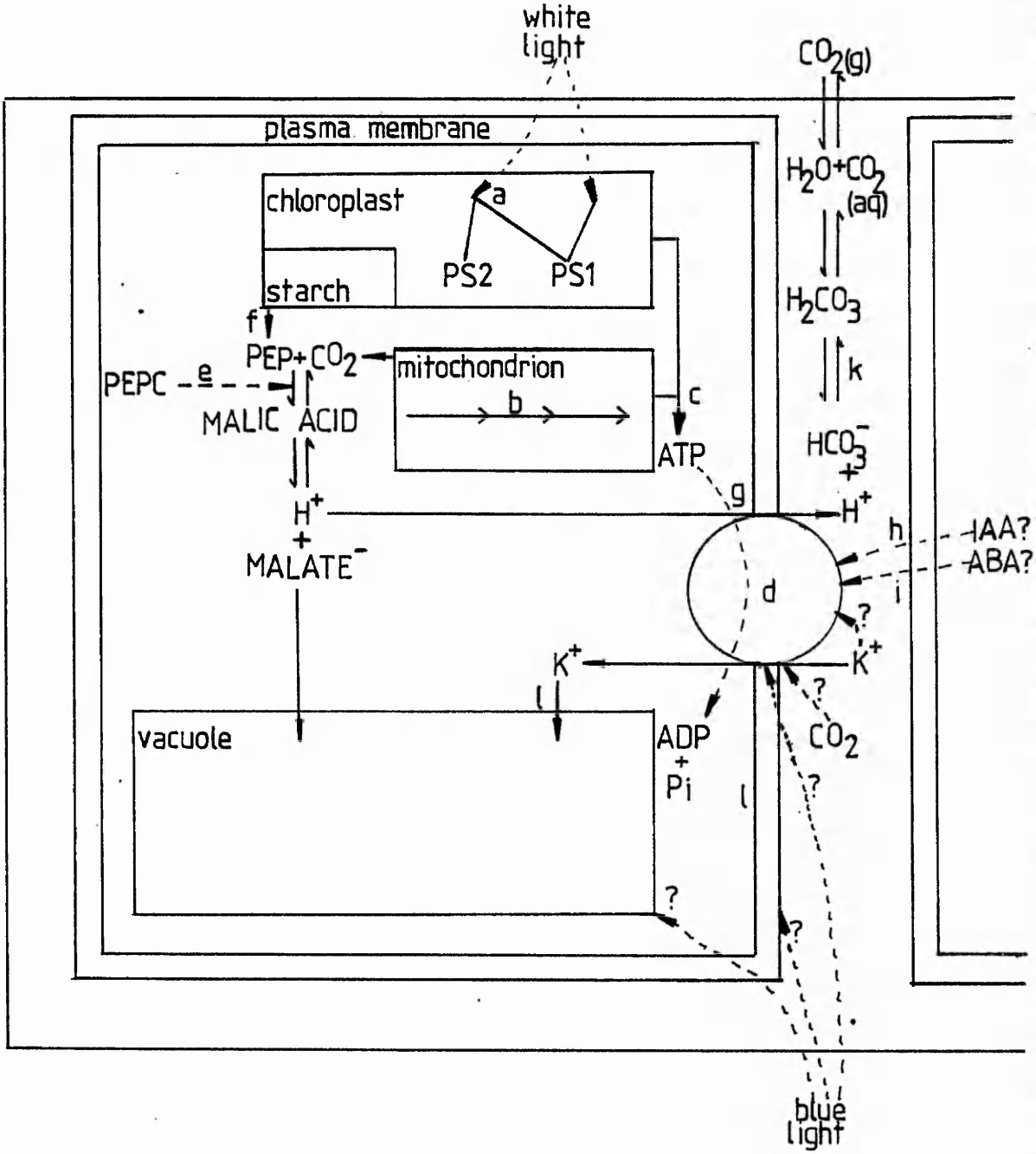
The nature of the bentazone molecule and its well-documented ability to interfere with chloroplast electron transport present a complex background for interpretation of the observed effects on C. album stomatal movement. The schematic representation of the metabolic processes thought to occur within the typical guard cell shown in figure 23 indicates that chloroplasts are often present as in this species and, hence, may be considered as a potential target for this herbicide (Fig. 23 a). Opposed to this suggestion, the stomatal response to bentazone is not characteristic of that to other photosystem II inhibiting herbicides, the action of

FIGURE 23

Schematic representation of possible metabolic processes involved in guard cell function with special reference to potential sites for bentazone action on C.album stomata

