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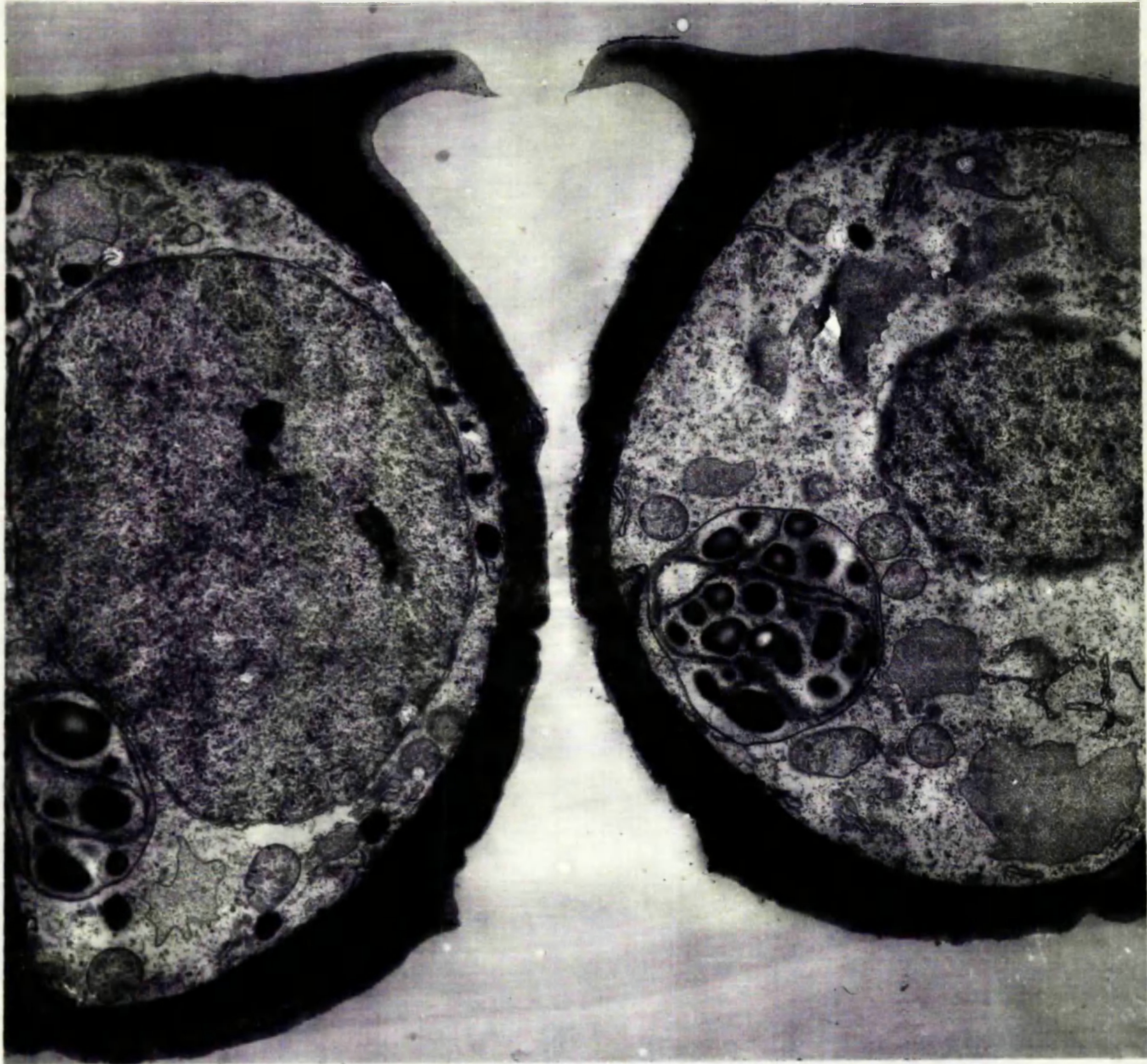
THE HORMONAL CONTROL OF STOMATAL APERTURE IN *COMMELINA COMMUNIS* L. BY ABSCISIC ACID

by

Jonathan D. B. Weyers, B.Sc.

December
1978



Frontispiece Stomatal throat of *Commelina communis* lower epidermis (TS *ca.* 10,000 x)

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to Mary,

Elspeth and John.

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ABBREVIATIONS

Abbreviations according to the Systeme International d'Unites (SI) as interpreted by Morris (1974) were used. Temperatures were recorded on the Centigrade (C) scale rather than tha Kelvin scale. The usual symbols for elements and molecules were used. Other specialised abbreviations included were -

ABA - Abscisic Acid
A.E.S. - Automatic External Standard
amu - Atomic Mass Units
bg - Background
cv. - Cultivar
D - Distribution Ratio
DE - Diethylaminoethyl Cellulose
d.w.(DW) - Dry Weight
E - Efficiency
ECD - Electron Capture Detection
Et_2O - Diethyl ether
Fig. - Figure
FID - Flame Ionisation Detection
f.w. - Fresh Weight
GLC - Gas-liquid Chromatography
IAA - Indole-3-acetic Acid
IR - Infra-red
Km - Substrate concentration at 0·5 maximum initial velocity of an enzyme-catalysed reaction (mol m^{-3})
λ - Wavelength
Me - Methyl
m/e - Mass-to-charge ratio
MES - 2(*n*-Morpholino) ethane Sulphonic Acid
MeOH - Methanol
MS - Mass-spectrometry
M.S.A. - Mean Stomatal Aperture
n - Number of Units Measured
NR - Non-random

PEGn	-	Polyethylene Glycol of chainlength n
pH	-	$-\log_{10}[H^+]$
PIPES	-	Piperazine-*NN'*-bis-2-ethane Sulphonic Acid
ψ	-	Water Potential
PVP	-	Polyvinyl Pyroltidone
R	-	Random
RH	-	Relative Humidity
S.E.	-	Standard Error
STP	-	Standard Temperature and Pressure (20 C, 101·3 KPa)
TIBA	-	2,3,5, triiodobenzoic Acid
TLC	-	Thin-layer Chromatography
$t_{\frac{1}{2}}$	-	Half-life
UV	-	Ultra-violet
var.	-	*Varietas*
Vmax	-	Maximum initial velocity of an enzyme catalysed reaction (mol s^{-1})

SUMMARY

The experiments of this thesis were concerned with the involvement of abscisic acid (ABA) in the control of stomatal closure following water stress. The hypothesis that this control is exerted in a truly hormonal manner was introduced by considering the evidence implicating wilt-induced ABA in certain post-stress stomatal responses in relation to present concepts of phytohormone action.

The results are presented in seven sections, each dealing with a specific aspect of the investigation. Section 1 was concerned with the development and characterisation of experimental systems whereby the effects of ABA on stomata of isolated leaf epidermis segments of *Commelina communis* could be studied. The importance of such factors as the ionic content of the incubation medium, the physiological state of the source leaf and methods of aperture measurement were assessed. Stomatal opening could be influenced by adding KCl to the basic 10 mol m^{-3} PIPES buffer (pH 6·8) incubation medium. Levels of the salt greater than 100 mol m^{-3} led to apertures in excess of 10 μm after 2 h, but when ABA treated (0·1 mol m^{-3}), closure was not complete. If segments were floated on 50 mol m^{-3} KCl buffer, adequate and reproducible opening was obtained, and when transferred to ABA-containing solution, rapid ($<$ 15 min) and complete ($<$ 0·3 μm) stomatal closure was observed. This medium was therefore used for all other experiments. Addition of Ca^{++} to the medium was found to result in closure of stomata and was not otherwise carried out. Senescent leaves yielded segments which were less sensitive to ABA than the youngest fully-expanded leaves normally used. Prior water stress to individual leaves or whole plants over short (0-5 h) and long (6 d) periods resulted in inhibited stomatal opening.

In Section 2, ^{14}C-labelled ABA was presented to epidermis tissue floating on buffer solution. The subsequent uptake of radioactivity was assayed quantitatively and qualitatively using the techniques of scintillation spectrometry, TLC, and soluble-compound microautoradiography. Uptake, which was a function of the presence of living cells, appeared to occur in such a fashion that radioactivity from ^{14}C-ABA accumulated in the guard cell region. Approximately 5·9 fmol ABA per stomatal

complex had been taken up at the time of stomatal closure. The efflux of radioactivity from tissues was relatively rapid and 3 exponential decay phases of efflux rate could be distinguished within 2 h. These appeared to correspond to efflux from free space and living tissue compartments. Efflux was correlated with stomatal re-opening, indicating that the ABA content of the tissue controlled stomatal opening potential. The kinetics of this re-opening were similar to those of stomata on epidermis from pre-stressed leaves. These results were discussed in relation to possible transport routes for endogenous ABA synthesised in the mesophyll.

Section 3 involved the use of $^{86}Rb^{+}$ as a tracer for K^{+}, the major cation involved in stomatal movements. $^{86}Rb^{+}$ was taken up by tissue into the guard cell region, and both the pattern of this uptake and the rate of subsequent efflux was found to be affected by ABA treatment. When supplied continuously, ABA resulted in a lower proportional uptake of $^{86}Rb^{+}$ into the guard cell region. The rate of efflux of the tracer after 2 h uptake was increased compared to controls if tissue was then placed in ABA-containing solution. This effect was confined to the first 10-15 min of efflux, correlating with the time taken for closure to occur. These results were discussed in relation to the mode of ABA action.

The fourth section described the development of a whole-leaf incubation system which was then used to examine the effects and movement of ^{14}C-ABA supplied *via* the transpiration stream. Autoradiographic evidence indicating guard-cell uptake of the hormone corroborated the conclusions of Section 2. In addition, it was possible to obtain 'upper limit estimates' of the sensitivity of *Commelina* stomata to ABA utilising the ability of this plant to yield epidermal peels. Thus, it was found that significant stomatal closure could be effected by as little as 45·5 amol ABA per stomatal complex. Complete closure (within 40 min) apparently required over 230 amol per complex.

Section 5 provided a measure of the ability of *Commelina* leaf tissue to synthesise ABA during a short-term (0-5 h) water stress. Levels of the hormone estimated by GLC-ECD rose from 31 to 178 ng per gramme fresh weight after 3 h incubation of tissue which had lost 10% of its fresh weight. Investigation of optimal ABA purification procedures was also carried out.

An attempt was made in Section 6 to examine the ultrastructure of *Commelina* epidermis. Plasmodesmatal junctions between cells were observed in all cell walls except those between guard cells and subsidiary cells. The relevance of this finding in relation to intercellular transport routes and mechanisms was discussed.

The seventh and final section described the transport of radioactivity from ABA in the phloem sap of *Ricinus communis*; Double-label techniques were used to study co-transport of ABA and sucrose. The hormone appeared to be readily translocated. GLC-MS was used to identify free ABA in *Ricinus* phloem sap.

In the General Discussion the results were considered in relation to the concept of the hormonal nature of wilt-induced ABA action, and areas of research deserving of further attention were identified.

GENERAL INTRODUCTION

This thesis is concerned with the hormonal nature of the involvement of abscisic acid (ABA) in stomatal closure following wilting. To introduce this theme it appears relevant, therefore, to discuss present concepts of plant hormones in general before assessing current knowledge about ABA in particular. This is followed by a survey of the evidence implicating wilt-induced ABA in certain stomatal responses, and comments on the nature and mechanism of guard cell movement. Accordingly, the introduction is split into four sections.

SECTION I. THE PLANT HORMONES

Advanced multicellular organisms integrate and correlate the relationships between organs, tissues, and cells. This fundamental requirement for complexity of construction and function is achieved during the processes of growth, development and reproduction, and in response to changes in the environment. Thus, the evolutionary success of a species is at least partly dependent on the establishment of efficient mechanisms whereby coordination can be effected (Barrington, 1964). It appears that the nervous and hormonal systems have evolved to fulfil this need.

In plants, hormonal control is believed to predominate, although 'nerve-like' plant responses have been described (Sibaoka, 1969). From the many molecules known to be synthesised by photosynthetic organisms, indole acetic acid, gibberellins, cytokinins, and abscisic acid have been given 'plant hormone' status in the literature, with the classification of ethylene debated. However, despite the wealth of experimental work on these compounds, little in the way of unequivocal evidence for physiological roles of the phytohormones has accumulated. In this section of the introduction methods of evaluating plant hormone action are discussed.

Historical

Credit for the initial concept of hormone action is generally attributed to Bayliss and Starling (1902, 1904). From experiments on animals they deduced that a blood-carried secretion of the intestinal wall stimulated pancreatic activity. They suggested that a number of

animal reflexes could be explained by the action of specific organic substances produced in one organ and transported in the blood stream to a site of action in another. Starling (1905) christened these chemical messengers 'hormones', from the Greek *hormaein*, to stimulate. Huxley (1935) reconsidered the original hormone concept taking into account later information on the variety of chemical control found in biological systems. He proposed the general term *activators*, defined as: 'chemical substances produced by the organisms, which exert specific functions in regard to correlation of differentiation'. This included 'parahormones', 'chemical transmitters', 'chemodifferentiators' and 'circulatory' and 'diffusion' hormones. The title *hormone* was reserved 'to cover all cases in which transport from tissue of production to tissue of action is found', whatever the *means* of translocation.

The great 19th century plant physiologists (e.g. von Goebel, Jost, Sachs, Pfeffer) discovered that many aspects of plant growth involved phenomena of correlation and irritability. Controversy existed (as it does in some cases now), concerning the importance of nutritional factors or the action of 'formative substances' carried in the 'saps'. It was through the study of tropic movements, however, that the idea of hormones in plants first developed. Darwin and Darwin (1880) showed that in coleoptile phototropism and root geotropism, the regions of stimulus perception and of adjustment growth were spatially separated. They concluded: 'some influence is transmitted from the upper to lower part, causing the latter to bend'. Later, Boyson-Jensen (1913) showed that the 'influence' could cross a wound gap and Paál (1919) demonstrated that it would pass a layer of gelatin, but not mica nor platinum foil. Went (1926, 1927) confirmed the chemical nature of the 'influence' by allowing it to diffuse from isolated coleoptile tips into agar blocks; when the blocks were asymmetrically applied to coleoptiles without tips, a curvature reaction occurred. Paál is said to have introduced the concept of a plant growth hormone, but he used the term 'correlation carrier'. In fact, Fitting (1909, 1910) had earlier mentioned the word 'hormone' to describe a substance or substances involved in post-flowering phenomena of tropical orchids. Nevertheless, study of tropic reactions stimulated the initial interest in plant hormones.

Nomenclature and Definition

The word 'phytohormone' was introduced at an early stage to distinguish plant hormones from those of animals, but several other names have since been preferred by specific authors. The generic synonyms include: *Plant regulators, plant growth regulators, plant growth hormones, plant growth substances* (see Tukey *et al.* 1954), and the more esoteric ergons (Larsen, 1955), *bios* (Löve and Löve, 1945), *correlation carriers* (Paál, 1919) and *semiochemicals* (Birch, 1974). An attempt was made to propose a uniform defined nomenclature (Tukey *et al.*, 1954), but agreement has been by no means universal (Larsen, 1955; Audus, 1972).

Whilst it is clear that restricted definitions limit practical usage (Van Overbeek, 1950), due regard must be made for established etymological meaning (Larsen, 1955) and scientific precedent (Huxley, 1935). The definition of 'hormone' which appears in Webster's Dictionary (1961) is: 'a specific organic product of living cells that, transported by body fluids or sap, produces a specific effect on the activity of cells remote from its point of origin'. Pincus and Thimann (1948) considered that a 'phytohormone' was: 'an organic substance produced naturally in higher plants, controlling growth or other physiological functions at a site remote from its site of action, and active in minute amounts'.

According to these definitions, man-made compounds not known to occur in plants cannot be termed hormones, and the term 'plant regulators' or 'plant growth regulators' was proposed by Tukey *et al.* (1954) to include natural *and* artificial compounds. They defined *regulators* as 'organic compounds', other than nutrients, which in small amounts promote, inhibit or otherwise modify any physiological process in plants'. However, this term was objected to by Larsen (1955), who claimed that the established definition of the verb *to regulate* was: 'to adjust so as to work accurately and regularly'. He maintained that many of the man-made compounds did not fulfil this definition in their effects, and proposed the terms *growth substance* and *growth hormone* to cover artificial and endogenous compounds respectively. Latterly, Cram (1976) has distinguished the terms

regulate and control, stating the *regulation* should refer to 'adjustment with regard to some law, standard or reference ....', whereas *control* describes in a more general sense: 'the exercise of restraint or direction upon'. The term 'regulate' should thus only be used to describe immediate effects on specific processes. As most experimental plant systems are complex, and the mode and site of action of phyto-hormones little understood, these restrictions seem to forbid the use of the term 'plant regulators'.

Hence, the name *plant growth substances* has come into general usage (e.g. Audus, 1972). Unfortunately, recent evidence suggests that the plant hormones and related substances do not *always* operate through the control of growth *per se*, or even through allied effects on differentiation or cell division. Several examples of hormone-mediated responses to changes in the environment not involving growth have been described (see Vaadia, 1976), and therefore use of this term may be misleading. Moreover, to use 'plant substances' would clearly be absurd, and whilst the simple term 'plant hormone' may be applied to naturally occurring substances suspected to act in the traditional manner, there is at present, no adequate agreed term for compounds such as fusicoccin and kinetin not known to be naturally synthesised by higher plants.

The difference between *hormones* and *vitamins* is indistinct in some cases (see Audus, 1972; Morton, 1974). One approach is to separate the two on the basis of type of action (Phillips, 1971). Thus Folkers *et al.* (1969) defined a *vitamin* as 'an organic substance of nutritional nature present in low concentration as a natural component of enzyme systems' which 'catalyses required reactions and may be derived externally to the tissues or by intrinsic biosynthesis'. Vitamins are thus, generally, enzyme cofactors, and whilst hormones may affect cellular proteins directly, this is probably in an allosteric fashion rather than at the site of action. The vitamin concept is more relevant to the heterotrophic animal and lower plant groups, since these must take in vitamins with food. However, aspects of the translocation of such cofactors as nicotinic acid, pyridoxin and thiamin between the shoot and roots of plants are hormonal (see Thimann, 1948).

Mer (1968) discussed the classification of inhibitory compounds, stating that a true *inhibitor* inhibits a process normally occurring

rather than one specifically promoted by another substance. For the latter case he suggested the term 'counteractant'. Larsen (1955) defined 'growth inhibitors' as 'substances which retard growth both in shoot and root cells and have no stimulatory range of concentrations'. Several authors consider the idea of 'inhibitory hormones' to be a contradiction in terms, since the word hormone is derived from the Greek 'to stimulate' (Phillips, 1971).

Jacobs' Rules

Notwithstanding the continuing controversy over nomenclature, remarkably little has been published on the criteria by which a substance can be considered to be a plant hormone, surely a crucial element in experimental design and interpretation. Jacobs (1959) formulated a set of six rules, with the mnemonic P.E.S.I.G.S., for critical appraisal of the question: 'What substance controls a given biological process?'. Other authors have discussed isolated aspects of this problem (Audus, 1972; Dennis, 1977; Wareing, 1977; Hillman, 1978; and Reeve and Crozier, in press), but no recent critical review has appeared.

Rule 1. *Parallelism*

This requires that a hypothetical controlling compound is shown to be 'normally present' and that 'the amount of structure varies in the intact organism in parallel fashion with the amount of chemical'. In recent times emphasis has been firmly placed on the demonstration of 'endogeneity'. It is increasingly obvious that levels of a compound in a tissue need not always be related to effect, and, as previously mentioned, hormone action appears not to be confined to the phenomena of organogenesis or growth.

Attention has thus moved to the standards by which specific organic compounds are judged to exist in a plant. Attempts to solve this problem, raised by Van Overbeek (1950), have led to the use of advanced physicochemical techniques in the search for adequate evidence (Hillman, 1978). However, it is apparent that absolute certainty in this context is impossible, and that results should be handled in a

statistical manner (Reeve and Crozier, in press). The latter authors, using the concepts of information theory, show that about 50 *bits* of information are required to specify, with high probability, the structure of an organic molecule of formula $C_vH_wO_xN_y$ (between the limits 100-1400 amu, x, y < 6). They conclude that full-scan mass spectrometry is the sole available technique to yield such informatinn in a single test.