KEY:

a	-	chloroplast electron transport
b	-	mitochondrial electron transport
c	-	ATP formation
d	-	electrogenic ion pump
e	-	PEPC activity
f	-	starch metabolism
g	-	nucleotide binding
h	-	pump effector binding
i	-	pump inhibitor binding
k	-	cell wall buffer system


white light
$CO_2(g)$
plasma membrane
H_2O+CO_2 (aq)
chloroplast
a
PS2
PS1
starch
H_2CO_3
f
PEP+CO_2
mitochondrion
k
PEPC
e
b
c
MALIC ACID
HCO_3^-
ATP
H^+
g
H^+
h
IAA?
ABA?
MALATE$^-$
d
i
?
K^+
K^+
?
j
ADP
+
Pi
CO_2
vacuole
l
?
?
?
blue light

which is often reversed by regulation of intercellular CO_2 concentration. Moreover, many of the diverse reactions of C. album stomata to bentazone were observed in darkness indicating that inhibition of guard cell photosynthesis is not essential for stomatal activity since mitochondria would be the primary source of energy under these circumstances.

The effect of bentazone on mitochondrial electron transport and ATP formation is unknown although whole plant data suggests some form of disruption. Thus, if this herbicide influences stomatal movement via uncoupling of respiratory ATP formation (Fig. 23 c) it might be expected to exert similar effects to DNP. Indeed, recent evidence suggests that bentazone and DNP may share a common binding site on the chloroplast membrance (Pfister and Arntzen, 1979) which may indicate a similar thylakoidal mode of action for these two chemicals. However, DNP inhibits light-opening of closed stomata and dark-closure of open stomata (Pemadasa and Koralege, 1977) whilst bentazone may accelerate both these processes under certain conditions and has not been found to inhibit the latter in any circumstances. Furthermore, the failure of exogenous ATP to counteract the bentazone-induced responses in the present study does not favour disruption of the energy supply as a probable mechanism. Even if a portion of the ATP supplied was involved in chelation of free Ca^{2+} ions the majority would still be present in the non-chelated form. Consequently, unless the guard cell membrane of C. album is totally impermeable to ATP some reversal of the effect of bentazone could be expected if the herbicide acts by uncoupling phosphorylation.

The influence of bentazone on chloroplast and mitochondrial energy supply within the guard cell may be an important factor in long-term phytotoxicity but it is unlikely to have a major effect during the immediate post-treatment period. Indeed, guard cells often have substantial starch reserves which may provide energy via glycolysis (Fig. 23 f) whilst experiments with other metabolic inhibitors have implicated cyclic photophosphorylation as a potential source of ATP. Bentazone is not known to directly inhibit either of these processes and the observed rapid stomatal responses are therefore more likely to result from influences of

this herbicide on guard cell turgor maintenance. However, although inhibition of energy supply may not be the cause of C. album stomatal responses to bentazone the possibility that the proton pumps responsible for ion movements are intrinsically linked to mitochondrial electron transport cannot be excluded. Thus, the lack of information regarding the effect of bentazone on the mitochondrial electron transport chain (Fig. 23 b) is prohibitive to further speculation.

Guard cell turgor is mediated by the processes responsible for accumulation of ions, including carboxylation of PEP to produce malic acid (Fig. 23 e) which dissociates to produce malate and protons for exchange with external potassium ions via membrane-bound electrogenic pumps (Fig. 23 d). Enhanced ion accumulation due to low intensity blue light or reduced inter-cellular CO_2 concentration is disrupted by bentazone but, since the mechanism for both these environmental stimuli is unknown, it is difficult to determine if PEP carboxylation is influenced by this herbicide. CO_2 is essential for regulation of the other major carboxylating enzyme in plants RubPC (McFadden, 1980), and may therefore control PEPC activity in a similar fashion. However, to the author's knowledge inhibition of PEPC by high CO_2 concentrations, necessary to explain reduced turgor under these regimes, has not been demonstrated either in C_4 plants or within guard cells. Indeed, most investigators have been unable to detect significant CO_2 fixation by PEPC of intact guard cells, although quite high rates occur in epidermal homogenates, suggesting that this enzyme may be relatively insensitive to extra-cellular CO_2. Hence, the high respiratory rates observed for guard cell protoplasts due to numerous mitochondria may be necessary to supply the CO_2 required for anion formation during stomatal opening.

The additional evidence that fusicoccin may stimulate malate formation and stomatal opening via a mechanism which over-rides the normal CO_2 response does not support direct control of PEPC by CO_2. Stimulation of electrogenic pumps by fusicoccin would be likely to increase cytoplasmic demand for protons and thus drive PEP carboxylation to produce extra malic acid. In the absence of this toxin PEPC activity may be dependent on proton demand from the membrane-bound electrogenic pumps which may themselves be

influenced by CO_2 concentration and, hence, a more likely target for bentazone.

The influence of blue light is difficult to assess since the pigment system responsible for absorbance in this region is currently unidentified. However, photosynthesis is not involved (Sharkey and Raschke, 1981). Furthermore, no specific requirement of PEPC for blue light has been reported and consequently it must be assumed that the most probable mechanism involves regulation of proton pump activity or guard cell membrane permeability to potassium ions. CO_2 may also act at this level but independent effects on the guard cell wall pH should first be considered.

It is now well established that IAA promotes stem extension by stimulating proton extrusion into the cell wall which increases cellulose plasticity and allows cell expansion due to increased turgor. A similar situation may exist in the epidermis since stomatal opening is considered to involve proton efflux into the intercellular spaces. However, in the presence of ambient CO_2 concentrations the following mechanism may lead to the formation of an effective buffering system in the epidermal cell wall:-

1) Atmospheric CO_2 comes into equilibrium with the cell wall aqueous phase:

$$CO_2\ (g) \rightleftharpoons CO_2\ (aq)$$

2) CO_2 (aq) may then form carbonic acid which may dissociate into its composite ions

$$CO_2\ (aq) + H_2O \underset{\text{Slow}}{\overset{\text{pka} = 6.38}{\rightleftharpoons}} H^+ + HCO_3^- \qquad pk_1 = 3.58$$

$$H_2CO_3$$

Thus, under atmospheric concentrations of CO_2 the pka of 6.38 will apply since CO_2 is present mainly as loosely dissolved CO_2 (aq). Indeed, at equilibrium HCO_3^- concentration will be directly proportional to ambient $[CO_2]$. Moreover, in the presence of excess potassium ions a mixture of salt and free acid may create an efficient buffer system since newly-introduced H^+ would automatically

form molecular H_2CO_3. Such a buffering medium would be effective at ambient CO_2 concentrations over the approximate pH range 5.38-7.38 if free exchange with the atmosphere is allowed to occur. However, drastic reductions in CO_2 concentration either during photosynthesis or induced artificially may disrupt the equilibria of the contributing reactions allowing sufficient pH fall to account for stomatal opening due to increases in cell wall elasticity according to the following sequence:

1) $[CO_2]$ (g) falls.
2) $[CO_2]$ (aq) falls.
3) $[H_2CO_3]$ falls.
4) $[HCO_3^-]$ falls.
5) $[H^+]$ rises.
6) Cellulose cell wall becomes more elastic.
7) Stomatal opening occurs.

Similarly, when K^+ is actively exchanged for H^+ via an electrogenic ion pump localised potassium deficiency may lead to a breakdown of the cell wall buffering capacity. Alkaline metal ions are essential to maintain the relative concentrations of the acid and salt components in the pH calculation for buffered solutions:

$$pH = pka + \log \frac{[salt]}{[acid]}$$

Hence, the postulated cell wall buffer system would respond to changes in both CO_2 and potassium concentration of the intercellular spaces. However, it would also be highly sensitive to agents causing excessive K^+ uptake or proton extrusion, and to anions derived from poorly dissociated acids stronger than H_2CO_3 which would effectively increase $[H^+]$. Support for the existence of such a system is provided by the observation that chemicals which enhance stem extension, including IAA and fusicoccin may also cause stomatal opening. However, the interaction of both these compounds with stomatal CO_2 responses (Travis and Mansfield, 1979 a; Snaith and Mansfield, 1982) may simply reflect breakdown of the buffer system at higher than usual CO_2 concentration resulting from increased proton extrusion and K^+ uptake by the guard cells.

Preliminary studies in this laboratory have indicated that bentazone may inhibit extension of Avena coleoptiles via a mechanism which is apparently antagonistic to IAA (Farr, unpublished). Indeed, the pka of bentazone is 3.5 (cf. 3.58 for H_2CO_3) which may create an artificially low pH in the epidermal intercellular spaces comparable to that postulated above at low CO_2 concentrations. Furthermore, this herbicide disrupts stomatal responses to both CO_2 and exogenous potassium ions in a similar fashion to that caused by fusicoccin. Consequently, bentazone may invoke stomatal movements via direct action on the electrogenic proton pumps (Fig. 23 d) or indirectly by disrupting the buffer system outlined above (Fig. 23 k).

Another chemical which produces differential stomatal responses is the gaseous pollutant SO_2 which may cause stomatal opening at low concentration and appreciable closure in high doses (Unsworth and Black, 1981) via a mechanism which also interferes with stomatal CO_2 responses (Majernik and Mansfield, 1972). Of course, SO_2 may form a weak acid in solution (pka 1.9) although it is considerably stronger than those produced by either CO_2 or bentazone. Consequently, this gas may disrupt cell wall buffering capacity and the relatively low threshold level between the opening and closure responses (0-500 ppb SO_2) may reflect the greater strength of the acid produced. Furthermore, SO_2 is a major by-product of bentazone photodegradation which occurs quite rapidly either in solution or as a thin film on glass (Nilles and Zabik, 1975). For example, after 24 hours irradiation in a photoreactor 15% of the original bentazone had been degraded to three different products all of which required evolution of SO_2 for their formation. Thus, the observed responses of C. album stomata to this herbicide in light may be indirectly caused by SO_2 emission resulting in a differential reaction dependent on the concentration of bentazone used. However, no such degradation has been reported in darkness suggesting that the herbicide itself may cause independent stomatal responses which are similar to those induced by SO_2 or separate characteristics of the bentazone molecule.