Quantification of hormone levels is necessarily linked to identification. Detection systems must be both sensitive and selective to separate and quantify the large number of compounds present at low concentrations in plant extracts. It follows that some assay methods such as bioassay, radioimmunoassay and colorimetric tests lack the required selectivity and accuracy required for hormone analysis. Quantification using these methods therefore involves assumptions that the measured entity is both the compound expected and a single substance. Whilst it is feasible to measure sensitivity, selectivity, accuracy and precision for a given series of techniques, it is unlikely that enough information will be accumulated unless some form of mass spectrometric verification of molecular identity is obtained (Reeve and Crozier, in press). Unfortunately, mass spectrometic methods may lack the desired sensitivity for measurement of hormone levels in individual plants or organs, and compromise may be necessary. The approach of 'successive approximation' may be appropriate (Reeve and Crozier, in press).

Normally, hormone levels are assessed in solvent extracts of the tissue of interest which are then purified using a range of techniques. As yet, no satisfactory evidence exists to suggest that extraction is a quantitative process. In pigmented tissues a common criterion used is the removal of all colour from the tissue (McDougall and Hillman, 1978). Others have analysed the hormone levels in successive extracts from a batch of tissue to determine a 'point of diminishing returns'. (Quarrie, 1978; Hemberg and Tillberg, 1978). The use of different solvents has been assessed (Milborrow and Mallaby 1975; McDougall and Hillman, 1978; Hemberg and Tillberg, 1978) and methanol appears to be most efficient. Milborrow and Mallaby (1975), cautioned that methanolysis of conjugated hormones might occur in methanol at alkaline pH. An analogous hydrolysis of conjugates may also occur during purification (Bandurski and Schulze, 1974). Remarkable increases in

extractable gibberellins found when detergents were used in extraction (Browning and Saunders, 1977) suggest that a proportion of the hormone pool is membrane bound: if the result is verified then this fraction may well be physiologically important.

After extraction, diverse methods are used to reduce the number of potentially interfering compounds (commonly assayed as 'dry weight'), whilst retaining the compound of interest. Inevitably, the efficiency of these processes is not perfect and (often variable) purification losses are incurred. Efficiency should be estimated, in every case, by the use of an identifiable purification standard, which should not be distinguished significantly by the purification procedures. This restricts the possibilities to isotopically labelled isomers of the compound of interest, which require to be discriminated and quantified only in the final stages. If a radioactive isomer is used, care must be taken that the method of radioassay includes some selective process (McDougall and Hillman, 1978) to assess breakdown during purification. Ideally, the criteria used in assay of a standard should be of equivalent rigour to those used for hormone identification.

Even after deliberation of the problems of hormone extraction and purification, a topic of fundamental importance remains - the physiological significance of any estimates of hormone levels. Although it is assumed that the molecular reactions in which hormones may be involved when acting *must* be affected by concentration (Audus, 1972), estimated whole tissue hormone levels probably do not express the *correct* concentration. Thus, the plant may be seen to be divided into 'compartments' in which hormone 'pools' have a concentration presumably related in some way to response (see Dennis, 1977). These may operate at the tissue level, or as seems more likely, at subcellular levels. Various attempts have been made to isolate organs, tissues and subcellular fractions before extraction to increase the relevance of results, but this may not be of great use unless much is known of the mode of action of the hormone. In any case, correlations between levels and tissue response do not prove causation alone, and must be seen as part of a larger group of information.

It is also possible to hypothesise that hormone concentration *per se*, even in the correct compartment, need not be related to effect. If true, the status of such a compound as a 'chemical messenger' would

be in doubt. Thus tissue response sensitivity could vary with, for instance, environmental conditions, and a constant concentration of hormone elicit a different response. Alternatively, only certain cells in a tissue may be normally responsive - a change in distribution of hormone within a tissue leading to response. Yet a further possibility to be considered is that metabolic rates are important in response (see Dennis, 1977), since rates of hormone turnover could vary while the free hormone pool remained constant. Attempts can be made to test the above possibilities using exogenous compounds and these will be discussed later.

The idea of 'competence' or 'target cells' (Osborne, 1977) is attractive in plants, as it is frequently found that groups of cells respond to hormone application rather than whole organs.

Rule 2. *Excision*

This requires that the source of a putative hormone be removed to 'demonstrate subsequent absence of formation of the structure' - perhaps better modified to simply: 'demonstrate lack of effect'. There are three approaches to test this rule. In the first, the tissue thought to synthesise the hormone is excised (e.g. the tip of a coleoptile) and the effect compared to controls. Into a similar category come treatments which involve diversion of the translocating system, like bark ringing or use of mica slips. The main problem associated with both these methods is the effect of wounding: it is known that ethylene, which has dramatic effects on plant growth and development at low concentration, can be released by injured cells (see Abeles, 1974). Removal of an organ which is a source or sink may also disrupt normal translocation patterns to give spurious results: of the transport systems, the phloem certainly is turgor dependent and may be (at least temporarily) affected by organ excision (Hall and Milburn, 1973). A second method is to apply a 'specific' inhibitor of the synthesis of the substance of interest. Such a compound should ideally act at the late stages of anabolism; cell-free studies should also be used to identify the pathway position and mode of inhibition. Complications include lack of specificity in action and precursor build-up, both of which may alter normal channels of metabolism. The precursors them-

selves may also have residual hormonal activity.

Genetic mutants provide a third method of 'removing' a compound. Of these, single-gene mutants having a completely inactivated synthesis are most useful. Some mutants may be multi-gene or involve only partial inactivation of synthesis: thus the mutant syndrome may involve the disruption of several pathways or may not involve complete lack of effect. Similar constraints concerning precursors apply, and clearly knowledge of the metabolic position(s) and type(s) of the block(s) should be acquired. A further problem may apply if the 'blocked' hormone is involved in a *sequence* of developmental phenomena because the cumulative effects of lack of hormone may not allow growth of an adequately developed experimental plant.

Rule 3. *Substitution*

This covers the application of synthetic hormone to the plant, substitution of an organ by synthetic compound, and substitution of an organ by an extract of the organ. Reapplication of extracts of organs after successive degrees of purification is a classic method of investigating hormone identity, particularly in animal physiology (Wade, 1978). However, a majority of the evidence obtained in recent years about plant hormones has simply involved the application of exogenous compounds. The results of such 'effectology' though, cannot be considered *in isolation* to prove that a substance acts hormonally, if at all, in the plant.

Difficulty has been experienced in hormone application and donation, it being often impractical to assess whether a compound has reached the site of interest, at what concentration it is present, the effects of osmotic and chemical factors, and the consequences of any wounding which might occur. Normally a substance is applied in organic solvent solution, lanolin paste, agar blocks or buffer solution to the whole or part of a plant and any effect compared to controls. Quantitative evidence strengthens the overall case (Jacobs, 1959) but this implies knowledge of the hormone concentration within the plant, which is usually not available. Nevertheless, there is a range of concentrations generally deemed to be 'hormonal' - roughly from 0.1 mol m^{-3} downwards.

In cases where radioactive analogues of a hormone are used it may only be possible to use the highest concentrations of this range as the specific activity is often not high enough to allow subsequent radio-assay, quantitative or qualitative. Nevertheless, by far the greatest problem encountered is concerned with whether the compound actually reaches the tissue cells or compartment of importance. This is found especially in studies of structure-function (see McWha *et al.*, 1972). It is often not known whether an applied compound without effect has merely not penetrated to the relevant compartment (possibly as a function of its different molecular structure), or is genuinely inactive. To some extent, radiotracers can provide evidence. These can also be used to test the possibility that a *positive* response is not due to internal metabolism of an analogue to the active substance.

Besides response selectivity, sensitivity can also be examined by applying exogenous compound: if different concentrations are applied, the minimum amount required to elicit response can be estimated, particularly if radiochemicals of known specific activity are used. Again, it is often presumed that the compound has reached the compartment of interest. Also, potential metabolism needs to be examined. The results obtained can be correlated with endogenous hormone fluxes.

Application of radiolabelled tracers is a traditional method of examining biochemical pathways and can thus be adapted for work on hormone synthesis and breakdown. Methods and associated problems have been reviewed by Brown and Wetter (1972). A neglected aspect is isotope discrimination (Goad *et al.*,1972), although elegant use has been made of stereo/radio isomers to yield information about pathways (see Cornforth, 1969). Other approaches include isotope dilution, isotope trapping and competition, short time-course tracer distribution studies and use of inhibitors (Brown and Wetter, 1972). The main disadvantage is the effects of artificially-high concentration. Cell-free systems may be valuable and tissue of suitable age and physiology should be chosen. It must also be stressed that physicochemical methods of metabolite identification are to be preferred.

In a similar fashion the concepts of enzyme kinetics have also been 'borrowed' from biochemistry to analyse hormone effects (Foster *et al.*, 1952). It is assumed that a crucial part of hormone action

requires hormone active-site interaction and that the comparative parameters (V_{max} and K_m; where $[s] \equiv [\text{hormone}]$ and reaction rate $\equiv$ response rate) are relevant. This so-called 'Michaelis-Menton approach' has been criticised because it over simplifies the systems involved (Audus, 1972). Such methods might be more useful were the site and mode of action of the hormones known.

Rule 4. *Isolation*

This requires that as much of the reacting system as possible should be isolated and the effect of the chemical shown to be the same as in the more intact organism. The aim is to reduce the probability that the compound acts primarily on another process. Unfortunately, interrelations between other systems may complicate the analysis of results. Explants have often been used in phytohormone studies, but disruption of aspects of whole plant physiology (such as nutrient and water supply) and the effect of wounding must be considered. Hence, the physiological relevance of many studies of hormone transport or effects on segments and explants is restricted.

Rule 5. *Generality*

This is meant in the sense that results should be confirmed with other species (or, if applicable, other types of organ). Selection of particular species (or even varieties) for specific experiments often has advantages and may even be a prerequisite for certain studies; this may mean that interspecies confirmation of results is difficult or impossible. In an extreme form, results may be variety or cultivar specific.

Rule 6. *Specificity*

This refers to the requirement that effects demonstrated should be restricted to a single compound. Study of molecular specificity involves comparison of the effects of hormone analogues, which have been discussed under rule three. The same cautions apply here. The problem is seen in exaggerated form in the case of the gibberellins, of which over fifty have been identified (Heddon *et al.*, 1978).

Other Considerations for Hormone Activity

In the formation of the above rules, Jacob (1959) did not use the word 'hormone'. If the definition of a compound affecting a biological process in plants is to include this word, expansion of his guidelines is required.

There are three explicit elements to the concept of hormone action of Bayliss and Starling: *synthesis*, *transport*, and *effect*. Problems associated with the demonstration of hormone endogeneity and action have already been discussed; the following section considers hormone transport, and, more generally, the control of hormone levels at their site of action. Clearly, a part of this control involves release and translocation systems, but it is axiomatic that some further action must occur in the 'target' tissue for the manifestation of effects (Robinson *et al.*, 1971).

In contrast to higher animals, where the bloodstream and lymphatic system constitute well-defined translocating pathways between organs that are usually discrete bodies, plants have three apparently inter-acting systems capable of mass transfer (distinct from the common possibility of diffusion). These are the phloem, xylem and symplastic pathways (Evans, 1976). Furthermore, plant tissue differentiation is less discrete, in that various specialised groups of cells (including those of translocating system) tend to merge and the background parenchyma exhibits degrees of specialisation according to position (Esau, 1965). This leads to difficulties in the physical distinction between areas, and consequently in the identification and isolation of synthesising and responding centres as well as the examination of hormone transport.

Transport: Spatial Separation

Van Overbeek (1950) commented that the distance between sites of phytohormone synthesis and action might vary from that between organs to that between molecules within a single cell. This open inter-pretation diverges from the original Bayliss and Starling concept. Indeed, the compounds controlling processes within cells have usually been specifically named under the umbrella term 'intracellular activators' (Huxley, 1935). Examples are messenger and transfer RNA (Jacob and

Monod, 1961); enzyme effectors (Monod *et al.*, 1963) and the naming of cAMP as 'second messenger' (Robison *et al.*, 1971).

Huxley's biological *activators* were classified according to distance and means of transport. *Local activators* act on the cell (*intracellular activators*) or tissue (*regional activators*) in which they are produced. *Hormones* (or *distance activators*) have effects on tissues and cells other than those in which they are produced and can be divided as *diffusion hormones* or *circulatory hormones* according to mode of translocation.

In plants, the 'organ-to-organ' action of Bayliss and Starling is restrictive in any case, since only four organs are recognised. These are: stem, leaf, root and reproductive zones (see Esau, 1965). It seems more suitable, both from morphological and functional considerations to divide plants into *tissues*, based on the *dermal*, *vascular*, and *fundamental* systems, but no simple all-purpose definition of tissue can be given (Esau, 1965).

According to Huxley's classification, some evidence of transfer of chemical between tissues is required to demonstrate true *hormone* action. The approaches to this topic in plant systems can be categorised as follows. In the first instance, a putative hormone should be shown to be present in the translocating system. For this, it is necessary to isolate the system or sample the contents of the translocating conduits. A range of methods have been used to do this for the phloem and xylem and specific problems have been reviewed recently (Fensom, 1975; Milburn, 1975; Peel, 1975; Van Die and Tammes, 1975; Ziegler, 1975; King, 1976; Pate, 1976). In contrast, few specific methods are known for analysis of the symplastic system. The 'diffusion' method allows collection of substances in agar blocks (see Dennis, 1977), but it has been questioned whether this type of transport is symplastic (Goldsmith, 1977).

Obviously, the criteria used to identify and estimate the levels of hormones in the transport 'fluids' should be as rigorous as for other plant extracts. In practice, a general lack of cellular contents confers an advantage: extracts may not need such extensive purification as those from whole tissues.

A second method is to demonstrate the translocation of applied

compounds, preferably between known sites of synthesis and action. This presents several technical difficulties, especially donation to the relevant translocating system. Methods of application are usually similar to those used for assessing hormone effects. Few, with the exception of xylem injection, are direct. Much use has been made of radioactively-labelled compounds, but it is important to analyse the molecular state of the label after transport.

A third group of methods provide an indirect approach. An assessment of mode of transport can be made by comparing the rate and distance of hormone transport with that found for other solutes in the system. Thus, 37% of a population of small organic molecules would diffuse 1 mm (in water at 20 C) in approximately 4.2 min but would take roughly 7 h to travel 10 mm and 8 years to move 1 m (calculated from Fick's Law, see Nobel, 1974). A characteristic of diffusion is that the apparent rate of transport diminishes according to the distance from the source, whilst physiological processes tend to maintain velocity independent of distance (Nobel, 1974). Symplastic transport (Tyree, 1970) appears to occur with a velocity of roughly 15 mm h^{-1} (Goldsmith, 1969). This transport may be polarised, indicating a metabolic process (Goldsmith, 1969). In the phloem solutes travel at 0.2-1.0 m h^{-1} (Nobel, 1974). Xylem transport, which is normally towards the leaves, occurs at rates, depending on environment, of up to 3.6 m h^{-1} (Nobel, 1974). Phloem and xylem transport are frequently distinguished by the use of stem girdles or bark ringing treatments (e.g. Hocking *et al.*, 1972). Movement of gaseous compounds such as ethylene could be by diffusion within intercellular spaces or in convection currents outwith the plant. Ethylene is also surprisingly soluble in pure water (*ca.* 40 mol m^{-3} at STP) and may be translocated like other soluble hormones.

Treatments such as anoxia and use of respiration inhibitors can indicate that an active process is involved. Specific compounds (e.g. TIBA for IAA) may appear to inhibit transport by competing for sites (Goldsmith, 1969).

In certain cases it may be possible to show that a particular tissue does not synthesise hormone in response to treatment when isolated, whereas an adjoining tissue can. If redistribution of

hormone after treatment of the intact system can be shown, it is logical to assume movement of hormone between the tissues (e.g. Loveys, 1977). Likewise, transient fluxes of hormone in connecting tissues would give indirect evidence of transport.

A debatable point is the extent to which phytohormones can be considered to be 'circulating hormones'. Much attention has been given to aspects of 'short distance' plant hormone transport, particularly in the case of IAA, and Goldsmith (1977) has discussed the mechanisms which may be involved. Recently, interest has been shown concerning hormone translocation in the 'long distance' transport systems - phloem and xylem (King, 1976). If the 'diffusion' of Huxley is taken literally, then symplastic or other apparently metabolic modes of short-distance transport must be considered 'circulatory'. On the other hand, Huxley himself (1935) classified plant growth hormones in the 'diffusion' category - perhaps due to lack of information on translocation.

Difficulty in isolation of the sites of synthesis and effect, combined with dearth of knowledge about precursor and catabolite identity, have meant that it is often difficult to establish whether a compound is transported itself or as a metabolite. Thus, hormone conjugates have been hypothesised as the transported moieties (see Wareing, 1977); some gibberellins are translocated whilst others are not (see Jones, 1973). It is not obvious in this case which molecule should have hormone status: the transported compound, or that which may appear to have effect after metabolism in the responding tissue.

Direct effects of phytohormones on the transport systems themselves have been postulated (Wareing, 1977) but knowledge of specific responses is limited (Moorby, 1977). Indirect effects on source and sink metabolism are better documented (Moorby, 1977).

Transport: Temporal Separation

Whilst it is clear that some element of *spatial* separation between sites of synthesis and action is necessary for hormone action as defined by Huxley, a corollary of this is that *temporal* separation of stimulus and response must also occur. This has led to the division of hormone responses into several events: perception of

stimulus, transformation of stimulus into biochemical information, transmission of information and response to information. The first two events are often grouped as 'presentation time'. A 'latent period' is considered to correlate with translocation of chemical messenger, followed by response during the 'reaction time'. Although partially covered by the *parallelism* of Jacobs, *temporal* separation of stimulus and effect may be seen as a consequence of hormone action. Wareing (1977) stated: 'spatial separation of the sites of growth-substance production and action is not an essential requirement of control involving temporal modulation', however, a substance corresponding to this description would presumably be classified as a *local activator* rather than a *hormone*.

Aspects of the temporal control of hormone levels have been discussed by Audus (1972) and Wareing (1977). Such control rests largely on the rates of biosynthesis and release, transport flux, and rate of inactivation of hormone at the site of effect. The kinetics of hormone synthesis can be studied by methods previously described, but the information accumulated thus far does not allow extensive speculation. There have been few investigations of loading and release of hormones into the translocating systems, and it is not known whether active loading occurs or whether hormones passively follow water movement.

Receptors

A further characteristic of hormone action is the uptake and recognition of hormone molecules by target tissue. Specific hormone receptor proteins have been postulated which combine reversibly with hormones to give a complex representing an 'activated structure' capable of inducing the primary hormone reaction (Kende and Gardner, 1976). The concept has found particular proof in the case of mammalian steroid hormones (O'Malley and Means, 1974). Kende and Gardner (1976) discussed a set of three criteria to assess the relevance of plant hormone binding studies. These were:

(a) receptors should be found primarily in cells of the target tissues and not in non-reacting zones,

(b) the 'kinetic parameters' (dissociation constant, etc.) of binding should be related to dose/response parameters of the biological system,

and (c) a relationship should be shown between hormone analogue binding ability and the biological activity of the analogue (with the exception of those which are non-competitive inhibitors).

Specific problems are mostly related to the interpretation of biological response variables, but the authors cautioned that: 'the existence of nonspecific binding sites in cell extracts is a very real possibility', and concluded that no single receptor protein for any of the plant hormones had been isolated. Notwithstanding, existence of specific receptors may be considered to be a *sine qua non* for hormone action (Robison *et al.*, 1971).

Genome and Environment

Studies of phytohormones should always take into account the possible permissive and conditioning roles of genome and environment. Thus, hormone-controlled processes may not be able to proceed without the appropriate environmental or genetic signal. Alternatively, availability of nutrients and energy might limit hormone-mediated development. In phenomena controlled by irradiation, the action of phytochrome may be important to trigger the synthesis or release of hormones (Wareing, 1977).

Substances and Phenomena

Many organic compounds have effects on plant systems. Even when those not known to occur in plants are rejected, the total is high (Steward and Krikorian, 1971; Gross, 1974). It seems almost absurd, in retrospect, to have selected from this number five groups of related compounds for rigorous study, and yet historical and methodological reasons for this can be identified. In the case of IAA, the early interest in tropic phenomena was a focus for study; research on the gibberellins was greatly aided by the availability of the GA-producing fungus *Gibberella fujikuroi*, several dwarf mutant

plants, and some semi-specific metabolic pathway inhibitors; interest in cytokinins has resulted from the fundamental nature of the processes, such as cell division and differentiation, in which they are supposed to act; with ethylene and ABA, research has been accelerated due to comparative ease of physicochemical identification.