High concentrations of SO_2 in plant tissue would ultimately lead to membrane damage which may contribute significantly to

phytotoxicity. Hence, bentazone may similarly disrupt the guard cell membrane tranport system via this mechanism (Fig. 23 l). However, experiments with Beta vulgaris discs have been conducted on several occasions in this laboratory and bentazone has not been found to cause betacyanin efflux under any of the conditions studied, suggesting that membrane integrity is unaffected by this herbicide. The rate of SO_2 emission from bentazone may therefore be insufficient to produce membrane damage at normal herbicide concentrations, but further study is needed for confirmation.

The remaining potential target of bentazone in the stomatal complex is at the proton pumps responsible for potassium transport. As mentioned previously bentazone, like fusicoccin may interact with IAA-enhanced coleptile extension due to proton extrusion. Consequently, all three chemicals may similarly influence proton pumps in guard cells although the involvement of IAA in stomatal movement in vivo is not confirmed. However, support for bentazone action at the proton pump is provided by the inhibition of the normally observed C. album stomatal response to exogenous nucleotides. Since such pumps are likely to be associated with ATP ase activity to provide energy the response to nucleotides may result from binding to this enzyme. Thus, interference by bentazone with stomatal movement induced by ATP or ADP suggests an alteration of the binding kinetics of those nucleotides (Fig. 23 g). Binding may be dependent on the presence of suitable effectors or inhibitors, which may include the plant hormones IAA and ABA (Fig. 23 h and i), to control stomatal responses to the environment. Hence, bentazone may interfere with guard cell ion transport by disrupting hormone activity. However, no specific requirement for either of these hormones has been demonstrated as necessary for the normal function of stomata in non-stressed plants.

Since bentazone interferes with the normally observed response of C. album stomata to light, CO_2 and KCl these factors may also be involved in the function of the pump. However, the complex nature of the interactions makes competitive inhibition unlikely since the mechanism of electrogenic ion transport in guard cells may depend on the concentration of several or, indeed, all of the factors outlined. Hence, bentazone may directly or indirectly disrupt the binding of various co-factors necessary for proton pump activity.

The elucidation of how this might occur is dependent on further studies of bentazone action using epidermal peels, guard cell protoplasts and isolated cell fractions to determine the precise influence of this herbicide on stomatal metabolism.

6.4 Proposals for further investigations

Considerable progress towards a fuller knowledge of the mode of action of bentazone as a herbicide for C. album has been achieved in this study. Moreover, the influence of this herbicide on stomatal movements may not only be an important contributor to phytotoxicity in susceptible species but also of considerable value in agriculture if the observed properties can be replicated under field conditions. However, several questions regarding both field use and the precise mechanism of bentazone action at the guard cell level remain unanswered and, hence, may form the basis of further research into this herbicide. Furthermore, it is suggested that any future studies should include 6- and 8-hydroxy bentazone since these metabolites may have great potential as non-phytotoxic anti-transpirants and consequently as tools for use in stomatal physiology. Indeed, investigation of these derivatives alone may constitute a major study worthy of detailed attention.

The species specificity of the observed effects of bentazone on stomata is unknown and it is possible that investigations into resistant and susceptible species may show differential responses which form a basis for selectivity as suggested for certain other herbicides (Das and Santakumari, 1975). Preliminary experiments need only monitor changes in LDR of different species in response to bentazone for a relatively short period with the porometer described in the present study. Indeed, differences in the reported transpiration response to bentazone treatment of resistant Glycine max and susceptible Cirsium arvense (Penner, 1975) indicate that such an approach may provide useful information. Thus, if the initial data showed stomatal activity then important species could be selected for detailed investigation of intact leaf photosynthesis and transpiration which, in combination with epidermal peel studies, may determine the involvement of the stomatal response in crop tolerance and pinpoint differences between

species in the function of these structures.

Further characterisation of the response to reduced doses is also desirable since the effects of application of 1/10 field-rate bentazone to C. album were not investigated beyond 24 hours when photosynthesis was little different to controls. Indeed, species which absorb the herbicide more easily may be more sensitive to sub-lethal doses and it is probable that the most suitable concentration may be dependent on the nature of the leaf surface and the quantity of surfactant additives used. Such information, therefore, would be useful as a background to field studies to determine if manipulation of LDR in a crop stand is a feasible proposition. Development of the ability of bentazone to modify LDR in the field would require accurate knowledge of stomatal status at application time which may be obtained using a porometer, although it is recommended that other types of porometer be evaluated for field use.