For other substances, it is often not possible from the available data about biological activity (usually from simple and limited bioassays) to tell whether a given compound might deserve 'hormone' status. The variety of organic molecules having effects is large and for particular phenomena defies nomenclature based on simple chemical affinity (Machlis, 1972). Some substances appear to belong to the *external activator* class of Huxley (1935) - compounds which diffuse or passively affect other plants. This phenomenon has been termed *allelopathy* (Evanari, 1961), and the compounds involved *pheremones* (see Birch, 1974). Classes of water-soluble and volatile plant pheromones have been described, but it is doubtful that ethylene normally works in this manner (Audus, 1972).

For the vast majority of substances, however, there is, at the moment, no specific physiological role proposed. In parallel, there are a number of physiological phenomena in which a chemical control is implicated, but for which the modulating compound is unknown or uncertain. Examples (depending on point of view) include vernalisation, senescence, apical dominance, differentiation, initiation of cambial activity, and others (see Went and Thimann, 1937; Thimann, 1948; Audus, 1972). Perhaps the best known is flowering, where a 'florigen' has been postulated for many years (see Audus, 1972; Zeevaart, 1976). Other named substances without definite chemical identity include 'wound hormone', 'leptohormone' (callus growth), rhizocaline (root formation), caulocaline (stem, bud growth), (see Thimann, 1948), and senescence factor (Osborne *et al.*, 1972).

A suggestion frequently made is that phenomena which do not seem to be controlled by a single plant hormone might be modulated by a combination of hormones acting in tandem (e.g. Khan, 1975; Wareing, 1977). The naming of joint effect of compounds on physiological processes has caused some controversy. In particular, the term *interaction* was objected to in certain cases by Drury (1969), who

favoured the statistical definition of the term, and stated that claimed 'interactions' were often statistically invalid. The terms 'counteraction', 'antagonism', and 'synergism' have also been used (Mer, 1968; Audus, 1972). The possibilities that two or more compounds act at the same intracellular site, or that a balance of hormones controls the overall process at different points of action, must be distinguished (Audus, 1972). The latter is an established principle of animal endocrinology, as is the action of a hormone on the synthesis, secretion or activity of other hormones (Frye, 1967).

Summary

To summarise, if *hormonal* action is hypothesised, a spatial separation of synthesis and action must be shown. Otherwise, a compound may belong to another of Huxley's categories. Additional information about hormonal action can be obtained from studies on temporal separation of synthesis and action, and the presence of receptors in the target tissue. Care must be taken to evaluate environmental and genetic factors.

It is indeed possible that the substances controlling physiological processes transcend the boundaries of even functional definitions such as Huxley's. Nevertheless, research in the field of plant physiology has virtually ignored his classification and there is only one appearance from a botanical journal in the Citation Index entries for Huxley's 1935 paper between the years 1964 to 1977. Outwardly at least, an ordered appraisal of the aforementioned rules and definitions does not usually seem to have been made before hormonal action is assumed. This may be due to the multiplicity of phenomena which each compound has hypothesised to act. In individual papers, such deductions have often resulted from poorly-controlled experiments concerning the effect of substance A on process B; very infrequently is a *direct* causal relationship indicated. Moreover, the responses observed are often ill-described and known to be affected by many other cellular or environmental parameters. It seems that intelligent application of guidelines might well aid not only the interpretation of results, but also the fundamental design of experiments.

Methodological and interpretational problems associated with the type of investigation required to demonstrate hormone action in plants have been discussed in the light of the original and modified definitions of this type of correlation. The components of phytohormone systems and some of the complicating factors are summarised in Fig.1.

In retrospect, the rules of Jacob (1959) underestimated certain problems, but, as yet, no general agreement has been reached on the criteria which should be imposed before hormone action is assumed. Plant physiologists are still not in a position to evaluate the importance of hormonal systems in growth, differentiation, and irritability.

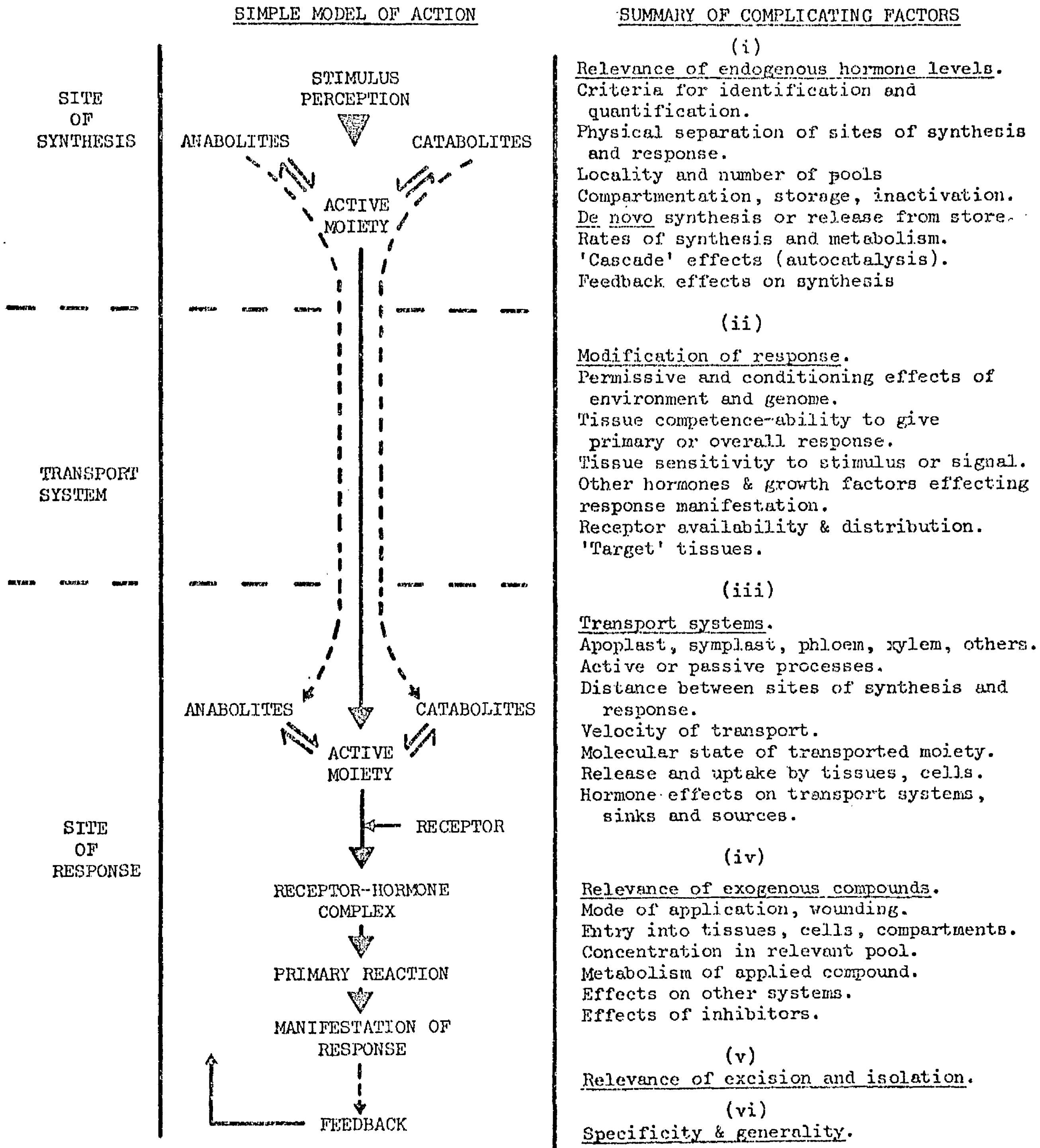
SECTION II. ABSCISIC ACID

The first isolation of abscisic acid (ABA) from plant material was performed by a group of researchers led by Addicott at the University of California. In a series of experiments they isolated and subsequently determined the structure of a substance thought to be responsible for the abscission of young cotton fruits (Liu and Carns, 1971; Ohkuma *et al.*, 1963, 1965). Parallel investigations in the U.K. into a postulated hormone causing dormancy in deciduous trees and a growth inhibitor present in yellow lupin fruits (see Addicott and Lyon, 1969, for review) culminated in the isolation of ABA from sycamore leaves (Cornforth *et al.*, 1965a) and lupin fruits (Cornforth *et al.*, 1966). The structure of naturally occurring ABA was confirmed by synthesis (Cornforth *et al.*,1965b).

Although there has been some variation in nomenclature (see Addicott and Lyon, 1969; Milborrow, 1974), the name *abscisic acid* is now taken to imply the compound shown in Fig.2a, having the full chemical name 3-methyl-5-(1'-hydroxy-4'-oxo-2',6',6'-trimethyl-2'-cyclohexen-1'-yl)-*cis*,*trans*,-2,4, pentadienoic acid. The molecule is capable of optical and geometric isomerisation and has other remarkable properties.

The asymmetric carbon atom at the 1' position confers optical activity; ABA obtained from plant sources rotates plane polarised light of wavelength 589 nm in a dextrarotatory manner (Cornforth *et al.*,

Fig. 1. Assemblage of the Components of Phytohormone Action.



SUMMARY OF COMPLICATING FACTORS

(i)

Relevance of endogenous hormone levels.
Criteria for identification and quantification.
Physical separation of sites of synthesis and response.
Locality and number of pools
Compartmentation, storage, inactivation.
De novo synthesis or release from store.
Rates of synthesis and metabolism.
'Cascade' effects (autocatalysis).
Feedback effects on synthesis

(ii)

Modification of response.
Permissive and conditioning effects of environment and genome.
Tissue competence-ability to give primary or overall response.
Tissue sensitivity to stimulus or signal.
Other hormones & growth factors effecting response manifestation.
Receptor availability & distribution.
'Target' tissues.

(iii)

Transport systems.
Apoplast, symplast, phloem, xylem, others.
Active or passive processes.
Distance between sites of synthesis and response.
Velocity of transport.
Molecular state of transported moiety.
Release and uptake by tissues, cells.
Hormone effects on transport systems, sinks and sources.

(iv)

Relevance of exogenous compounds.
Mode of application, wounding.
Entry into tissues, cells, compartments.
Concentration in relevant pool.
Metabolism of applied compound.
Effects on other systems.
Effects of inhibitors.

(v)

Relevance of excision and isolation.

(vi)

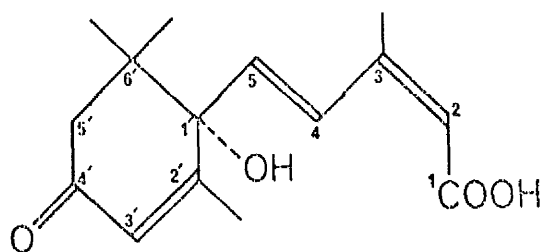
Specificity & generality.

1966). Applying the Cahn - Ingold - Prelog (1966) rules, natural ABA is (+)-S-ABA. Chemically synthesised ABA, on the other hand, is a racemic mixture of the two optical enantiomers (Figs. 2a and 2b) and thus does not rotate plane polarised light. It has different properties from natural ABA (e.g. melting point). Milborrow (1974) has reviewed the biological activities found for these two isomers. He suggests the similarity generally found may be due to a spatial symmetry of the molecule about the 1' carbon, or alternatively, that the hydroxyl group plays no part in ABA action in certain systems.

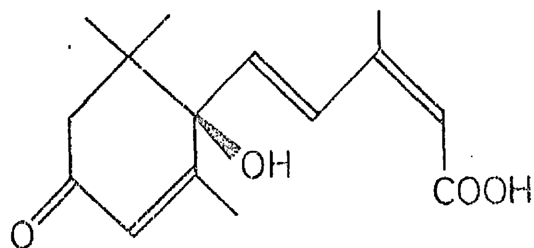
Geometric isomerisation of the ABA molecule can also occur by rearrangement at the unsaturated bond between the second and third carbon atom. It is a matter of some debate whether 2-*trans*-ABA (*t*-ABA, Fig.2c) occurs naturally in plants. The two isomers are interconvertable on exposure to visible and ultra-violet radiation (Mousseron - Canet *et al.*,1966; Lenton *et al* ., 1971; Ciha *et al.*, 1977), and it is conceivable that *t*-ABA is lost during extract purification. This possibility of differential purification efficiencies for the two isomers should be considered when using standards during purification. Some authors report the presence of *t*-ABA in extracts (Milborrow, 1970; Jones *et al.*, 1976), whilst others (avoiding irradiation of extracts) do not (Gaskin and McMillan, 1968). The biological activity of the geometric isomers is also the subject of controversy. Milborrow (1974) suggested that activity of *t*-ABA and *trans* isomers of analogues was due to rearrangement when illumination was given during assay.

Methods of analysis of ABA have been recently reviewed by Milborrow (1974), Saunders (1978), and Reeve and Crozier (in press). Many authors have used bioassay methods to quantify ABA levels. These are mostly based on the 'inhibitor-β' of Bennet-Clark and Kefford (1953), a zone found in paper chromatograms of plant extracts which is inhibitory to *Avena* coleoptile segment straight growth. This region was shown to contain ABA using physicochemical techniques (Cornforth *et al.*, 1965a). Milborrow (1967) claimed that ABA contents of plant extracts were similar when measured by optical rotatory dispersion or by wheat embryo bioassay, but other authors have found that physicochemical and

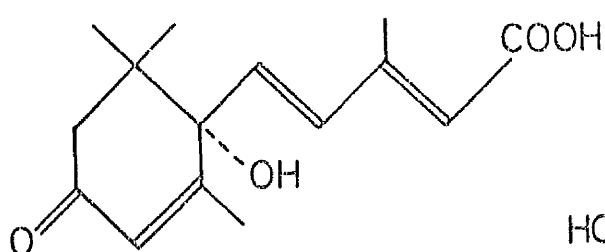
Fig. 2. Fischer Projections of ABA and Analogues.



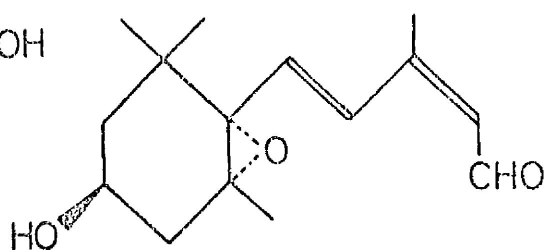
a. (+)-ABA (with numbering system for carbon skeleton)



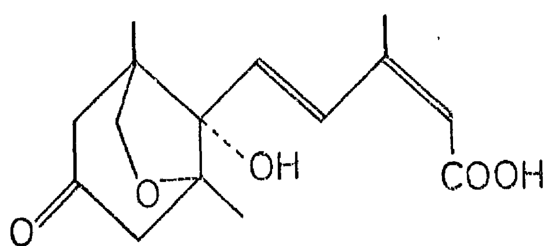
b. (-)-ABA



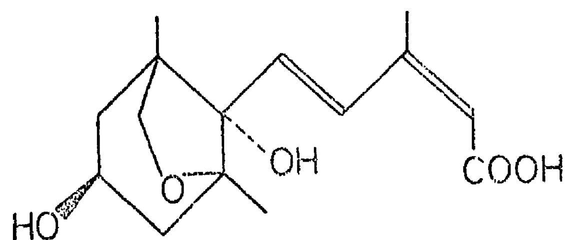
c. *t*-ABA



d. Xanthoxin



e. Phaseic acid



f. Dihydrophaseic acid

bioassay estimates differ substantially (Lenton *et al.*, 1972; Alvim *et al.*, 1976). Saunders (1978) states that ABA represents a small fraction by weight of the inhibitor-β fraction. Certainly, the complex appears to contain many other active compounds, such as phenolic and aliphatic acids, which might affect bioassay response (e.g. Varga and Ferenczy 1957; Housley and Taylor, 1958; Robinson and Wareing, 1964; Kefeli and Kadyrov, 1971; Saunders, 1978). Furthermore, other ether-soluble acidic plant hormones such as gibberellins may be present and interfere with the bioassay response to ABA (Carr *et al.*, 1964; Coombe *et al.*, 1967; De Lange, 1971; Wareing and Saunders, 1971). The stomatal aperture bioassay of Ogunkanmi *et al.*, (1973) seems the most selective, being unaffected by other hormones. Nevertheless, the accuracy of bioassay results must ultimately depend on the purification procedure used. This may explain some of the discrepancies found in the literature.

Physicochemical methods for ABA determination are thus to be preferred (Saunders, 1978). Again, it must be emphasised that none guarantees absolute identification or quantification. Methods which have been used and the least amount of ABA required to quantify a response are shown in Table 1.

Spectropolarimetry (optical rotatory dispersion, ORD) and circular dichroism (Cornforth *et al*., 1966; Milborrow, 1967) are methods which utilise the optical properties of the ABA molecule. Although reasonably precise and selective these methods are not especially accurate, suffering from interference from other compounds present in extracts (Addicott and Lyon, 1969; Saunders, 1978).

Davis *et al.*, (1968) and Lenton *et al.*, (1968) investigated gas-liquid chromatography (GLC) of the methyl ester of ABA (MeABA) using flame ionisation detection. Saunders (1978) discusses the stationary column packings which can be used. A more sensitive method of detection is electron capture detection (ECD), which was invented by Lovelock (1958). It operates on the principle that the conductivity of gases in an ionisation chamber is very sensitive to changes in the gas composition. With important modifications (Lovelock, 1963), it can be used in conjunction with GLC to estimate levels of organic molecules containing certain electron-attracting

Table 1: *Limits of detection of various* physicochemical *detectors for ABA.*

Method of detection	Limit of detection	Reference
Optical Rotatory Dispersion	500 ng	1
Circular Dichroism	200 ng	2
Flame Ionisation Detection	10-100 ng	3
Mass Spectrometry 1. Scan 2. Fixed $^{M}/_{E}$	100 ng 1 ng	4,5
Electron Capture Detection	1-10 pg	6
UV Spectrometry (fixed λ)	100 pg	5
IR Spectrometry (scan)	100 ng	5

References. 1. Cornforth *et al.*, 1966.
2. Milborrow, 1967.
3. Lenton *et al.*, 1968.
4. Gaskin and MacMillan, 1968.
5. Reeve and Crozier, in press.
6. Seeley and Powell, 1970.

species such as halogens and conjugated keto groups (Lovelock, 1961). Seeley and Powell (1970), noting that ABA had two such conjugated keto groups, were the first to use ECD for the hormone. They obtained ABA from purified apple juice and claimed limits of detection of between 1-100 pg, with linearity extending to 1 ng. Besides its low limit of detection, ECD has good selective properties. In plant extracts, where a small amount of ABA may be present in an excess of weakly absorbing material, it can be particularly useful.

Additional information can be obtained from GLC-ECD chromatograms by exposing the sample to UV irradiation for a period to cause formation of *t*-ABA, which is normally separated by most GLC columns from ABA (Lenton *et al* ., 1971; Saunders, 1978).

Some workers have attempted to lower the limits of detection of ECD for acidic hormones by forming chlorinated derivatives (e.g. Bittner and Even-Chen, 1965), but the selectivity of the detector is reduced by derivatisation of previously undetected compounds.

Although the information content of GLC-ECD traces is low (even with isomerisation techniques) the method represents very high selectivity for such a simple system (Reeve and Crozier, in press); successive approximation can be used to verify accuracy. The low limit of detection means that individual plants, leaves or organs can be assayed. Indeed, Beardsell and Cohen (1975) developed a technique to estimate ABA levels in 125 mg samples of leaf tissue, and Quarrie (1978) in samples equivalent to 30 mg leaf fresh weight.

Liquid chromatography has been used, usually with UV detection for analysis of ABA (Sweetser and Vatvars, 1976; Ciha *et al* ., 1977). The method can be used for preparation prior to gas chromatography-mass spectrometry, and is well suited to early stages of purification as it has a high sample capacity.

Mass spectrometric methods, particularly full-scan detection (Gaskin and MacMillan, 1968; Gray *et al*., 1974), provide unparalleled information about ABA extracts (Reeve and Crozier, in press). Mass fragmentography (Hillman *et al*., 1974; Railton *et al* ., 1974) has been used as a specific GLC detector for ABA, but the information content is similar to that of GLC-FID traces unless several ions are monitored (Reeve and Crozier in press). Other high information

techniques used include infra-red and nuclear magnetic resonance spectra (see Milborrow, 1974). The common disadvantage of such methods is the large sample size required for analysis.