The enhanced penetration of bentazone in the presence of Actipron is also worthy of further attention since the actual mechanism resulting in improved uptake is not known. It is suggested that "dual-label" experiments using ^{3}H-Actipron and ^{14}C-bentazone may be usefully employed to determine if the adjuvant moves into the leaf with the herbicide or acts at the cuticle to modify the morphology of the epicuticular wax or the structure of cutin matrix to improve absorption of the herbicide only. Similar investigations with other surfactants may also be valuable as the contribution of mineral oils to the phytotoxicity of herbicide mixtures may be appreciable. Indeed, information derived from such studies may help to clarify some of the apparent contradictions in the literature regarding the mode of action of these additives as a means of enhancing herbicide penetration and improving weed control.

Modification of stomatal aperture is clearly involved in the complete mode of action of the herbicide bentazone. However, whilst the present study has characterised the influence of bentazone on C. album epidermal peel stomatal movement in relation to potassium ion availability and certain environmental parameters, further studies are essential, especially in other species, to determine the mechanism of these responses. Firstly, since inhibition

of chloroplast electron transport or photodegradation of bentazone may be involved in the observed stomatal activity, it is considered that additional epidermal peel experiments in light are necessary to complete the investigations conducted in darkness only. Moreover, fluorescence studies of guard cell chloroplasts would provide a simple means of observing herbicide effects on photosynthesis without recourse to complex extraction procedures required for investigations of O_2 exchange. In addition, other studies may employ epidermal peels to study antagonism between plant hormones, fusicoccin, bentazone and other metabolic inhibitors with more "precise" targets.

The involvement of the guard cell wall matrix in stomatal responses to CO_2, potassium and bentazone, outlined earlier in this Chapter, may be excluded by experiments on guard cell protoplasts. These bodies, recently extracted by enzyme degradation of the epidermis have been found to swell in blue light and shrink in the presence of ABA (Zeiger, 1981) suggesting that they are functional. Indeed, protoplasts have become a popular experimental system for stomatal physiologists in the study of, for example, ionic relations and enzyme activity of guard cells. Moreover, these structures may provide easier access to subcellular organelles which may then be used to study bentazone activity. Thus, chloroplasts, mitochondria, nuclei, vacuoles and various membrane fractions may be extracted from guard cell protoplasts and purified to allow metabolic investigations previously impossible within the confines of epidermal peel studies.

The possibility that bentazone affects guard cell proton pump activity is perhaps the most difficult area for future study since pure plant membrane preparations are rare. The origin of such fractions is notoriously difficult to determine (Leonard and Hodges, 1980) and whilst the structure of ATPases associated with bacterial, mitochondrial and chloroplast transport systems is now quite well-defined, little progress has been made for those associated with the tonoplast and plasma membranes. Moreover, proton pumps on these membranes are more likely to become uncoupled during extraction since they are not enclosed within an organelle. Indeed, a more detailed investigation of the effect of bentazone on IAA enhanced

coleoptile extension may yield more relevant information regarding the proton pump activity of this herbicide. However, the continuing development of guard cell protoplast techniques may soon yield a method for the preparation of membrane vesicles with sufficient ATPase activity to permit studies of the binding kinetics of hormones, nucleotides, potassium ions and CO_2 as influenced by bentazone. Hence, it is considered that further elucidation of the mechanism of stomatal activity of this herbicide may be possible in the near future.

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THE INVOLVEMENT OF STOMATA IN BENTAZONE ACTION IN CHENOPODIUM ALBUM, L.

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Summary. Leaf diffusive resistance porometry has shown that in a controlled environment stomatal movement in C. album follows a circadian rhythm with pre-dawn opening, pre-dusk closure and partial midday closure. Application of bentazone caused stomatal closure and disruption of this rhythm, particularly when applied during the mid-morning period when the stomata were open. This response was less apparent following midday applications and the corresponding herbicidal efficacy was also reduced. Penetration of ^{14}C-bentazone into C. album leaves was found to be relatively slow but also influenced by the time of day of application. However, the addition of the surfactant Actipron to the formulation enhanced penetration regardless of application time. Further data has shown that bentazone exerts rapid and significant reductions in both intact leaf photosynthesis and transpiration, and it is suggested that stomatal closure is of importance in the complete mode of action of bentazone in this species. Herbicide, fat hen, porometry, penetration, transpiration, photosynthesis.

INTRODUCTION

The susceptibility of weeds to bentazone is influenced by both the prevailing environmental conditions (Davies et al., 1979) and the time of day of application (Doran and Andersen, 1976). Stomatal movement is also under tight environmental and physiological control (Jarvis and Mansfield, 1981), and in C. album bentazone has been shown to significantly influence stomatal movement in epidermal peels (Taylor et al, 1980).

This paper examines the effect of time of day of bentazone application in relation to stomatal movement and herbicide penetration in intact leaves of C. album, and also monitors leaf photosynthesis and transpiration in the presence or absence of the herbicide.

METHODS AND MATERIALS

(1) Plant growth conditions: In all experiments the youngest fully expanded leaf was used from 4-6 week old C. album plants grown in a 1:1 mix of J. Arthur Bowers potting compost and John Innes No. 2 compost in Fisons 600G3 growth cabinets. A 16h photoperiod was provided by Atlas Grolux and Warmwhite fluorescent tubes at a photon flux density of 200$\mu E.m^{-2}.s^{-1}$ (P.A.R.) and temperatures maintained at 20°C (day) and 12°C (night).

(2) Measurement of leaf diffusive resistance: Adaxial leaf diffusive resistance (LDR) was determined in the growth cabinet using a Crump Automatic Diffusive Resistance Porometer, which functions by measuring the time taken for the humidity in the sensor cup surrounding the leaf to rise between two pre-set levels. The porometer was calibrated using perforated plates before each set of readings was taken. Up to 20 readings of different leaves could be obtained in a 30 min period, although no single plant was measured more than twice in one day to avoid leaf damage. Thus, for example, 96 plants were monitored over a 10d period to obtain the data in Fig. 1. For herbicide studies, 20 plants were sprayed with field-rate bentazone (3l in 280l water. ha^{-1}; formulation BAS 3517H, containing 48% (w/v) a.i.bentazone) at either 0930-0945h or 1215-1230h using a Binks-Bullows gravity-feed sprayer at 1 bar pressure

whilst a further 20 plants were left untreated to act as controls. LDR was monitored at 0900-1030h and 1200 - 1330h for 6d before and 5d after when chlorotic symptoms began to develop.

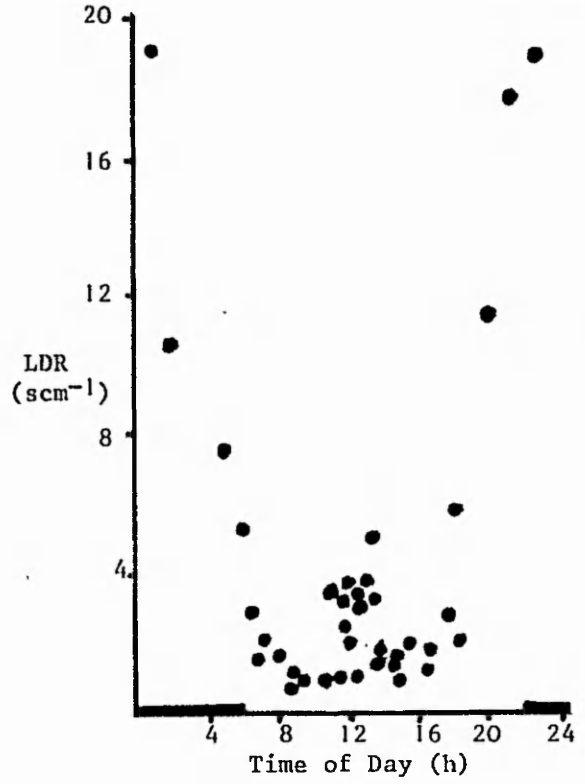
(3) ^{14}C-bentazone uptake by C. album leaves: Penetration of ^{14}C-bentazone into the youngest fully expanded leaf of known LDR was determined according to Pallett and Caseley (1981) at either 0930 or 2030h. Five 0.5µl drops of field-rate bentazone containing 2.5 x 10^5 dpm total activity, were applied to each of 4 leaves using a Burkard microsyringe. After 0, 24, 48, 72 and 168h in the growth cabinet the treated leaves were excised and washed in 10cm^3 0.5% (v/v) Actipron, and the radioactivity in the washings determined by liquid scintillation counting.

(4) Measurement of C. album photosynthesis and transpiration: C. album leaf photosynthesis was continuously measured for 5h after application of field-rate bentazone at 0930h using an Infra-Red Gas Analysis (IRGA) circuit, as described by Dunleavy (unpubl.). Light intensity was maintained at 750µE.m^{-2}.s^{-1}, temperature at 20°C and relative humidity at 60 ± 10% during measurement. Photosynthesis was calculated by determining CO_2 depletion rates according to Gaastra (1959). Transpiration was measured in a similar fashion by monitoring increase in r.h. using a Vaisala HMP 13ST sensor.