Using physicochemical methods, a large number of higher plant species have been shown to contain ABA. These include angiosperms, gymnosperms, a fern, a horstail, and a moss (see Milborrow, 1974). ABA distribution is not restricted to any particular part of the plant, and the hormone has been identified in leaves, roots and reproductive organs (Ohkuma *et al.*, 1965; Milborrow, 1967).

Shortly after the first isolation of ABA from plant tissues, two independent studies, using 'inhibitor-β' estimates of the hormone, showed that the activity of this inhibitory zone increased after wilting the tissue used for extraction. (Pustovoitova, 1967, 1970, 1972; Wright, 1969). Wright and Hiron (1969), using ORD, confirmed that ABA levels rose some 40-fold after a water stress. They correlated this observation with the known stimulation by ABA of stomatal closure (Mittelheuser and Van Steveninck, 1969) and the reduction of stomatal aperture known to be associated with water stress (see Meidner and Mansfield, 1968), and their discovery has since dominated research on the role of ABA in plants. Discussion of this aspect of ABA physiology will be reserved for the following section.

The biosynthesis and metabolism of ABA has been reviewed by Milborrow (1974) and few additions to the subject have appeared since. From consideration of the molecular structure of ABA (e.g. geminal methyl groups, degree of unsaturation), it appears to have a typical isoprenoid structure, and, having a fifteen-carbon skeleton, it is classified as a sesquiterpenoid. Radioactively-labelled mevalonic acid has been incorporated into ABA by cell-free chloroplast preparations (Milborrow, 1973). Faster rates of mevalonate incorporation were obtained when the preparation was lysed, suggesting that ABA is normally formed within the chloroplasts (Millborrow, 1973). However this may have been due to a direct effect of the osmoticum used for lysis. Better evidence was obtained by Loveys (1977), who showed that ABA content of isolated chloroplasts could account for most of that present in turgid leaves. Endogenous ABA had previously been identified

in chloroplast preparations (Railton *et al.*, 1974), and the fact that ABA can be synthesised by Avocado mesocarp etioplasts (Milborrow, 1973) and root systems (Walton *et al.*, 1976) suggests that ABA synthesis is a property of plastids in general. This is consistent with other terpenoid pathways (Goodwin, 1973).

Two routes of synthesis have been proposed. The first involves established terpenoid anabolism to the sesquiterpenoid precursor farnesyl pyrophosphate and then by further enzyme-catalysed steps to ABA, and the second degradation of tetraterpene pigments *via* xanthoxin. Taylor and Smith (1967) found that the carotenoid violaxanthin gave rise to an inhibitor of cress germination after strong illumination. This was later identified as xanthoxin (Fig.2d) which can be converted to ABA *in vivo* (Taylor and Burdon, 1970, 1973). Nevertheless, several pieces of evidence favour the first pathway : $[^{14}C]$-labelled phytoene (a carotenoid precursor) was not metabolised to give ABA (Jeffries 1972), and known inhibitors of carotenoid biosynthesis had no effect on mevalonate incorporation into ABA.

The rate of synthesis of ABA from labelled mevalonic acid increases during treatments causing low turgor (Milborrow and Robinson, 1973). This probably accounts for most of the wilt-induced ABA; release from 'bound' forms such as the glucose ester is possible, but these normally account for less than one third of shoot ABA content (Milborrow, 1974). It has been suggested that 'bound' ABA is a rapid storage or inactivation product of wilt-induced ABA (Hiron and Wright, 1973).

Phaseic acid (PA) and dihydrophaseic acid (DPA) (Figs. 2e and 2f) have been identified as major metabolites of ABA, both from studies of metabolism of the labelled hormone and from endogenous measurements (Walton and Sondheimer, 1972a, 1972b; Walton *et al.*, 1973; Loveys and Kriedemann 1974; Harrison and Walton, 1975). Since these metabolites have low biological activity, the 'DPA pathway' may have a role in regulation the hormonal activity of ABA (Gillard and Walton, 1976). Results of Harrison and Walton (1975) suggest that rates of synthesis and metabolism of ABA both rise during water stress. PA inhibits photosynthesis, whilst ABA does not (Kriedmann and Loveys, 1975). These authors consider that the metabolite may control photosynthetic responses to water stress.

Milborrow (1974) reviewed the biological activity of ABA metabolites and analogues. It appeared that few had activity approaching that of ABA itself in the assay systems investigated. The metabolism rates reported for the hormone indicated that it could penetrate cells quite rapidly. Essential requirements for activity appeared to be a complete pentadiene side chain in *cis*, *trans* configuration, having a carboxyl group or a group which could be converted to it, in the 1 position. The ring could have various modifications without effect, although a 4' double bond enhanced activity. Milborrow suggested that many active analogues might well be converted to ABA *in vivo*.

The data most relevant to effects on stomatal action were published after Milborrow's review. Orton and Mansfield (1974), using the Commelina stomatal bioassay of Ogunkanmi *et al.*, (1973) obtained similar results to those of McWha *et al.*, (1972), although they found that the 4 double bond in the side chain could be substituted by a triple bond.

Before assessing the role of wilt-induced ABA, it is necessary to consider other aspects of plant physiology for which ABA involvement has been hypothesised. Space, however, does not permit a comprehensive review, and this can be obtained in several articles which will be cited.

ABA was first isolated in the course of research on the control of dormancy and abscission, so it is not surprising that these aspects figure highly in the literature. Indeed, the name abscisic acid implies abscission activity. Addicott and Wiatr (1977) summarised the effects of abscisic acid on abscission, as did Milborrow (1974). The latter author argued that since very high ABA concentrations were necessary to obtain effects, these unphysiological amounts might stimulate ethylene production. Ethylene is very active in promoting abscission (see Abeles, 1974) and enhanced production of the gas has been shown following ABA application (Gertman and Fuchs, 1972). Nevertheless, good correlations between ABA levels in cotton fruit and dehiscence and premature abscission were found by Davis and Addicott (1972). Seed germinability was also affected in inverse relationship to ABA levels. Correlations between water stress and leaf fall have been noted (Milborrow, 1974) and a role for stress-induced ethylene has been proposed (Jackson and Osborne, 1970).

To some extent the original postulated role of ABA ('dormin') in dormancy has been discredited by later physicochemical measurements of hormone content. Lenton *et al.*, (1972), using GLC techniques, found that an increase in inhibitor-β levels in birch twigs in response to short-day treatment, shown by Eagles and Wareing (1964), was not due to increased ABA levels, but rather to an increase in other inhibitory compounds. Similarly, Alvim *et al.*, 1976) found discrepancies between inhibitor-β and ABA determinations. Difficulty has been experienced in repeating early work which suggested ABA could induce dormant buds (Hillman *et al.*, 1973). Some correlations between ABA levels and dormancy have been found (see Milborrow, 1974; Alvim *et al.*, 1976). ABA causes the formation of dormant 'turions' in Lemna (Stewart, 1969).

ABA inhibits seed germination in many systems. Its effects on the barley aluerone system are probably the most extensively investigated (see Milborrow, 1974). Levels of ABA may be correlated with stratification (see Milborrow, 1974) and ABA levels of lettuce 'seed' fell 30-fold after 24h imbibition (McWha, 1973). Kahn (1975) proposed that seed dormancy was controlled by a balance of inhibitor(s) and 'promotor(s)'. Often, ABA has been assumed to be the inhibitor, but recent evidence suggests that short-chain fatty acids may be important (Berrie *et al.*, 1975).

Inhibition of whole plant growth or coleoptile and root extension is one of the most frequently cited ABA effects (Milborrow, 1974), but there is little direct evidence to suggest control of growth phenomena by ABA. There is no known inhibitor of ABA synthesis available to test the effects of ABA removal on growth. Certainly, *flacca*, the wilty tomato mutant with lowered ABA levels does not exhibit spindly growth (Milborrow, 1974). Simpson and Saunders (1972) found no differences in the ABA contents of tall and dwarf varieties of pea, either turgid or wilted. Quarrie and Jones (1977) compared the effects of ABA application and water stress treatments on wheat growth and found they were similar in many respects. It is possible that ABA effects on *whole plant* growth are secondary to effects on stomatal aperture. However, rapid changes in coleoptile and root growth upon ABA application cannot be explained in this way. Marré (1977) has

postulated a common site for hormone action in such phenomena at the membrane level.

ABA has been proposed as the inhibitor of root extension which undergoes lateral transport in positive root geotropism (see Wilkins, 1977), but there is a lack of direct evidence concerning such transport of applied ABA. Indeed Hartung (1976) found *upward* movement of label from radioactive ABA rather than the expected downward movement. Furthermore, the roots of *flacca* behave in normal fashion (Itai,pers.comm.). Xanthoxin and other inhibitors have been suggested as alternatives to ABA (Wain, 1977; Dörffling, 1978).

ABA has been implicated in the control of senescence (Colquhoun, 1974; Milborrow, 1974), but most experiments have been carried out on leaf disc systems rather than whole plants. Although ABA has effects on pigment loss from radish leaf discs within one day, over a six-day period it appears to retard chlorophyll degradation (Colquhoun and Hillman , 1972). The 'senescence factor' of Osborne, which accelerates leaf ageing, does not appear to be ABA (Osborne *et al* ., 1972). Ethylene may be important in control of senescence (Milborrow, 1974), and gibberellins have also a possible rôle (Davies *et al*., 1977).

Effects of ABA have also been noted on fruit set and development, flowering, membrane permeability, growth *stimulation* and apical dominance (Milborrow, 1974).

Taking into account the often contradictory and equivocal evidence cited above, and the criteria for hormone action discussed in the first section, it appears that the evidence implicating ABA in control of these phenomena is poor. Many experiments have been described involving the application of high concentrations of the hormone: this obviously highly active molecule may consequently penetrate to pools and compartments where it is not normally present, to act in a manner irrelevant to natural plant physiology. It is also often difficult to decide whether effects described are direct or indirect; in many cases responses observed can be reinterpreted in the light of subsequent knowledge of the effects of wilting on ABA levels and ABA involvement in stomatal movement and ion transport. Thus the small increase in spinach leaf ABA content following 'long-day' treatment may be due to slight wilting (Zeevaart, 1971); the reduction in whole plant growth

after ABA application may be due to lack of CO_2- fixation products following stomatal closure (Quarrie and Jones, 1977).

In the next section the large amount of evidence implicating ABA in plant recovery from stress via stomatal closure and other effects will be reviewed.

SECTION III. ABA, WATER STRESS AND STOMATAL RESPONSES

An area of plant physiology which has seen much expansion in recent years is that of phytohormone involvement in plant water relations. In particular, it has become evident that plants have hormonal mechanisms by which they can respond to conditions of water deficit stress.[1] This subject has been reviewed by Itai and Benzioni (1976), Vaadia (1976), and Mansfield *et al.* (1978), and it clear that two important groups of compounds in this context appear to be the cytokinins and ABA plus related compounds. A large body of information has accumulated concerning the hormonal role of ABA in the modulation of plant responses to water stress *via* effects on stomatal aperture and the following 5 lines of research have contributed significantly to knowledge of this phenomenon.

1. *The after-effects of stress on stomatal aperture*

A large number of parameters of plant growth and development are affected by water stress (Kozlowski, 1968) and it has been known for many years that following stress, stomata are slow to recover full aperture despite watering. In their respective reviews, Iljin (1957) and Hsiao (1973) cite several examples.

A number of studies have indicated that there is a leaf water potential (Ψ) threshold above which stomatal opening remains relatively constant, other conditions being equal. Below this value, however, leaf resistance has been shown to rise sharply, increasing 20 to 30 fold for a drop in Ψ of 0.5 MPa (see Hsiao, 1973). The fact that this lowering in stomatal aperture continued after adequate watering indicated that some physiological adaption had taken place (Allaway and Mansfield 1970). Fischer (1970) placed epidermal strips from non-

1. Throughout this thesis, the contracted phrase 'water stress' is taken to imply 'water deficit stress'.

stressed *Vicia* leaves on the mesophyll of stressed leaves and *vice versa*. Strips transferred to stressed mesophyll exhibited depressed stomatal opening, whilst strips from stressed leaves had increased stomatal apertures after transfer to unstressed mesophyll. Stress did not appear to affect response of stomata to CO_2-free air or CO_2 compensation point. Earlier, Stålfelt (1961) showed that mild water stress could reverse stomatal opening elicited by CO_2-free air, indicating an independence of the two factors. Fischer (1970) concluded that a major part of the stomatal adaption to stress lay in an effect on the guard cells.

The hypothesis that inhibition of stomatal opening might be due to wilt-induced ABA was first proposed by Wright and Hiron (1969) who stated "...some of the physiological responses induced by water stress in the field (that is, temporarily reduced growth rate, closure of stomata and so on) might be regulated by an increase in the content of (±)-abscisic acid (sic) in the plant tissues".

Adaption to water stress has many facets. The initial reactions to such stresses as leaf excision appear to be mechanical (*hydropassive*) in origin (see Meidner, 1965; Raschke, 1975a). Thus slight initial stomatal closure is often followed by opening, apparently governed by release of xylem tension. Only after several minutes does stomatal closure occur which cannot be reversed rapidly by supplying water (*hydroactive*). In the longer term an osmotic adjustment may occur (Hsiao, 1973; Dodoo, 1977). Clearly, these factors must be considered in relation to hypothesised rôles for ABA.

2. *ABA effects on stomatal aperture*

The first indication that ABA might affect stomatal movements came in a short communication from Little and Eidt (1968). In the course of experiments on the effects of the hormone on bud break, they observed that treated plants transpired less than controls. Soon afterwards, Mittelheuser and Van Steveninck (1969) reported that ABA fed through the transpiration stream to wheat leaves caused a reduction in stomatal aperture and a decrease in water loss. They confirmed earlier results of Livne and Vaadia (1965) that kinetin had the opposite effect. Meidner (1967) had shown that kinetin appeared to act by

depressing intercellular-space CO_2 levels, but Jones and Mansfield (1970) showed that the ABA effect was not due to changes in leaf CO_2 status. Furthermore, Horton (1970), using isolated epidermal strips, showed that whilst ABA prevented stomatal opening, kinetin, benzyladenine and GA_3 had no significant effect. This evidence suggested that ABA had a direct effect on the stomatal apparatus, whereas kinetin affected stomata indirectly.

Mansfield and Jones (1971) initiated work on the mode of action of ABA. They showed that ABA treatment resulted in a fall in guard cell osmotic pressure, whilst the value for the subsidiary cells remained constant. Using histochemical techniques, they found that the numbers of starch bodies increased, and potassium ion levels decreased, in guard cells on epidermis treated with the hormone. Horton and Moran (1972) confirmed that ABA inhibited potassium influx to guard cells. These, then, were similar effects to those observed during stomatal closure caused by high CO_2 levels, darkness, or natural rhythms (see Meidner and Mansfield, 1968).

The interdependence between CO_2 and ABA in causing closure was further investigated by Loveys *et al.* (1973), who showed that CO_2 treatment did not alter whole leaf ABA levels in grape. Raschke (1975b) noted that the response to *Xanthium* to ABA was dependent on the CO_2 concentration (and *vice versa,* in his experiments). Mansfield (1976a) found a delay in the response of stomata to ABA in CO_2-free air but no statistical interaction between ABA and CO_2. He concluded that their mode of action was different, but that there were conditions in which they could mutually interfere.

ABA has been shown to affect stomatal aperture when applied in many ways. Closure has been noted when whole plants were sprayed with ABA (Tal and Imber, 1970), when the hormone was supplied to epidermis tissue in buffer (Willmer *et al.*, 1978), and, most commonly, after application *via* the transpiration stream (Mittelheuser and Van Steveninck, 1971). A wide range of species are affected by the hormone (see Milborrow, 1974; Raschke, 1975a). Lancaster *et al.* (1977) reported that stomata of one plant, *Lupinus luteus,* seemed remarkably insensitive to ABA, although it did have effects.

The speed of the closing reaction in most plants ranks with the fastest known plant hormone responses (Milborrow, 1974) occurring

within 5-10 min in some systems (Cummins *et al.*, 1971; Kriedemann *et al.*, 1972). Moreover, very little ABA is required to elicit a response. The hormone is active at 100 nmol m^{-3} (Ogunkanmi *et al.*, 1973) and Kriedemann *et al.* (1972), using radioactive ABA, calculated that as little as 90 fmol mm^{-2} leaf area was present in French bean leaves at the initiation of closure.

Although effects of leaf sprays on aperture and transpiration persist for days (Mizrahi *et al.* 1974), short-term transpiration stream application effects were rapidly reversible (Cummins, 1973). This was apparently not due to metabolism, so Cummins concluded that some compartmentalisation had occurred, and that non-epidermal leaf tissue may have acted as a sink for the hormone.

Recent investigations on the effects of ABA have concentrated on its mode of action and these are reviewed in the next introductory section. To summarise the above evidence, it seems that ABA application can mimic some of the 'hydroactive' effects of water stress on stomatal aperture.

3. *Wilt-induced ABA and correlations with stomatal aperture*

The pioneering work of Pustovoitova and Wright which showed that ABA was produced in shoots during wilting (Pustovoitova, 1967; 1970; 1972; Wright, 1969; Wright and Hiron, 1969) was later corroborated in many other laboratories, using a wide variety of plant species and plant parts. A selection of representative results is shown in Table 2.

Particular emphasis has been placed on correlations between stress, ABA levels, and stomatal aperture or leaf resistance. Hiron and Wright (1973) described a stress treatment in which French bean seedlings were exposed to a continuous stream of air at 38 C. The plants were observed first to wilt, and then to gradually recover full turgor. During the stress, leaf resistance to air flow increased rapidly at the onset of wilting. Leaf ABA levels were estimated by bioassay, and exhibited a remarkable parallelism with leaf resistance. Leaves at different stages in the stress were excised and incubated for 4 h to see whether the ABA-accumulating system was 'switched' on or off. The results indicated that when in a wilted state, synthesis

Table 2: *Comparison of Endogenous ABA Levels Before and After Water Stresses.*

Reference	Plant Material used	Treatment given for stress	Endogenous levels found/ µg kg.f.w.$^{-1}$ mg m^{-3} for fluids	
			Control	Stressed
Hiron and Wright (1973)	*Phaeolus vulgaris* leaves	warm air stress (4 h)	40	301
Hoad (1973)	*Ricinus* leaves	water withheld from plants	31	410
Milborrow and Robinson (1973)	Excised *Helianthus* roots	30% f.w. loss	27	85
Walton *et al.* (1976)	Roots from whole *P. Vulgaris* plants	PEG 6000 - 0.4 MPa stress in root medium	6	62
Walton *et al.* (1976)	Excised *P. Vulgaris* roots	30% f.w. loss	19	81
Davison and Young (1972)	Xylem sap from many species	collected from field	ranged from 0.1 to	10
Hoad (1975)	Xylem sap from *Helianthus*	PEG 1000 - 1.2 MPa stress in root medium	1.5	40
Hoad (1973)	Phloem sap from *Ricinus*	water withheld from plants	105*	900*
Zeevaart (1977)	Phloem sap from *Ricinus*	water withheld from plants	200	2300

* Mean values

was high, but when turgid, synthesis was low. Furthermore, the apparent free ABA level in 'recovered' leaves dropped during the incubation, indicating metabolism of the ABA. This may have been, at least in part, conversion to a conjugate of some form.

Largely from these results, Milborrow (1974) considered that there were three phases to the regulation of ABA levels during stress:-

(i) *Rapid synthesis triggered by turgor loss.*

From the results of Hiron and Wright (1973) he inferred that ABA levels started to rise seven minutes after treatment. Kriedemann *et al.* (1972) obtained values for the least amount of ABA required to cause stomatal closure and compared them to endogenous levels of the hormone (both on a whole leaf basis). They calculated that a mere doubling of leaf ABA levels would be required to cause closure. In fact, levels have been found to rise by up to forty times upon stress (Wright and Hiron, 1969, and Table 2).

(ii) *Feedback inhibition of synthesis when sufficient ABA had been formed.*

This was supported by results of Milborrow and Noddle (1970) who found no biosynthesis of ABA by wilted plants previously fed on a labelled analogue of the hormone. However, Harrison and Walton (1975) proposed that both synthesis and metabolism were elevated during stress, and the relative rates of these processes determined the ABA level.

(iii) *Gradual lowering of free ABA levels after turgor recovery.*

Levels of free ABA remaining in plants after a stress seem to depend on the stress given and the plant species but may remain elevated for days.

Later studies on correlations between whole leaf ABA levels and stomatal closure have not revealed such a close relationship between the two parameters as found by Hiron and Wright (1973). In long-term stress experiments (0-10 d), Beardsell and Cohen (1975) found a disproportionate rise in leaf resistance compared to whole leaf ABA levels. In shorter experiments (0-1 h), Walton *et al.* (1977) found that whole leaf ABA levels did not alter significantly before stomatal closure occurred after a stress. 5 h later, however, the variables were well correlated. This led Beardsell and Cohen (1975) and

Mansfield *et al.* (1978) to postulate that a small, but significant, *redistribution* of ABA occurred upon stress, *followed* by higher *synthesis* rates. So far, evidence has not been forthcoming to support this hypothesis. Possible hydropassive effects of wilting must be taken into account (see Raschke, 1975a). Certainly, since ABA seems to affect the stomatal *apparatus* when acting (see later discussion), then the levels of ABA present in the *epidermis* must *a priori* be considered to be more important than those of the *whole leaf*. This approach was adopted by Loveys (1977) who provided elegant evidence on the sites of ABA synthesis in leaves of *Vicia*, a plant in which the epidermis and mesophyll tissues can be separated. Isolated leaf epidermis was incapable of ABA synthesis when stressed, whereas isolated mesophyll tissue did synthesise the hormone. Furthermore, levels of ABA were elevated in epidermis removed from stressed whole leaf tissue. Loveys concluded that transport of wilt-induced ABA had occurred from the mesophyll tissue to the epidermis, but did not correlate this with stomatal closure. Other results of Cummins (1973) and Raschke (1975b) also indicate an intra-leaf compartmentation of ABA pools.

It has already been noted that a leaf water potential threshold exists for stomatal closure in water stress. Zabadal (1974) and Beardsell and Cohen (1975) investigated the Ψ threshold for ABA production, and found that levels rose at a distinct point. Raschke (1975a) noted that both Ψ and time of exposure varied in these experiments, so confirmation was needed. This was provided by Walton *et al.* (1977). They obtained a distinct threshold for ABA production after exposure to different Ψ for 2 h. Brown *et al.* (1976) found that preconditioning cotton plants to water stress made them less reactive in terms of stomatal closure when desiccated, compared to controls. They presented evidence that an osmotic adjustment had occurred in the leaves. This implies that it may be misleading to relate ψ to endogenous ABA, and leaf turgor may be more appropriate. In tomato plants given lowered water regimes, Rasmussen (1976) found that ABA levels between controls and treatments did not differ after 18 d. This may be linked to secondary long-term adjustment in the plants.

ABA levels have also been found to rise as a result of a number of

other stresses besides direct water deficit stress e.g. waterlogging, root cooling or heating, lack of root aeration, mineral deprivation, excess salinity, boron toxicity and fungal attack (see Mizrahi and Richmond, 1972a; 1972b; Hiron and Wright, 1973; Milborrow, 1974; Boussiba *et al.*, 1975). But all of these generally cause a reduction in leaf turgor by increasing root or xylem resistance to water flow. Boussiba *et al.* (1975) hypothesised that ABA might control plant 'cross adaption' to stress, the phenomenon whereby prior stress improves or modifies reaction to later alternative stress. Thus, ABA application modified response to subsequent stresses (Mizrahi and Richmond, 1972b).

To summarise, *whole leaf* ABA measurements do not always correlate with stomatal movements or leaf resistance during stresses. In addition to short-term hydropassive movements and long-term osmotic adjustments, inter-tissue pooling effects must be considered.

4. *The 'wilty' mutant of tomato*

Tal (1966) described a tomato mutant *flacca* which Stubbe produced by X-irradiation of the cultivar Rheinlands Ruhm. This single-gene mutant had a permanently wilty appearance which Tal showed to be due to high levels of transpiration caused by open stomata. It did not respond to stress by stomatal closure movements. Subsequent investigations by Tal and his co-workers into the reasons for this excessive stomatal opening revealed an inability to synthesise ABA. Imber and Tal (1970) showed that ABA applied to *flacca* both as a foliage spray and in the root culture solution could cause phenotypic reversion of the mutant syndrome, though this was never complete even at high concentrations of the hormone. Tal *et al.* (1970) noted that another symptom of *flacca* was a lowered rate of root pressure exudation (0.35 of control). Mutant sap exudate had a higher osmotic potential (*ca.*2x) than Rheinlands Ruhm. These parameters could also be altered to near normal by ABA application (Tal and Imber, 1971). Bioassay measurements of 'hormone-like activity' were carried out by Tal and Imber (1970) and Tal *et al.* (1970) which indicated that the mutant had greatly lowered levels of ABA-like activity, increased levels of kinetin-like activity and increased levels of auxin-like substances. Due to possible interference between hormones in the plant extracts, these assays must be

considered unreliable, but Tal and Nevo (1973) measured ABA by GLC-ECD and confirmed that levels in *flacca* plants were roughly 17% of controls. Nevo and Tal (1973) investigated whether this difference was due to higher metabolism rates and demonstrated that the mutant had a decreased rate of ABA synthesis. The genetic lesion was assumed to be between farnesyl pyrophosphate and ABA. Obviously the block was not complete, since some ABA was found in the mutant. Their results indicate that ABA synthesis is controlled by a single major pathway (in tomato). To date, no studies on mevalonate incorporation have been published. These might reveal the exact point of lesion in the ABA synthesis pathway from precursor build-up.

On the basis of the bioassay estimates of hormone activity, it has been concluded (e.g. Tal and Nevo , 1973) that high levels of auxin and kinetin-like activity found were a secondary effect of lowered ABA synthesis. If these results were confirmed with appropriate techniques, *flacca* would prove a useful tool to investigate the interrelationships between hormones in normal plants. If not, careful study of the mutant morphology and habit may also indicate or deny rôles for ABA in other physiological phenomena (e.g. root water and ion uptake, root growth).

5. *Other effects of ABA possibly related to Stress Adaption*

Glinka and Reinhold (1971; 1972) have reported that application of ABA to plant tissues increases their permeability to water. Tal *et al.*(1970) noted that ABA treatment restored *flacca* root exudation rate and Glinka (1973) demonstrated that an ABA-induced increase in root exudation rate was mostly due to an increased permeability to water of the root membranes. ABA also has effects on ion uptake and release by roots (see Pitman *et al.*, 1974a), but temperature and nutrient status can modify results (accounting for conflicting reports in earlier publications).

Pitman *et al.* (1974b) showed that wilting of barley plants caused a reduction in transport of ions from root to shoot but did not alter ion uptake, effects which were paralleled by ABA treatment and substantiated by observations on *flacca* before and after ABA treatment (Tal *et al.* 1970a). Pitman *et al.* postulate that ABA might act as part of a 'fine control' of ion transport.

If wilt-induced ABA can affect roots during stress, the question arises of its origin and possible movement to the root from the shoot. It must be emphasised that a stress applied to any part of a plant should quickly affect all other parts because of the inextensibility of water in the xylem conduits, i.e. a warm air stress to the leaves will also stress the roots. Although isolated roots do synthesise ABA in response to a stress (Walton *et al.*, 1976), the levels attained in the short term are small compared with those of leaf tissue (see Table 2). Transport of ABA may occur from the shoot, but the results of Walton *et al.* (1976), using phloem girdling do not support this contention over a treatment period of 2 h. Those of Hoad (1975) indicate the reverse over the same period. Results with ^{14}C -ABA suggest that shoot-root transport can take place (Hocking *et al.*, 1972).

ABA has been found in xylem sap and root pressure exudate (Davison and Young, 1972; Hoad, 1975), and its origin and possible physiological function is of interest. Some investigators have postulated a rôle in bud dormancy, others in growth inhibition, but the possibility that it is involved in the water relations of plants has been largely neglected. After stress, the ABA concentration in sunflower root pressure exudate rose to 40 mg m^{-3} (151 µmol m^{-3}, Hoad, 1975), 3 orders of magnitude higher than the lowest known concentration to have effect (Ogunkanmi *et al.*, 1973). In view of the fact that ABA applied *via* the transpiration stream has dramatic effects on stomatal aperture (albeit usually at slightly higher concentrations), the possibility of a longer term effect of ABA in xylem sap is intriguing.

Another interesting hypothesis is that wilting might cause increased levels of ABA in seeds or the maternal tissues of fruit. ABA has been implicated in seed dormancy and wilt-induced ABA may thus enhance dormancy properties of the seed and fruit. If this occurs during drought conditions the advantage to the seed is obvious, being better able to withstand the prevailing conditions until the drought is over. Evidence that transport of wilt-induced ABA to grains occurs was obtained by Goldbach and Goldbach (1977).

Other Hormones and Compounds in Stress Adaption

Besides ABA, the other plant hormones have all been ascribed rôles

in stress adaption. Unfortunately, endogenous measurements may be unreliable and effects unspecific. Particular caution must be expressed with respect to results which concern the cytokinins. Firstly, analysis of the levels of 'cytokinin-like activity' have generally been carried out using callus growth bioassays. In contrast, levels of wilt-induced ABA have been measured using a variety of accurate and selective physiochemical techniques. Inaccuracy of bioassay because of interference of other compounds in the extract has already been discussed, and Milborrow (1974) cites four references in which ABA inhibited callus growth. Thus, unless adequate precautions were taken to ensure that cytokinins and ABA were separated during extract purification, the results of callus growth assays may not reflect true cytokinin contents of tissue during stress, but rather the spurious effect of wilt-induced ABA. Secondly, whilst synthetic ABA, isotope-labelled ABA and ABA analogues have been available, this has not been so for the endogenous group of cytokinins. In addition, the species distribution of compounds such as zeatin is unknown. Experiments on the effects of cytokinins on stress-related phenomena, such as transpiration, have frequently been carried out using the chemical 6-fufurylaminopurine (kinetin), which, although relatively potent, *has never been isolated from plants*. It follows that inferences from the application of kinetin to plant systems may be unreliable. Research on cytokinin involvement in stress adaption has therefore been limited by the techniques and chemicals available at the present time.

Despite these *caveats*, the possibility of the involvement of other hormones in stress adaption cannot be ignored. Synthetic cytokinins affect stomata indirectly (Meidner, 1967) and also have effects on root systems (Tal *et al.*, 1970). IAA and gibberellins also have effects (see Vaadia, 1976), but not on stomata of isolated epidermis tissue (see Raschke, 1975a). The concept of a 'hormone balance' which controls plant water relations has been discussed by many authors.

Apart from the so-called 'hormones', two other sets of compounds have been shown (a) to increase in levels during wilting and (b) to have effects on stomatal aperture in isolated systems. These are all-*trans* farnesol (Wellburn *et al.*, 1974) and short-chain fatty acids

(Fenton *et al.*, 1976; Willmer *et al.*, 1978). At the moment, relatively little is known of the physiology of these compounds. Relevant measurements of farnesol have been restricted to *Sorghum*. When applied to epidermis tissue both compounds seem to result in loss of membrane integrity and even guard cell death (Fenton *et al.* 1977; Willmer *et al.*, 1978). These reports correlate in an interesting manner with observations of Stålfelt (1961) and Allaway and Mansfield (1970) that the numbers of living guard cells in epidermis material were lower if a previous water stress had been given.

Xanthoxin has been attributed a rôle in stomatal closure. Although the compound had effects when fed *via* the transpiration stream, it did not affect epidermal strips (Raschke *et al.*, 1975) and levels did not increase after wilting (Zeevaart, 1974). Nevertheless, it can be metabolised to ABA (Taylor and Burdon, 1973).

Levels of ethylene are known to rise during water stress (Jackson and Osborne, 1970; El-Beltagy and Hall, 1974; Aharoni, 1978), but the gas has apparently no effects on stomatal aperture (Pallaghy and Raschke, 1972; El-Beltagy and Hall, 1974; Aharoni, 1978).

Does ABA act 'Hormonally' during Stress?

Having reviewed the main evidence that ABA has a rôle in stomatal responses to water stress, it is now possible to evaluate whether this rôle fulfils the previously discussed criteria for control of a biological phenomenon, and more precisely, for true hormone action. To avoid lengthy discussion, the evidence is presented in tabulated form (Table 3). This Table will be considered in relation to the experimental work of this thesis in the General Discussion.

SECTION IV. ABA AND STOMATAL MECHANISMS

In the past few years a number of advances in our knowledge of the means by which stomata open and close have been made. These have been discussed in several recent reviews (Meidner and Willmer 1975; Raschke, 1975a; 1976; 1977; Thomas, 1975; Hsiao, 1976). The

Table 3. *Summary of Evidence that ABA Controls Stomatal Aperture during Water Stress.*

A. CRITERIA FOR CONTROL OF A BIOLOGICAL PROCESS (AFTER JACOBS, 1959)

CRITERION OR RULE	MAIN EVIDENCE	REFERENCES	CORROBORATIVE EVIDENCE (CE) AND CONTRA-INDICATIONS (CI)	REFERENCES
1. PARALLELISM				
(a) *COMPOUND PRESENT IN ORGANISM*	(a) ABA PRESENT IN PLANTS (MS IDENTIFICATION)	E.G. GASKIN AND MACMILLAN (1968)	-	-
(b) *RESPONSE, AMOUNT OF COMPOUND VARY IN PARALLEL FASHION*	(b) LEVELS OF ABA IN WHOLE PLANT PARALLELED RESPONSE DURING AND AFTER STIMULUS	E.G. HIRON AND WRIGHT (1973)	(CE) OTHER EXAMPLES OF RELATIONSHIP BETWEEN WILT-INDUCED ABA AND DROUGHT TOLERANCE	E.G. LARQUE-SAAVEDRA AND WAIN (1974; 1976)
			(CI) WHOLE LEAF ABA LEVELS DID NOT ALWAYS PARALLEL RESPONSE	E.G. BEARDSELL AND COHEN (1975) WALTON *ET AL.* (1977)
2. EXCISION				
REMOVE SOURCE OF COMPOUND AND DEMONSTRATE LACK OF EFFECT	*FLACCA*. SINGLE-GENE MUTANT OF TOMATO RHEINDLANDS RHUM: LOW LEVELS ABA, PERMANENTLY OPEN STOMATA	TAL (1966); TAL AND NEVO (1973)	-	-
3. SUBSTITUTION				
EXOGENOUS COMPOUND SUBSTITUTES FOR STIMULUS	APPLIED ABA CAUSED STOMATAL CLOSURE	E.G. LITTLE AND EIDT (1968); MITTELHEUSER AND VAN STEVENINCK (1969); TUCKER AND MANSFIELD (1971); MIZRAHI *et al*. (1974)	(CI) *LUPINUS LUTEUS* HAD APPARENT LOW SENSITIVITY TO ABA	LANCASTER *ET AL.* (1977)
4. ISOLATION				
ISOLATE REACTING SYSTEM, DEMONSTRATE EFFECT OF COMPOUND	EPIDERMAL SEGMENTS AFFECTED BY ABA GUARD CELL PROTOPLASTS SHRINK ON ABA TREATMENT	TUCKER AND MANSFIELD (1971); OGUNKANMI *ET AL.* (1973) SCHNABL *ET AL.* (1978)	-	-
5. GENERALITY				
RESULTS CONFIRMED WITH SEVERAL SPECIES	MANY SPECIES EXHIBITED WILT-INDUCED ABA FORMATION, RESPOND TO ABA	SEE REFS. IN SECTION III ALSO MILBORROW (1974); MANSFIELD *ET AL.* (1978)	(CI) SEE RULE 3	LANCASTER *ET AL.* (1977)
6. SPECIFICITY				
SINGLE ACTIVE COMPOUND	FEW ABA ANALOGUES HAD ACTIVITY ON EPIDERMAL SEGMENTS	ORTON AND MANSFIELD (1974)	(CE) SAME SITUATION FOR OTHER BIOASSAY SYSTEMS	SEE MILBORROW (1974)

B. CRITERIA FOR HORMONE ACTION (AFTER HUXLEY, 1945)

CRITERION OR RULE	MAIN EVIDENCE	REFERENCES	CORROBORATIVE EVIDENCE (CE) AND CONTRA-INDICATIONS (CI)	REFERENCES
1. SPATIAL SEPARATION *OF SITES OF SYNTHESIS AND RESPONSE*	(a) SITE OF ABA SYNTHESIS APPEARED TO BE PLASTIDS	MILBORROW (1974); RAILTON *ET AL* (1974); LOVEYS (1977)	-	-
	(b) SITE OF ACTION EPIDERMIS	HORTON (1970)	-	-
2. TRANSPORT BETWEEN TISSUES				
(SITES OF SYNTHESIS AND RESPONSE IN DIFFERENT TISSUES)	ABA NOT SYNTHESISED BY ISOLATED EPIDERMIS DURING STRESS BUT APPEARS IN EPIDERMIS IF ISOLATED AFTER STRESS TO WHOLE LEAF	LOVEYS (1977)	(CE) EPIDERMIS TISSUE PLACED ON STRESSED MESOPHYLL EXHIBITED DEPRESSED STOMATAL OPENING	FISCHER (1970)
3. TEMPORAL SEPARATION				
BETWEEN STIMULUS AND RESPONSE	HYDROACTIVE STOMATAL CLOSURE OBSERVED AFTER DELAY FOLLOWING STRESS	SEE RASCHKE (1975A)	(CE) RATES OF ABA SYNTHESIS AND RESPONSE DELAY TIMES COMPARABLE	SEE MILBORROW (1974)
4. RECEPTORS				
EXISTENCE OF SPECIFIC RECEPTORS IN TARGET CELLS	-	-	-	-

following comments briefly summarise the latest discoveries with specific regard to the effects of ABA in stomatal closure.

Stomatal movements are a function of relative changes in the turgor of guard cells and subsidiary or epidermal cells. Increased guard cell turgor results in stomatal opening due to non-uniform extensibility in the guard cell wall structure (see Meidner and Willmer 1975; Raschke, 1975a). It is now apparent that a net uptake of potassium combined with chloride influx and/or malic acid formation is largely responsible for a reduction in guard cell solute potential which leads to a turgor increase. In addition, guard cell proton extrusion appears to be involved in the maintenance of electroneutrality. Whilst our knowledge of environmental parameters affecting stomatal aperture is good and information on the events of movements is improving, our knowledge of how the osmotic changes are controlled is poor at present. There is no rigorous evidence that any single process controls all the others; this means that further studies of mechanisms at the molecular level cannot easily be undertaken. It seems that the fundamental processes are membrane mediated. Ions entering or leaving guard cells must pass the plasmalemma and probably also the tonoplast, since plasmodesmata between guard cells and epidermal or subsidiary cells appear to be scarce at maturity (Carr, 1976). Membrane-bound, cation-transporting ATPases have been hypothesised in analogous theories to those invoked in studies of root and algal cell ion uptake. As yet, direct evidence for these is extremely thin. If energy-requiring 'pumps' are hypothesised, then energy availability would seem a likely point of control. Even constant maintained apertures seem to require energy expenditure to maintain uptake-efflux equilibrium (Penny and Bowling, 1974).

Stomatal Opening

The evidence for guard cell K^+ uptake during opening came from three main sources. In the first place, solutions of potassium salts were found to cause stomatal opening in epidermal strips. Staining techniques revealed that this resulted in guard cell K^+ uptake (see Hsiao, 1976). Secondly, guard cell $^{86}Rb^+$ or $^{42}K^+$ uptake was related to stomatal aperture (Fischer, 1972), and thirdly, opening was quantitatively related

to endogenous guard cell K^+ content using the electron microprobe (Humble and Raschke, 1971) and ion-selective microelectrode (Penny and Bowling, 1974). In both the second and third methods, estimation of guard cell K^+ content (with an associated anion) accounted for observed increases in osmotic potential and turgor.