RESULTS

Fig. 1

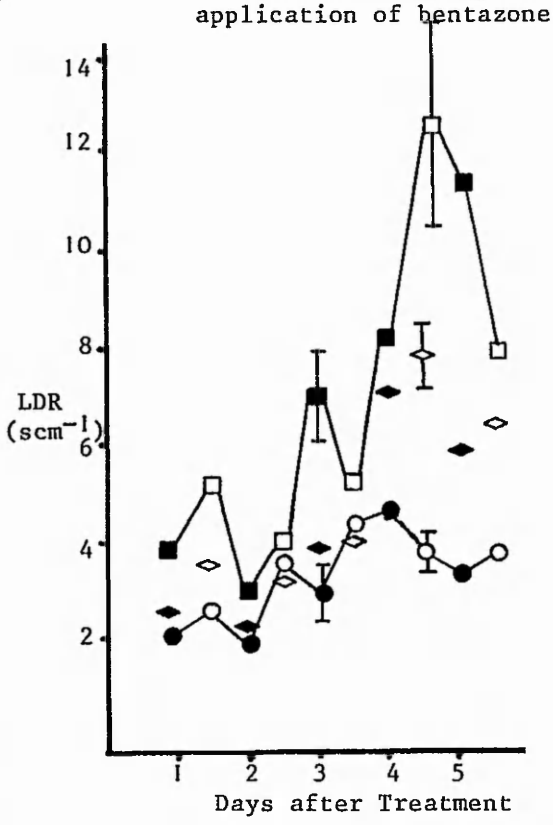
Relationship between C. album LDR and time of day



Each point represents the mean of upto 20 measurements of different leaves taken within a 30 minute period.

Fig. 2

Changes in C. album LDR after application of bentazone



Closed symbols measured 0900-1030h; open symbols measured 1200-1330h; ●—○, control; ◆—◇, sprayed 1230h; ■—□, sprayed 0930h. Bars represent S.E. values, n = 15.

The relationship between LDR and time of day in C. album leaves (Fig. 1) infers a circadian rhythm of stomatal movement in this species when grown under the conditions described, with pre-dawn opening, pre-dusk closure, and a tendency to close around midday. Minimum LDR, and hence maximum stomatal opening, is observed both before and after the midday closure period. Fig. 2 shows the response of C. album LDR to bentazone application. Although control plants show midday closure throughout the period of observation, plants sprayed with field-rate bentazone at 0930h show departures from this rhythm in two major respects. Firstly, treated plants show higher values than the controls, and secondly, the rhythm in LDR is interrupted by bentazone application. Thus, plants treated at 0930h show a marked increase in LDR one day after treatment and after day 2 the expected midday closure is lost, culminating in further stomatal closure by day 6. However, plants sprayed during the midday closure period (1230h) show a more gradual increase in LDR, and only begin to deviate from control values 4 days after treatment. Furthermore, fresh and dry weight determinations show that more damage is shown in plants sprayed at 0930h than at 1230h (Table 1).

Table 1

Fresh and dry weights of plants before and 7 days after treatment with field-rate bentazone or water at either 0930 or 1230h

Time of day of Treatment	Days after treatment 0		7	
	Fresh Weight	Dry Weight	Fresh Weight	Dry Weight
Control	1.16 ± 0.5	0.116 ± 0.050	3.83 ± 1.3	0.415 ± 0.160
0930h	-	-	0.98 ± 0.37	0.154 ± 0.030
1230h	-	-	3.07 ± 0.50	0.303 ± 0.100

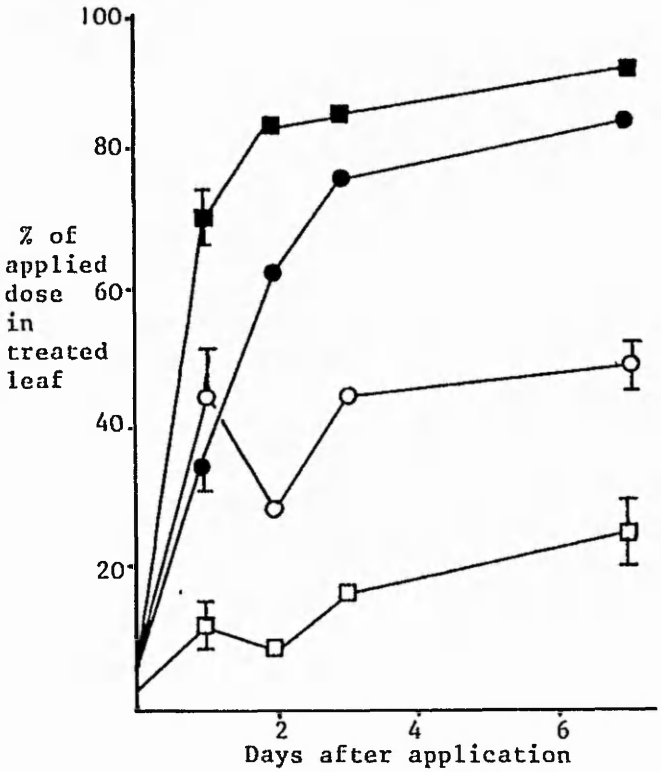
(Weights in g; values expressed as means ± S.D. where n = 8)

Fig. 3 shows that the penetration of ^{14}C-bentazone into intact C. album leaves is approximately doubled if the herbicide is applied at 0930h when mean LDR is 1.67s.cm^{-1} and stomata open, than if applied at 2030h when mean LDR is 15.6s.cm^{-1} and the stomata effectively closed. However, the addition of 0.5% (v/v) Actipron to the formulation ensured a more rapid penetration and reversed the time of day response.