Two main anions have emerged as candidates for the neutralisation of the positive charge which would otherwise result from K^+ influx to guard cells: These are Cl^- and malate. Increases in chloride ion concentrations have been observed by histochemical methods and the electron microprobe (Humble and Raschke, 1971; Raschke and Fellows, 1971). Malate levels increased in guard cells during opening (Allaway, 1973), and appeared to be formed by the carboxylation of phosphoenol-pyruvate (PEP) after starch glycolysis (Willmer *et al.*, 1973). [In fact, guard cell CO_2 fixation (Shaw and MacLachlan, 1954; Willmer *et al* ., 1973) does not seem to involve photosynthetic reduction in the Calvin cycle (Raschke and Dittrich, 1977; Outlaw and Kennedy, 1978). Rather, carboxylation of PEP occurs. As a consequence, the epidermis is heterotrophic for carbon and appears to obtain sugars from the epidermis (Dittrich and Raschke, 1977a).] Recent results of Outlaw and Lowry (1977) indicate that at least in *Vicia,* guard cell citric acid levels may also rise upon opening. In their tissue, citrate levels were quantitatively as important as malate. Dittrich and Raschke (1977b) reported that citric acid was formed from applied ^{14}C-malate in *Commelina* epidermis. Results of most authors, however, suggest that the rise in malate levels alone is adequate to compensate for the charge associated with K^+ not neutralised by Cl^- (eg. Schnabl, 1978).

The relative importance of chloride and malate appears to be species and nutrition dependent. Thus Cl^- accounted for 0-20% of the K^+ taken up by guard cells of *Vicia* (Humble and Raschke, 1971) whilst in *Zea,* Cl^- neutralised from 40-100% of the K^+ transferred from subsidiary cells during opening (Raschke and Fellows, 1971). In onion, which has no guard cell starch, Cl^- compensated entirely for the K^+ influx (Schnabl and Ziegler, 1977) and the stomata did not open in the absence of chloride (Schnabl, 1978). In *Vicia*, stomata can open in the absence of chloride (Raschke and Humble, 1973) and van Kirk and Raschke (1978a) showed that the amount of malate formed in the

epidermis varied according to Cl^- supply. Osmond (1976) described three documented types of regulation of malate synthesis in response to the balance of inorganic ion uptake. These were: control by means of cytosol pH, feedback regulation, and control by means of Cl^- concentration. It is possible that these may all operate in the case of guard cell malate formation.

If potassium influx is neutralised to any extent by organic anion formation, then either the guard cell pH will fall, or H^+ extrusion (or OH^- uptake) must occur. Numerous observations have indicated that guard cell sap pH *rises* during opening (Meidner and Mansfield, 1968), so the latter alternative seems more likely. Evidence for this was obtained by Raschke and Humble (1973) who showed that *Vicia* guard cells excreted H^+ into the medium during K^+ uptake. In the absence of Cl^-, the amounts of H^+ secreted by epidermal strips were some 10 times larger than required for an observed guard cell pH rise, confirming the quantitative importance of anion synthesis.

Several authors have suggested that H^+ extrusion and concomitant cytoplasmic pH changes are the primary events of stomatal movement (e.g. Raschke, 1975a, Smith and Raven, 1976).

Stomatal Closure

In essence, the events of closure caused by darkness, rhythms, or high CO_2 concentration, seem to be the reverse of those of opening (Meidner and Mansfield, 1968). Thus, closing and opening may result in a 'shuttle' of ions between guard cells and subsidiary or epidermal cells. Evidence for this was obtained in *Zea* by Raschke and Fellows (1971) and in *Commelina* by Penny and Bowling (1974) and Penny *et al.* (1976). In both species, movement of Cl^- accounted for about 40% of the K^+ taken up by guard cells on average. Bowling (1976) hypothesised that movement of monovalent malate might occur between guard and epidermal cells in *Commelina*. The malate efflux data of Dittrich and Raschke (1977b) and van Kirk and Raschke (1978b) provided limited evidence for this. In *Commelina*, the total epidermal level of malate did not change whether stomata were open or closed (Bowling, 1976), but in *Vicia*, contents *did* alter according to aperture (Allaway, 1973; van Kirk and Raschke, 1978a). Also, ^{14}C-malate incorporation into starch was greater in the dark than in conditions conducive to

opening (Willmer and Rutter, 1977). These results suggest that Bowling's hypothesis is not wholly correct.

Results of Humble and Hsiao (1970), using $^{86}Rb^+$ as a tracer for K^+, indicated that K^+ efflux from guard cells occurs during closure induced by darkness. These studies, and those of Raschke and co-workers cited above, were carried out using epidermal strips floating on solution; it should be remembered that transfer between cells may be short-circuited in such systems. On the other hand, extracellular ion storage sites have been hypothesised (Stevens and Martin, 1977; E. MacRobbie, pers. comm.). Results of Saftner and Raschke (1978) indicate that the ion exchange capacity of the guard cell wall is large.

Effects of ABA

Early observations of the mode of action of ABA (eg. Mansfield and Jones 1971) revealed a similarity in the events of closure to those obtained by other means. This has not been contradicted by later research. Indeed, ABA is often used as an experimental tool to obtain closed stomata (eg. Dittrich and Raschke, 1977b; van Kirk and Raschke, 1978b). It thus seems that effects of ABA are similar, but faster, than those caused by other factors. Since ABA-induced closure occurs relatively quickly (5-10 min), the guard cells must dispose of accumulated solutes rapidly. Recent results of MacRobbie *et al* . (1978) demonstrated that efflux of $^{86}Rb^+$ from preloaded guard cells was transiently higher during closure caused by ABA. Studies of Dittrich and Raschke (1977b) showed that malate efflux was higher after ABA treatment (5 min) than after a darkness treatment (30 min) which also caused closure. The hormone may affect the passive permeability of the guard cell plasmalemma to those and other solutes. Alternative sites of action include inhibition of guard cell ion uptake and activation of an efflux pump (Hsiao, 1976) and Marrè (1977) has suggested that ABA might inhibit H^+ efflux pumps sensitive to fusicoccin. However, inhibition of ion uptake or efflux seems inconsistent with rapid action (Hsiao, 1976). Nevertheless, it is possible that ABA acts at several guard cell sites.

There is also limited evidence that the hormone may act at several cell sites in the epidermis. Results of Penny and Bowling (1974) indicated that active uptake of K^+ by epidermal cells occurred after closure, although their methods have been disputed by Moody and Zeigler (1978). Itai and Meidner (1978a; 1978b) hypothesised that ABA-induced closure involved the epidermal cells. They confirmed evidence of Mansfield and Jones (1971) and Horton and Moran (1972) that the hormone affected solute uptake and distribution between epidermal and guard cells. Closure did not occur if epidermal and subsidiary cells were killed, but this is not conclusive since the effects of epidermis turgor relations must be considered (Squire and Mansfield, 1972).

To summarise, the symptoms of ABA-induced stomatal closure appear to parallel those for closure triggered by other environmental factors, only faster. However, there is a paucity of unequivocal information. ABA seems to affect membrane permeability but may act at several sites.

MATERIALS AND METHODS

PLANT MATERIAL

The experiments reported in this thesis were carried out using two plant species: *Commelina communis* L. and *Ricinius communis* L. *var. Gibsonii.*

Plants of *C. communis* L. were grown from seed initially supplied by Dr. C. Willmer (Stirling University, U.K.) and subsequently collected from plants which were allowed to flower. These were ripened in dry conditions at room temperature for three or more months prior to sowing. Approximately 20 seeds were planted 1-5 mm deep in a 125 mm rim diameter pot. Coleoptile emergence took place after 7-10 d and seedlings were transferred to individual pots (100 or 125 mm) after 14 d. If planted in 100 mm pots, the seedlings were transferred to 125 mm pots after a further 14 d. Staking of plants was carried out about 1 month after seedling emergence and plants were used for experimentation after this time and before flowering commenced (*ca.* 7 weeks after potting). Such plants had from 6-9 fully expanded leaves on the leading stem. Branching was frequent, apical dominance being lost after 3-4 leaves had expanded fully. Mature plants thus had 3-6 branches yielding leaves of comparable area and physiological age.

Plants of *R. communis var. Gibsonii* were grown from seed (Daggs Ltd., U.K.) stored at 4 C before use and planted *ca.* 15 mm deep, one per 100 or 125 mm pot. The cotyledons appeared above soil surface after 14 d. If grown in 100 mm pots, the seedlings were transferred to 125 mm pots after primary leaf expansion. Plants were used when 1-2 alternate leaves had expanded.

Seed and plants of both species were grown in a standard compost (peat:sand:loam, 1:1:1; by volume) containing either John Innes Base (Lindsey and Kesteven Fertilisers Ltd., 3.2 kg m^{-3}) or Osmokote controlled-release fertiliser (Sierra Chemical Europe B.V., Heerleen, Holland, 1.1 kg m^{-3}). Watering was carried out twice daily at 0800 and 1700 h. Once-weekly, Fisons Parks general purpose liquid feed (Fisons, Cambridge, U.K.) was applied at the manufacturer's recommended dilution. All the plants used were grown in a glasshouse.

Temperature was not strictly controlled but night heating was provided in winter and the houses were well ventilated on summer days. Between September and April, supplementary illumination was given for 16 h daily from Atlas Kolorlux 400 W MBFR/U high-pressure mercury vapour lamps placed 800 mm apart 1 m above bench height. The irradiance from these lamps, in the band 400-1000 nm, varied from 5-10 W m^{-2} at pot height to 20-40 W m^{-2} at upper leaf canopy height for mature *Commelina* plants. A detailed analysis of the spectral distribution of the illumination from these lamps is given by White (1973).

The following pesticides were used at irregular intervals as required.

A. Draza Slug Killer (May and Baker Ltd., Dagenham, U.K.)

B. Liquid Malathion (for aphids), Murphy Chemicals Ltd., St. Albans, U.K.

C. Gammexane Smoke Generator (for aphids) ICI Plant Protection Division, Fernherst, U.K.

D. Morestan Smoke Generator (for Red spider mite), PBI Ltd., Watham Cross, U.K.

Specimen plants were identified at the species level (*Commelina*) and *varietas* level (*Ricinus*) using the following macro-characteristics (Bailey, 1925).

Commelina communis L.

A. Capsule two-celled and four-seeded.

B. Flower size greater than 12 mm across.

C. Stems erect exhibiting little adventitious rooting.

Ricinus communis L. *var. Gibsonii*

A. Striped seed coat markings.

B. Oval cotyledon shape.

C. Shape and distribution of nectarines on petioles.

CHEMICALS AND GLASSWARE

(i) *Gases*

Cylinder gases were supplied by B.O.C., Glasgow, U.K, and B.O.C. Special Gases, London, U.K. The supplier's specifications were as follows:

'Air' was collected near Polmadie, Glasgow and had a CO_2 concentration of *ca.*300 vpm. 'CO_2-free air' was made from liquid nitrogen and oxygen. The oxygen content of the gas was 19.5 ± 1.5%. Other impurities tested (CO, NO_x and total hydrocarbons) were all less than 1 vpm. 'Nitrogen' used was oxygen-free grade.

(ii) *Solvents*

Water was glass-distilled and stored in polyethylene tanks. Methanol was supplied 'purissima' grade by Beveridge Ltd., Edinburgh, U.K. Solvents supplied in drums (e.g. diethyl ether) were always redistilled; ethyl acetate was always freshly redistilled before use. Other solvents were used as supplied.

(iii) *General chemicals*

General laboratory chemicals were supplied by:

Hopkins & Williams, Ltd., Chadwell Heath, U.K.,
May & Baker Ltd., Dagenham, U.K.,
and B.D.H. Ltd., Poole, U.K.

(±)-ABA (Grade 1, 95 + % purity) was supplied by Sigma Chemical Co. Ltd., St. Louis, U.S.A.

(iv) *Radiochemicals*

The radiochemicals used are listed in Table 4. Specific activities stated are those of the supplier except in the case of tritiated ABA. The specific activity of this compound was estimated by transferring an aliquot of source solution to a methanol-rinsed glass bottle of known weight. The solvent was evaporated until the weight of the container was constant to the nearest 20 μg. The weight difference was then used to calculate the specific activity assuming 100% purity.

Table 4 *Radiochemicals*

Radiochemical	Supplier	Supplier's Batch Designation	Specific Activity TBq mol^{-1}
(±)-2-^{14}C-ABA	Radiochemical Centre, Amersham, U.K.	Code CFA.557	0.2257
(±)-2-^{14}C-ABA	New England Nuclear, Boston, U.S.A.	Lot 882-210	1.328
(±)-G-^{3}H-ABA	Radiochemical Centre, Amersham, U.K.	Code TRQ.702	17.168
(1,2)-^{3}H-*n*-hexadecane	"	Several batches	Varied
1-^{14}C-*n*-hexadecane	"	Several batches	Varied
^{86}RbCl	"	Code 133BA 3BA	Varied due to short $t_{\frac{1}{2}}$ of ^{86}Rb
U-^{14}C-sucrose	"	Code CFB4 Batch 56	0.3182

Radiochemical purity of 2-^{14}C-ABA and G-^{3}H-ABA was estimated by TLC in appropriate solvent systems at various intervals during the use of the chemical. A single peak containing over 95% of extracted radioactivity was found in all cases. Chemical purity was not directly assessed.

The Radiochemical Centre estimates of the chemical and radiochemical purity of labelled n-hexadecane were in excess of 97%. No estimates of purity of ^{86}RbCl were available. The Radiochemical Centre estimate of the radiochemical purity of U-^{14}C-sucrose was 99%.

(v) *Glassware*

Glassware was routinely washed by soaking in warm 'dilute' Pyroneg (Diversey Ltd., Cockfosters, U.K.) detergent solution, scouring with a bottle brush and rinsing in tap water. Glassware retained for work involving radioactivity was washed by steeping in 'strong' Pyroneg

solution for at least 24 h, rinsed with tap water, rewashed as per routine glassware, and rinsed with methanol after drying.

EXPERIMENTAL METHODS

General experimental conditions

All experiments on live plant material were performed in a 10.4 m^3 walk-in growth room. This had two side benches each equipped with a bank of five 1.5 m 80 W white fluorescent tubes (Atlas Ltd., Glasgow, U.K. and Omega Lampworks Ltd., Stockport, U.K.) placed 650 mm above bench height. The room was illuminated for 16 h daily (0800-2400 h). The air in the room was circulated and maintained at 23 ± 1.5 C. Humidity was uncontrolled but was generally in the region 60-80% RH. The air was frequently changed by leaving the door open for extended periods outwith experimental times.

Plants were transferred to the growth room from the glasshouse one or more days before use in experiments. Watering was carried out as in the glasshouse unless otherwise stated. Other experiments, e.g. hormone purification, were carried out in a laboratory at ambient temperatures (*ca.* 20-25 C.)

Most experiments were repeated at least twice. If not, this fact is mentioned in the text.

Treatment of plant material

(i) *Preparation of epidermal tissue*

Epidermis tissue for incubation studies: Epidermal strips were obtained from the abaxial surface of the fully expanded leaf closest to the apex. The following procedure was adopted to optimise epidermal cell viability and minimise mesophyll cell contamination.

The chosen leaf was first cut with a razor blade into strips of manageable width (3-4 mm). This was usually done between the parallel major veins of the leaf (Fig.3), since rupture of the epidermal strip and/or mesophyll contamination was often observed during peeling at points where veins crossed the strips. Two leaf strips could be obtained from each leaf, one each side of the midrib. During peeling of epidermis from the first of these, the rest of the leaf was placed

Fig. 3. Site of Leaf Strips Taken for Peeling.

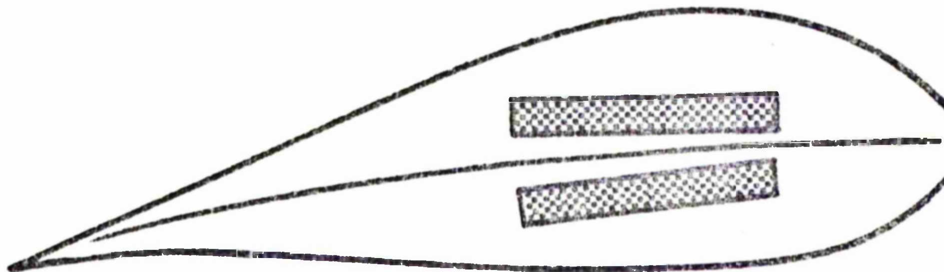
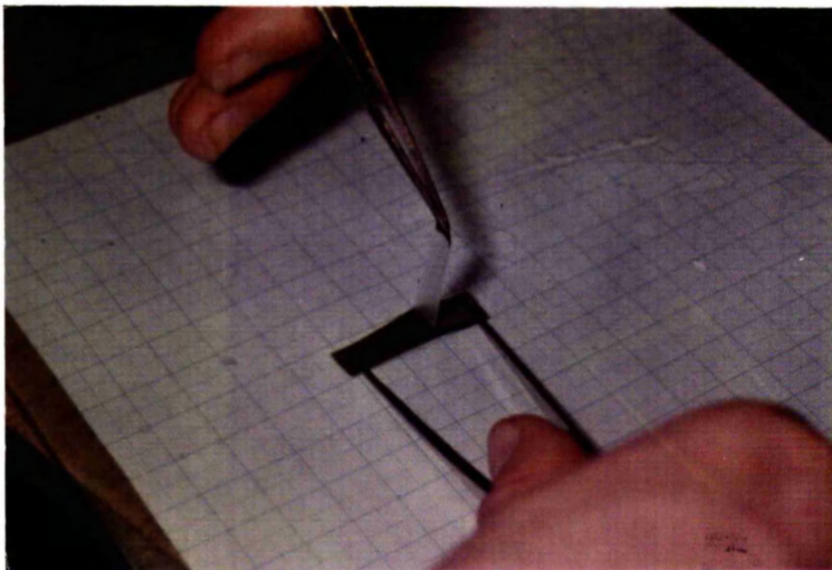


Fig. 4. Method of Peeling Epidermal Strips.



adaxial surface up, on distilled water, to prevent desiccation. Having obtained a leaf strip, a razor cut was made at the leaf tip end through the upper epidermis without damaging the lower epidermis. The 'tab' thus created was pulled vertically away from the mesophyll tissue using tweezers (Fig.4). After peeling, the epidermal strips were cut into squares or other shapes for identification (= segments). This tissue was manipulated using tweezers. When large numbers of segments were required those already peeled were floated on distilled water prior to use. The distribution of segments between treatments from such a 'pool' was assumed to be random (designated 'R' in figure legends). If incubation of strips was staggered between treatments, the allocation was non-random as different leaves, although physiologically equivalent, were used for each batch of tissue required (designated 'NR' in figure legends).

In order to test the relationship between epidermal cell viability and mesophyll contamination the angle of peeling (here defined as the angle between the separating tissues) for different leaf strips was varied from about 0.1 -3.05 rad ($5-175^{\circ}$).

Epidermis tissue from pretreated leaves: Epidermis for experiments involving pretreated leaves (on or off plant) was removed as above with the exception of that which was required for radioassay by scintillation spectrometry, where several modifications were employed. Firstly, the width of leaf strips was carefully measured (normally 7 mm); subsequent cuts at right angles to the length of the epidermal strip were restricted to 7, 14, or 21 mm to yield segments of area approximately 50, 100, or 150 mm^2 respectively. Where used, mesophyll plus upper epidermis tissue corresponded to the lower epidermis tissue removed from the leaf strip. When both sides of the lamina were sampled, the second was placed between moist 'Kleenex' tissue during sampling of the first. A further modification was that the angle of peeling was normally slightly greater than normal in order to limit further the possibility of mesophyll contamination. If epidermal tissue was required for stomatal aperture measurement, this was removed from areas adjacent to those sampled and either freeze-dried or measured immediately.

'Dead' epidermis tissue: 'Dead' epidermis material was obtained by the 'freeze-thaw' method. Tissue was placed under liquid nitrogen for 5-10 s and thawed by placing in distilled water.

Freeze-drying of epidermis: Epidermal tissue which was freeze-dried for autoradiography and/or fixing of stomatal aperture was first applied to slides subbed using the following method. Methanol-rinsed slides were dipped in subbing solution (5 g gelatin dissolved completely in 10^{-3} m^3 hot water before adding 0.5 g chromium potassium sulphate) and allowed to dry. Subbed slides were stored in boxes at 4 C. Epidermis was floated on a small drop of water on the slide. The fluid was then removed with a piece of 'Kleenex' tissue. The slide with strip attached was then quickly immersed in liquid nitrogen (-196 C) and left until freeze-drying in an Edwards Freeze Dryer Model EF2 (Edwards High Vacuum, Crawley, U.K.). Slides were transferred to the chamber after precooling the coil to -30 C. Freeze-drying was then carried for at least 3 h and normally 12 h or more. Freeze-dried material was stored in perspex boxes containing anhydrous silica gel.

(ii) *Staining of epidermal tissue*

Neutral Red: Epidermal material was floated on 1 kg m^{-3} aqueous solution of neutral red indicator for 2-4 min, and was then transferred to distilled water for a variable period. To study uptake of the dye by epidermal cells (i.e. non-subsidiary cells) the strip was examined microscopically after a rinse of about 2 min. In the case of subsidiary or guard cells this period was extended to 5-20 min.

Cells which took up dye appeared pale purple in colour and those which did not were orange-red (Willmer and Mansfield, 1969). Such cells were designated 'alive' and 'dead' respectively. Estimation of the proportion of live to dead cells was made by counting the number of these cells whose outline bisected an eyepiece graticule at 100x magnification. Estimation of the proportion of stomatal complexes with no living subsidiary cells was made by counting the number of live and dead complexes in half the field of view at 100x magnification. The proportion of living guard cells was estimated in the same way.

Several of the above estimates were combined for each segment.

Uptake of neutral red dye by the stomatal complex as a function of time was studied by photography of slide-mounted segments at various intervals after staining.

Evan's Blue: Epidermal material was floated on a drop of aqueous solution of Evan's Blue (2.5 kg m^{-3}) placed on a slide. A cover slip was applied and excess dye removed under gentle pressure with a 'Kleenex' tissue. According to Gaff and Okongo-ogola (1971), the dye is not taken up by living protoplasm, but penetrates the cell wall if the contents are ruptured or dead. Consequently, cells which remained colourless were designated 'alive' and those which appeared blue were said to be 'dead'. Estimation of the proportions of living cells was by the same method used with neutral red.

(iii) *Incubation Conditions for Epidermal Segments*

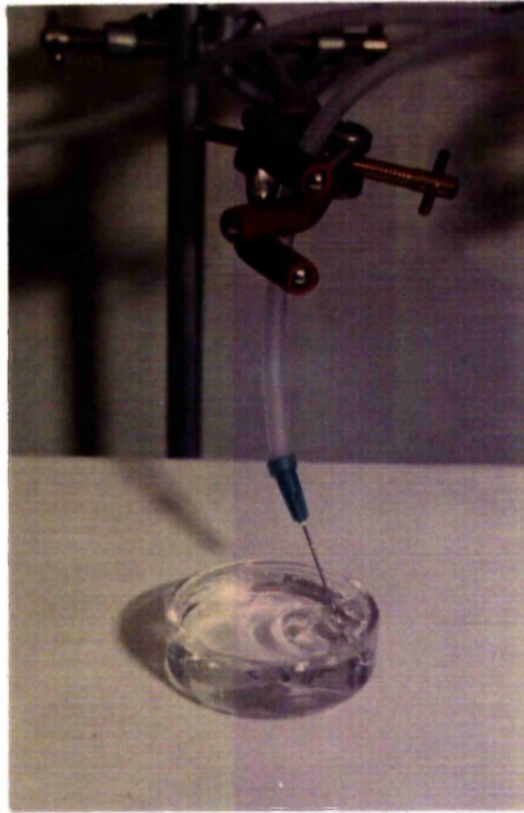
In all incubation experiments, a rigid timetable was adhered to. Solutions were pre-incubated in the growth room for 0.5 h starting between 1130 -1215 h. This allowed equilibration of the medium, in terms of temperature and CO_2 concentration, before adding epidermis tissue, which was therefore placed in buffer from 1200-1245 h. If staggered incubations were required by the experimental design, staggered preincubations were employed.

Two incubation systems were used in these experiments: the method used in individual cases is shown in the figure legends.

System 1: In this method, 20 x $10^{-6} m^3$ buffer solution was placed in a 25 x $10^{-6} m^3$ glass petri dish 300 mm below a bank of fluorescent lamps on a board covered with Benchkote. At this level the direct irradiance in the band 400-1000 nm lay between 16-19 W m^{-2} . CO_2-free air was bubbled through the solution using hypodermic needles at a rate of 1.7 x $10^{-6} m^3 s^{-1}$ (see Fig.5). Up to 12 dishes were used per experiment.

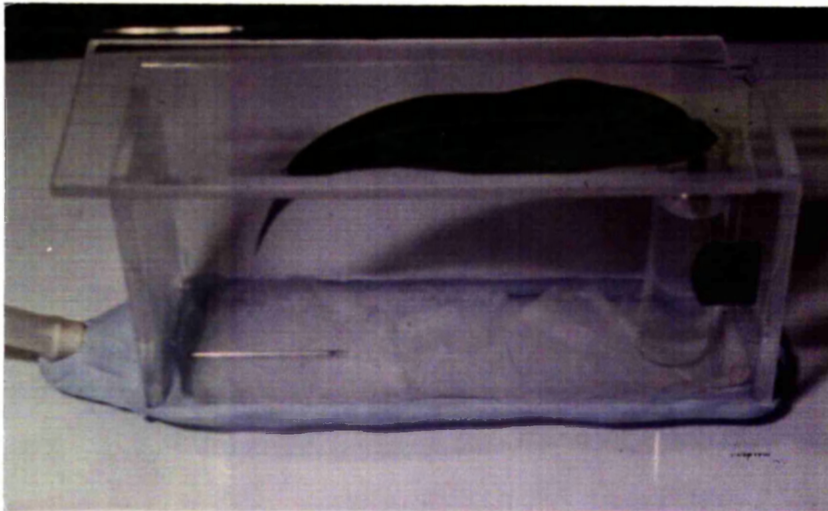
System 2: This method was preferred for work with radioactive solutions as aeration was not carried out and therefore splashing and vapour

Fig.5. Incubation of Epidermal Segments: System 1.



The petri dish was 50 mm in diameter

Fig.6. Incubation of whole leaves.



The perspex box was 110 mm long.

production were minimised. $5 \times 10^{-6}\ m^3$ solution, was incubated in a well of a clear perspex 6-welled Linbro Multi-dish (Linbro Scientific Co., Inc., Hamden, U.S.A.). The wells were covered with a perspex lid and placed on cartridge paper on a slow orbital shaker (Gallenkamp Ltd., London, U.K.). This was placed such that the dishes received the same irradiance as those of system 1.

When transfer from dish to dish was required for treated epidermis tissue, equivalent disturbance was given to the control tissue, although this was not always transferred to a new dish.

Uptake of label by epidermal strips: Uptake of labelled ABA or ^{86}Rb was examined by floating epidermal tissue on buffer solution containing the tracer. After the desired incubation period free-space label was removed from the tissue by a suitable rinsing procedure. Epidermal material was then used for radioassay.

Efflux of label from epidermal strips: Efflux of label from epidermal tissue incubated as above as a function of time was studied by sequentially transferring segments between efflux solutions. The buffer used was similar with the exception of label content to the uptake medium. 500 mm^3 was allowed per epidermal strip. The amount of label efflux was estimated by radioassay of the efflux medium.

Comparison of the efflux of ^{86}Rb from strips in solutions of buffer and buffer with ABA was made by transferring strips to dishes containing these solutions (system 1) and sampling the solutions by micropipette at various intervals. Since the volume sampled was 200 mm^3 from an initial total of $10 \times 10^{-6}\ m^3$, the cumulative efflux was given by

$$\frac{T_n = C_n(50 - n) + \sum_{1-n} C_i}{\text{area of tissue}}$$

where n = no of samples taken, T_n = cumulative efflux at nth sample, C_i = radioactivity in ith sample, C_n = radioactivity in nth sample.

Free-space Rinsing: The free space rinsing period was calculated from study of the efflux characteristics of the relevant label.

For ^{14}C-ABA treated tissue, the rinsing treatment consisted of 6×15 s washes in buffer using incubation system 2.

In the case of ^{86}Rb, the rinsing treatment varied: the total rinse time is noted in figure legends. Generally, segments were floated on buffer (system 2) for several minutes and then submerged in fresh buffer solution to remove label on the cuticle surface for the remainder of the rinse period.

For both isotopes, the rinse buffer used was identical, with the exception of tracer content, to that used for incubation. 500 mm^3 buffer per segment was allowed in each rinse.

(iv) *Solutions used for incubation of epidermal tissue*

These solutions were made up from stock buffer solutions as required and used once only. Stock solutions based on PIPES or MES (B.D.H. Ltd., Poole, U.K.) were adjusted to the correct pH with KOH before making up to volume. The pH was then rechecked. All stock solutions, particularly those containing ABA, were replaced regularly and were stored at room temperature in actinic glass bottles. The following buffer systems were used.

PIPES/KCL pH 6.8 buffer: These were based on 10 mol m^{-3} PIPES pH 6.8 and made from the following stock solutions:

A. 500 mol m^{-3} KCl in 10 mol m^{-3} PIPES, pH 6.8.

B. 10 mol m^{-3} PIPES, pH 6.8.

C. 0.2 mol m^{-3} ABA in 10 mol m^{-3} PIPES, pH 6.8.

As example, a solution of 100 mol m^{-3} KCl/10 mol m^{-3} PIPES/0.1 mol m^{-3} ABA was made from stock solutions A, B, and C in the proportions 2:3:5 (v/v).

When cold RbCl was used instead of KCl, the solutions were made up from a 500 mol m^{-3} RbCl in 10 mol m^{-3} PIPES pH 6.8 stock solution in place of solution A. Calcium and strontium were supplied as chlorides and solutions of the salts were made by serial dilution of a 1 mol m^{-3} stock solution containing 50 mol m^{-3} KCl and 10 mol m^{-3} PIPES at pH 6.8.

PIPES/Na NO_3 pH 6.8 buffer: The PIPES/$NaNO_3$ buffer system of Ogunkanmi *et al.* (1973) was used. This was also based on 10 mol m^{-3} PIPES pH 6.8. Incubation solutions were made from the same stock solutions as above but with 500 mol m^{-3} $NaNO_3$ instead of 500 mol m^{-3} KCl. Solutions containing different concentrations of ABA were made by serial dilution.

MES pH 4.5 buffer solution: This buffer was used for the selective killing of cells in epidermis tissue (Squire and Mansfield, 1972). KCl was not added.

Citrate pH 5.5 buffer solution: 10 mol m^{-3} Citrate buffer at pH 5.5 containing 10 mol m^{-3} KCl (Willmer *et al.*, 1978) was made from citric acid and Na_3 citrate stock solutions (100 mol m^{-3}) in the approximate proportion 3:7 (v/v) diluted ten times with distilled water. ABA was dissolved in the solution at a concentration of 0.1 mol m^{-3}.

(v) *Preparation of whole leaves*

Leaf material: Leaves were obtained from mature plants with several branches. Healthy first or second fully expanded leaves nearest to the apices were selected. After detaching a leaf with some 50 mm of stem below the point of insertion, the portion of stem above the leaf was excised and the end of the lower part cut rapidly with a razor under water at least five times. This procedure reduced the possibility of air embolism occurring in xylem conduits due to the release of xylem tension (J.A. Milburn, pers. comm.). The stem was then trimmed to 40 mm. Care was taken not to dampen the lamina during this procedure.

Incubation of leaves: Leaves with 40 mm stem attached were incubated by placing the stem in vials of distilled water or aqueous ABA solution inside perspex chambers 150 x 10^{-6} m^3 in volume (Fig.6), through which air or CO_2-free air of high humidity was passed.

In order to increase the humidity of the gas supply, it was first passed through two Dreschel bottles of water in series, and then into the base of the perspex boxes *via* hypodermic needles. The bottom of each chamber was lined with damp 'Kleenex' tissue and a perspex lid was

placed on top of the box in such a way that the gas supplied passed over the leaf before leaving the chamber. The gas flow rate was regulated at $2{\cdot}5 \times 10^{-6}$ m^3 s^{-1} $chamber^{-1}$, which was approximately one chamber volume per minute. The boxes were placed on a glass plate and sealed with 'Blu-tack' (Bostik Ltd., Leicester, U.K.). Up to 15 chambers were in use at any given time.

All experiments using this method were carried out between 1000-1340 h. If transfer of leaves from control to treatment solutions was required, untreated leaves were given equivalent disturbance.

(vi) *Water-stress Treatments*

Short-term stresses (0-5 h): For short-term stresses, treatments measured by fresh weight loss were used. Leaves were removed from well-watered plants in the greenhouse and weighed in the laboratory. Control batches were immediately placed in clear polythene bags from which the air was removed by suction before sealing. Treated leaves were placed on a top-loader balance and dried until 10% fresh weight loss had occurred. A hair dryer (airstream temperature < 35 C) placed 1 m from the leaves was used to accelerate this process. After reducing fresh weight, the stressed leaves were bagged on a similar fashion to controls. Sets of control and treatment leaves were left in the bags under fluorescent lighting (5-10 W m^{-2}, in the band 400-1000 nm) for 1,3 and 5 h. The time taken for reducing fresh weight was included in treatment times.

Long-term stresses (0-5 d): For long-term stresses, treatments which involved reducing or withholding water from potted plants were used. Individual watering regimes are described with the relevant experiment.

(vii) *Transport of Radio-tracers in the Phloem Sap of Ricinus*

These experiments were carried out in the growth room. The adaxial surface of first or second alternate leaves of *Ricinus* seedlings was abraded gently with Aloxite Optical Smoothing Powder (The Carborundum Co., Ltd., Manchester, U.K.) on an area of approximately 10 mm diameter, using damp cotton wool. Abrasion was stopped when anthocyanin pigments (present in gland cells of the epidermis) were

observed to have been removed. The area was washed with distilled water, dried and then used for the application of tracers in one of the following ways:

A. A 2 mm thick, 10 mm diameter cylinder of 1.5% (v/v agar containing G-^{3}H -ABA was applied to the abraded leaf surface and covered with a 15 mm diameter glass coverslip.

B. A 25 mm^3 drop of aqueous solution of tracer(s) (with or without buffer) was applied to the abraded leaf surface by micropipette and covered with a 20 mm square film of polyethylene.

A 5-10 mm lateral bark incision was then made immediately and the phloem sap collected in glass microcapillary tubes or in methanol-rinsed glass pipettes of suitable size. The time of collection and volume collected (where relevant) were noted. In some cases two or more ascending lateral incision were made. Full details of the leaf abraded, method of tracer application, activity of tracer(s) applied, point of incision and number of incisions, are given in the individual figure legends.

For analysis of the molecular state of tritium label from labelled ABA present in the sap, the exudate was adjusted to pH 2 with 1 kmol m^{-3} HCl and partitioned twice against ethyl acetate (1 x 5 volumes, 1 x 1 volume). The ethyl acetate was evaporated from the combined fractions and the sample used for TLC analysis.

Circulation of label from applied ABA into the xylem sap was studied by collecting root pressure exudate using the method of Wieringa and Bakhuis (1957).

The effect of abrasion on cell structure and leaf anatomy was examined by preparing sections, for light microscopy, of abraded and control areas of leaf.

(viii) *Silicone Rubber Stomatal Impressions*

Flexico 'Silflo' silicone impression material and 'Silflex' catalyst (J. & S. Davis Ltd., London, U.K.) in the proportion 10:1 (v/v) were mixed thoroughly with a plastic spatula. The fluid was

applied gently to the leaf surface (generally abaxial), allowed to harden for several minutes, and then peeled off. A 'positive' was made by applying the impression to a thin layer of No.7 clear nail varnish (Boots Ltd., Nottingham, U.K.) coated on a slide. The varnish was then allowed to dry thoroughly (0.5 h) before peeling away the impression. The positive was viewed by light microscopy, care being taken to ensure that the stomatal throat was in focus.

Measurements

(i) Estimation of stomatal aperture and epidermal segment areas

Stomatal apertures on live epidermis tissue, freeze-dried epidermis and from stomatal impressions were measured by light microscopy. A magnification of 400x was employed and the pore throat width (at its widest point) was estimated by eyepiece graticule to the nearest 0.75 µm. As a general rule, 25 measurements from each of two or three segments were combined for each mean shown. The total number of aperture measurements, n, and number of replicate segments, s, is shown in individual figure legends.

In order to 'randomise' observations and yet achieve speedy measurement, a compromise method was used whereby five groups of five stomata were measured from individual segments by 'random' movement of the stage. Stomata at the edges were not used. If an assistant wrote down verbally-communicated measurements, the estimation of 50 apertures could be completed in 90 s.

When segment areas were required, they were estimated by eyepiece graticule at 25x magnification and were accurate to the nearest 0.1 mm^2. When required, estimates of contaminating mesophyll cells were obtained from several counts in half the field of view at 100x magnification.

(ii) Radioassay

Liquid scintillation spectrometry: Radioactivity in samples was measured using a Packard 3380 liquid scintillation spectrometer. $20 \times 10^{-6} m^3$ plastic vials (A. & J. Beveridge Ltd., Edinburgh, U.K.) were used with $10 \times 10^{-6} m^3$ scintillation cocktail ('scintillant').

(a) *Estimation of ^3H and ^{14}C activity*

Scintillants

Two types were used:

A. 4 Kg m^{-3} PPO (2,5-diphenyloxazole) in toluene.

B. Two parts A plus one part Triton X-100
(A. & J. Beveridge Ltd., Edinburgh, U.K.).

Scintillant A was used with toluene-soluble organic solvent samples and scintillant B with aqueous samples.

Counting efficiency: The efficiency of counting was estimated using the Automatic External Standard (A.E.S.) feature of the spectrometer. This was calibrated for each scintillant using ^{3}H-hexadecane or ^{14}C-hexadecane in the following manner.

A plastic vial was weighed to the nearest 10 μg and reweighed after adding *ca.* 20 mm^3 of labelled hexadecane. Scintillant was added immediately. The theoretical amount of radioactivity present was calculated from the weight difference using the specific activity of the hexadecane. The vial was then counted in the relevant channel(s) on the spectrometer and the A.E.S. ratio noted. Greater degrees of quenching (and therefore lower A.E.S. ratios) were obtained by adding drops of a methanol extract of French bean leaves to the vial. The counting efficiency was calculated at each A.E.S. ratio from the formula.

$$\text{Efficiency} = \frac{\text{count obtained}}{\text{theoretical activity present}}$$

The relationship between A.E.S. ratio and efficiency was estimated by fitting the points obtained to least square curves using an HP67 calculator (Hewlett Packard, Corvallis, U.S.A.). Linear, exponential, logarithmic and power curves (of equation $y = a^x + b$, $y = ae^{bx}$, $y = a \ln x + b$ and $y = ax^b$ respectively, where y = efficiency and x = A.E.S. ratio, $o < x, y < 1$) were fitted and that giving the highest coefficient of correlation accepted.