Fig. 4 clearly illustrates the time-course of inhibition of both whole leaf photosynthesis and transpiration by field-rate bentazone. Total inhibition of photosynthesis occurred after 4h of measurement and no subsequent recovery was observed, whereas the inhibition of transpiration took only 2.5h.

Fig. 3

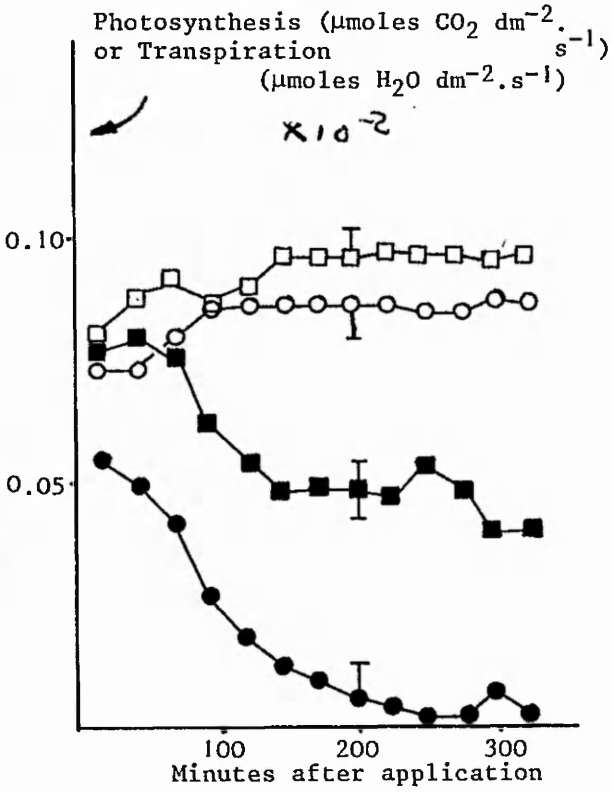
Penetration of ^{14}C-bentazone into C. album leaves in the presence or absence of 0.5% (v/v) Actipron



Open circles, bentazone, applied 0930h; closed circles, bentazone & actipron 0930h; Open squares, bentazone, 2030h; closed squares, bentazone & actipron, 2030h. Bars represent S.E. values.

Fig. 4

Effect of bentazone on C. album leaf Photosynthesis and Transpiration



Open circles, control leaf photosynthesis; closed circles, treated leaf photosynthesis; open squares, control leaf transpiration; closed squares, treated leaf transpiration. Bars represent S.E. values.

DISCUSSION

The data presented above clearly shows that when C. album is grown in a controlled environment, leaf adaxial stomata show a pronounced circadian rhythm of movement, and that LDR is altered by bentazone application. Some supporting data has been obtained with field-grown C. album, although rapid changes in leaf external environment make porometer readings more variable and prone to error (Dunleavy,(unpubl.). However, this study, and a previous one using abaxial epidermal peels (Taylor et al., 1980), clearly show that stomatal movement in this species is affected by bentazone.

The observation that ^{14}C-bentazone penetrates C. album leaves more readily following 0930h application than at 2030h presents further supporting evidence for stomatal involvement in bentazone uptake. However, more than 30% of the applied dose could be washed off the treated leaf 7 days after application, indicating that bentazone uptake is a slow process in this species (c/f < 10% after 24h in the case of rapid ^{14}C-difenzoquat plus Agral uptake in wheat, Pallett and Caseley, 1981). Nonetheless, uptake was considerably improved in the presence of 0.5% (v/v) Actipron.

Previous studies in this laboratory have shown that C. album leaf surfaces are well endowed with water-repellent epicuticular wax platelets but that the guard cells

and antechambers of the stomatal complexes appear virtually wax free (Taylor et al., 1981). Direct bentazone passage through the stomatal pore is considered unlikely since actual pore dimensions are very small (1-2μm), although the presence of Actipron may facilitate this by reducing the surface tension of the herbicide formulation (Schönherr and Bukovac, 1972). It is considered more likely, however, that the important role of Actipron in this case is to provide better coverage of the leaf surface and thus expose a greater proportion of the epidermis to the herbicide. Indeed, the clear evidence that bentazone causes an inhibition of transpiration more rapidly than of photosynthesis (Fig. 4) supports the earlier data in Fig. 2, and suggests that the initial effect of bentazone application may be to close stomata thus limiting the CO_2 supply for photosynthesis. Later cumulative penetration of the herbicide may then inhibit photosynthesis at the chloroplast level by blocking electron transport, and eventually plant death.

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