Calculation of radioactivity present per vial: The factory pre-set channels were used to obtain ^{3}H, ^{14}C and double-label efficiency curves

for the different scintillants. The radioactivity present in each vial counted was then calculated using a programme for the HP67 calculator which utilised the efficiency curves and the following formulae:

Tritium

$$\text{radioactivity} = \frac{\text{counts in } ^{3}\text{H channel} - \text{bg}}{^{3}\text{H efficiency in } ^{3}\text{H channel}}$$

Carbon 14

$$\text{radioactivity} = \frac{\text{counts in } ^{14}\text{C channel} - \text{bg}}{^{14}\text{C efficiency in } ^{14}\text{C channel}}$$

Double-labelling

$$^{14}\text{C radioactivity} = \frac{\text{counts in } ^{14}\text{C(H) channel} - \text{bg}}{^{14}\text{C efficiency in} ^{14}\text{C(}^{3}\text{H) channel}}$$

$$^{3}\text{H radioactivity} = \frac{\text{counts in } ^{3}\text{H(}^{14}\text{C) channel} - \text{bg}}{^{3}\text{H efficiency in } ^{3}\text{H(}^{14}\text{C)channel}} - \frac{^{14}\text{C radioactivity}}{\text{isotope ratio}}$$

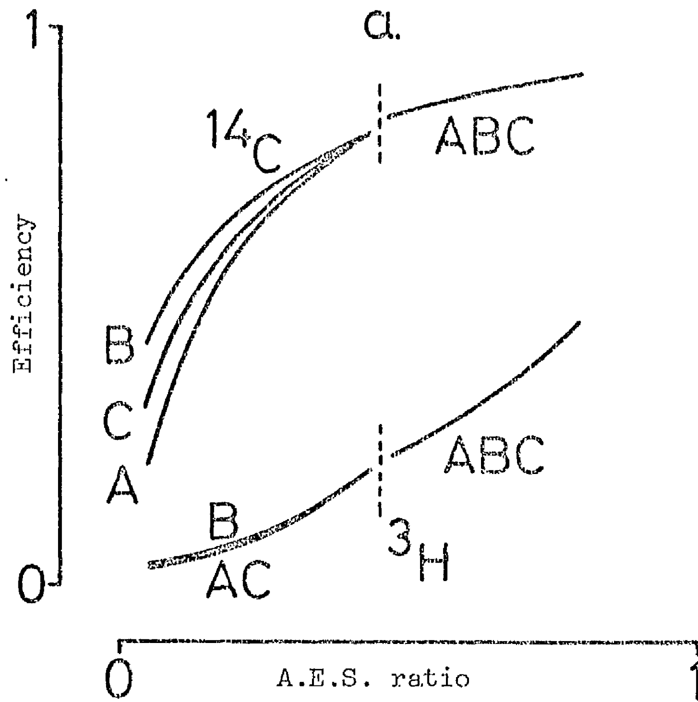
$$\text{isotope ratio} = \frac{^{3}\text{H efficiency in } ^{3}\text{H(}^{14}\text{C) channel}}{^{14}\text{C efficiency in } ^{3}\text{H(}^{14}\text{C) channel}}$$

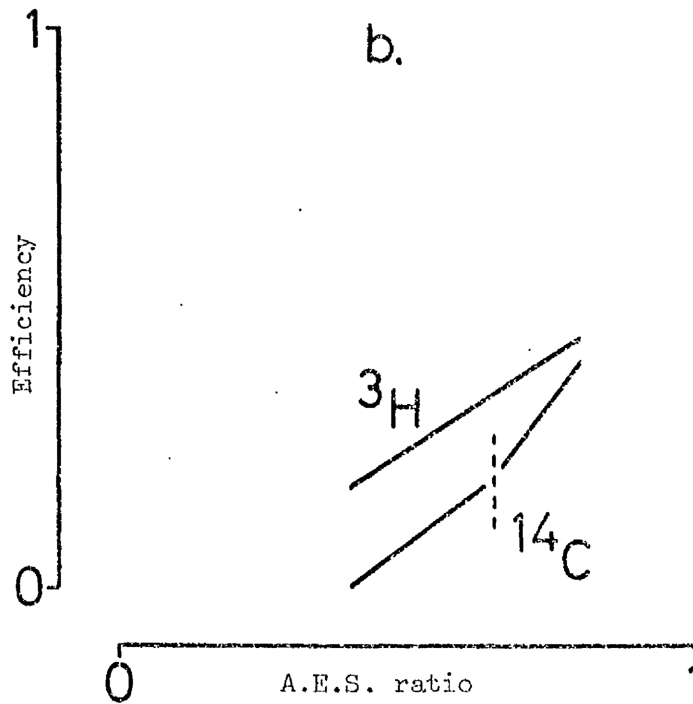
The efficiency curves used in these analyses are illustrated in Figure 7. Background (bg) was obtained by using scintillant only in the vials. It did not vary significantly with quench.

Sample preparation

If required, samples were prepared by rendering them soluble in either a suitable organic solvent or water. Radioactivity was extracted from epidermal segments by placing the tissue in a scintillation vial with 10^{-6} m^3 methanol or methanol:ether (1:1. v/v) mixture for 3 h or more. The same procedure was used for sections of plastic TLC plates.

Fig. 7. Efficiency Curves Used for Scintillation Spectrometry.





For Curves used see following page.

Fig. 7. Efficiency Curves Used for Scintillation Spectrometry

a. Single Label Calculations.

Scintillants A, B as text $C = A + 1 \times 10^{-6}\ m^3$ MeOH: Et_2O.

Equations used:-

Isotope	A.E.S. range	Scintillant	Equation
^{14}C	A.E.S. = 0·45 – 0·8	A,B,C	$y = 0{\cdot}957 + 0{\cdot}145 \ln x$, $r = 0{\cdot}969$, $n = 17$
	A.E.S. = 0 – 0·45	A	$y = 1{\cdot}05 + 0{\cdot}277 \ln x$, $r = 0{\cdot}996$, $n = 14$
		B	$y = 0{\cdot}949 + 0{\cdot}170 \ln x$, $r = 0{\cdot}991$, $n = 34$
		C	$y = 0\ 987 + 0\ 218 \ln x$, $r = 0\ 992$, $n = 16$
^{3}H	A.E.S. = 0·45 – 0·8	A,B,C	$y = 0{\cdot}09e^{2{\cdot}1x}$, $r = 0{\cdot}998$, $n = 17$
	A.E.S. = 0 – 0·45	A,C,	$y = 0{\cdot}23e^{5{\cdot}2x}$, $r = 0{\cdot}992$, $n = 29$
		B	$y = 0{\cdot}03e^{4{\cdot}4x}$, $r = 0{\cdot}998$, $n = 37$

b. Double Label Calculations.

Only scintillants A, B used

Equations for both:-

Isotope	A.E.S. range	Equation
^{14}C in $^{14}C(^{3}H)$	A.E.S. = 0·4 – 0·65	$y = 0{\cdot}720x - 0{\cdot}27$, $r = 0{\cdot}996$, $n = 17$
	A.E.S. = 0·65 – 0·8	$y = 1{\cdot}396x - 0{\cdot}704$, $r = 0{\cdot}999$, $n = 4$
^{3}H in $^{3}H(^{14}C)$	A.E.S. = 0·4 – 0·8	$y = 0{\cdot}686x - 0{\cdot}09$, $r = 0{\cdot}998$, $n = 22$
Isotope ratio	A.E.S. = 0·4 – 0·8	$y = 5{\cdot}094x^{2{\cdot}548}$, $r = 0{\cdot}995$, $n = 9$

(b) Estimation of ^{86}Rb activity

Scintillant

^{86}Rb β-emission was counted using the Cerenkov radiation produced by this isotope in water. Hence, 10×10^{-6} m^3 distilled water was added to each sample as scintillant.

Counting efficiency

As the compound used for ^{14}C and ^{3}H A.E.S. ratio determinations did not produce significant Cerenkov radiation in water, this method was not used for efficiency determinations. It was assumed that the variability in quenching between samples was negligible. The optimum efficiency in the 'high energy' channel was found by using a sample of ^{86}RbCl of known activity and maximising the counts obtained by altering the window settings and amplification. The highest efficiency obtained was 0.38 at 100% amplification with the window set at 300-1000 units.

Calculation of radioactivity present per vial

This was calculated from

$$\text{radioactivity} = \frac{\text{counts in high energy channel - bg}}{0.38}$$

Sample preparation

If necessary, samples were prepared by rendering them soluble in water. For segments, this was achieved by adding 100 mm^3 perchloric acid to the tissue in each vial for 3 h or more prior to the addition of 10×10^{-6} m^3 H_2O.

Allowance for radioactive decay

Owing to the short half-life of ^{86}Rb (18.66 d), allowance was made for this in the calculation of the activity present in solutions at any given time. The equation

$$A_t = A_o e^{-\lambda t}$$

was used, where A_o = original or reference amount of activity,

A_t = activity remaining at time t, λ = decay constant (for ^{86}Rb = 0.03715 d^{-1} or 0.00155 h^{-1}).

Microautoradiography

The microautoradiographic technique was a modification of that of Willmer *et al.* (1973).

(a) *Application of photographic film*

Pieces of Kodak Pan-F 18 DIN/50 ASA film (35 x 20 mm) were cut in the dark. The emulsion side was applied directly to the tissue and held in place firmly with a plain glass slide which was then taped to the subbed slide with freeze-dried tissue. 20 mm width 'Sellotape' was used for taping, but it was important to cut this into strips prior to film application because static sparking occurred as it was unpeeled. Controls for positive and negative chemography were always included. That for positive chemography consisted of unlabelled tissue with unexposed film. For negative chemography, exposed film was used with unlabelled tissue. Tapered slides were placed in a cardboard or plastic light-tight box and covered with black polythene and/or aluminium foil to exclude light.

(b) *Exposure*

Exposure of microautoradiographs was carried out at -15 C. The exposure period was calculated from estimates of the amount of radioactivity in the tissue on the basis that latent image formation was governed by an inverse relationship between tissue radioactive content and exposure time. Thus tissue radioactivity, in Bq mm^{-2}, multiplied by exposure time, in days, was predicted to equal a constant, K, for optimal exposure. Empirical evidence suggested that K ≈ 4.5 d Bq mm^{-2} was satisfactory for ^{14}C β-emission. This value was also found to hold for ^{86}Rb β-emission. Exposure times therefore varied from days to months between experiments depending upon the estimated radioactive content of the tissue.

(c) *Development Protocol*

In darkness, the light-tight box was opened and a razor blade used

to cut the tape between the slides. The pieces of film were removed and a number of notches cut in the side with scissors for identification. The film was then placed in a beaker after bending over the corners to prevent adhesion in solution. The slides were stored separately in an order corresponding to the film notches. A piece of muslin was then placed over the beaker and held in place with an elastic band. Development and fixing was carried out in the beaker with PQ Universal developer and Hypam fixer (Ilford Ltd., Ilford, U.K.) following the manufacturer's recommended procedure. The film was subsequently rinsed for at least 30 min in tap water and dried.

(iii) Analysis of molecular state of tracers

Radiochemical purity and the molecular state of radioactively labelled compounds was examined using thin layer chromatography in appropriate solvent systems.

TLC was carried out at 20 ± 1 C in sealed glass tanks lined with blotting paper and containing 150×10^{-6} m^3 solvent. 'Polygram' 50 x 200 mm plastic TLC plates precoated with 0.25 mm gypsum-free silica gel were used. An aliquot of radioactive sample in a suitable volatile organic solvent (e.g. diethyl ether, methanol, ethyl acetate) was applied as a spot or streak 20 mm from the base of the plate. Standards were applied in two ways.

A. As distinct spots separated by a longitudinal score in the support medium.

B. Standard was incorporated into the sample application solvent as the double-label analogue e.g. ^{14}C -ABA standard with ^{3}H - labelled sample.

The TLC solvent was allowed to run to a transverse score 160 mm from the origin. After drying the plate, it was cut into 20 eight-millimetre segments which were assayed by liquid scintillation spectrometry.

Results were expressed as percent of total recovered radioactivity present per segment. In each case illustrated, the total radioactivity recovered is noted in the figure legend.

(iv) Diffusive Resistance Measurements

These measurements were carried out using a Li-cor Li 20S Diffusive Resistance Meter (Li-cor Ltd., Lincoln, U.S.A.).

Calibration. This was carried out according to the manufacturer's instructions in a clear celluloid cage in which damp tissues had been placed. Readings were not taken unless the humidity was greater than 90% RH, or if the temperature of the growth room differed by more than 0·5 C from 27 C. Points obtained at this temperature were converted to 25 C-equivalent readings using the supplied conversion curve.

Readings. Measurements of transit time were taken with a stop-watch to the nearest 0.1 s. Leaf and ambient temperatures were noted. Transit times were converted to 25 C-equivalents and the resistance estimated from the calibration curve. Conductance was calculated as the reciprocal of resistance.

(v) Chlorophyll content of Commelina leaves

Relative senescence of *Commelina* leaves was estimated by measuring leaf chlorophyll content. As micrometer measurements indicated that leaf thickness did not vary significantly after expansion, chlorophyll levels were measured and expressed per unit area of leaf.

Leaf discs (53.5 mm^2) were taken by cork borer and boiled in methanol for 15 min. The volume of methanol was made up to $2 \times 10^{-6} m^3$ and the absorption spectrum measured using a Pye Unicam SP8000 UV Recording Spectrophotometer (10 mm pathlength, 0-1.0 scale). The absorbance (A) at 665 and 650 nm was measured and used to calculate the total chlorophyll content from

$$\text{total chlorophyll} = 25.5\, A_{650} + 4.0\, A_{665}$$

(Šesták *et al.*, 1971, after MacKinney, 1941).

In experiments where epidermis tissue was taken to assay stomatal reaction to ABA, samples of lamina for chlorophyll estimation were taken from the opposite side of the midrib. For this reason, only leaves having apparently even distribution of pigment within the leaf were used.

(vi) Measurement of irradiation

An Optometer Light Measuring Instrument Model 40X (United Detector Technology Inc., Santa Monica, U.S.A.) was used with the radiometric filter and diffuser unit supplied.

(vii) Humidity Measurement

Relative humidity was measured using a 0-100% hair hygrometer (Fischer Ltd., Dreback, W. Germany). When long-term measurements of temperature and humidity were required a thermo-hygrograph (Cassella Ltd., London, U.K.) was used.

(viii) Estimation of endogenous ABA content of Commelina tissue

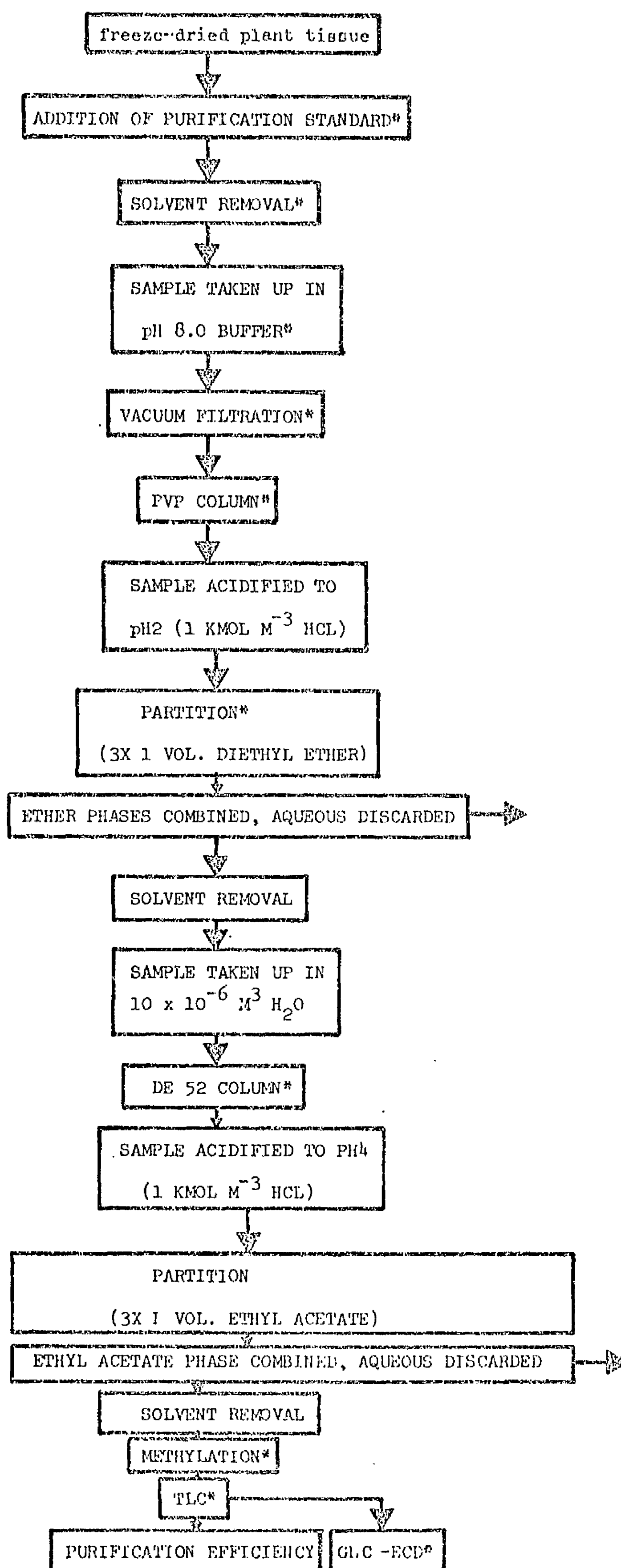
Extraction and purification of the methanol-soluble ABA-containing fraction of freeze-dried *Commelina* tissue was carried out using the procedure illustrated in simplified form as a flow diagram in Fig.8. Individial steps requiring elaboration are marked with an asterisk and details of these stages are given below. At all stages in the procedure the extracts were kept away from direct sunlight. Samples were stored between operations at 4 C.

Extraction: Freeze-dried tissue was extracted with methanol (approximately 10x fresh weight, v/w) at 4 C. The solvent was replaced twice at 24 h intervals and the 3 extracts were then combined for purification. Blank extracts (methanol only) were added at this point.

Purification standard: A standard of 2- ^{14}C -ABA was added to the pooled methanol extracts of each sample. This consisted of an amount of labelled ABA roughly equivalent to the estimated ABA content of the tissue (calculated both from the specific activity of the standard:- 0.2257 TBq mol^{-1} ≡ 0.855 Bq ng^{-1}, and from published or previously obtained estimates of ABA content). Equivalent samples were taken for radioassay at the time of addition and at the end of the purification an estimate of the total amount of radioactivity remaining in the extract was made: these two figures were used to estimate the purification efficiency.

Solvent removal: Solvents were removed from extracts by either of two methods. For large samples (> 5 ml), the solvent was removed by rotary film evaporation at <40 C, whilst smaller samples were dried under a stream of nitrogen.

Fig.8. Purification Procedure for Abscisic Acid.



Procedures marked with an asterisk are discussed further in the text.

pH 8.0 buffer: 66.7 mol m^{-3} pH 8.0 phosphate buffer was used. This was prepared from 66.7 mol m^{-3} monosodium phosphate and 66.7 mol m^{-3} disodium phosphate solutions in the proportion 26·2:1 (v/v). Samples were taken up in buffer by scouring the evaporation vessel using methanol-rinsed boiling stones with 10 x 10^{-6} m^3 buffer. This was repeated twice to give 30 x 10^{-6} m^3 buffer.

Vacuum filtration: This was carried out on extracts in buffer solution using Whatman's No.1 filter paper. A 'chase' of 10 x 10^{-6} m^3 buffer was given.

PVP columns: These were prepared from PVP powder (Polyclar AT - polyvinyl pyrollidone, Sigma Chemical Co. Ltd., St. Louis, U.S.A.) which was washed once with water and twice with pH 8.0 phosphate buffer to remove fines. 5 mm of glass beads (40 mesh) were placed on the sinter of a 150 x 10^{-6} m^3 Pyrex glass mercury filter tube (sinter porosity 1, height 210 mm, I.D. 30 mm) and 60 mm PVP layered on top. Columns were then washed with 100 x 10^{-6} m^3 phosphate buffer.

Samples (in buffer) were allowed to run through the column before being eluted with a further 100 x 10^{-6} m^3 buffer. The first 20 x 10^{-6} m^3 eluate was discarded and the next 60 x 10^{-6} m^3 eluate collected and retained. Columns were used once only.

To test column efficiency and other elution characteristics, samples of buffer containing G-^{3}H -ABA were passed through the column and eluted with buffer. 2 x 10^{-6} m^3 samples were collected for radioassay.

Partition: Partition of solvents was carried out such that adequate mixing and separation of phases occurred. In investigations of the efficiency of separation, G- ^{3}H -ABA was incorporated into the relevant phase and efficiency was calculated by sampling the phases after partition. Where extracts of plant tissue were required for dry weight determinations. These were obtained by extracting 100 g *Ricinus* leaves and purifying the extract to the first sample acidification stage. 30 g f. w.-equivalents of the extract in pH 8.0 buffer were used. The pH was then adjusted using HCl and KOH to 2.0, 4.0, or 8.0. 'Artificial' phloem sap was made from 15% (w/v) sucrose in water, and was adjusted to pH 2 with HCl. Partition was carried out using equal

volumes in each phase. To determine dry weight separation efficiencies, dry weights of samples were determined by heating, freeze-drying or vacuum desiccation; carbonisation was specifically avoided. In separate determinations, radioactive ABA was incorporated in the buffer solutions, and tracer separation efficiencies determined by sampling after partition, care being taken to avoid chemiluminescence radioassay artifacts.

The following values were determined for the solvents *n*-hexane, diethyl ether, chloroform, ethyl acetate and water saturated butanol (after Fifield and Kealey, 1975), assuming that equilibrium had been reached after partition, and that radioactivity measured represented ABA. The distribution of dry matter or tracer between the aqueous and organic phases was given by:

$$\text{Distribution ratio, } D = \frac{(CA)_o}{(CA)_{aq}} \quad ,$$

where (CA) = total concentration of all forms of solute A, o = organic phase, aq = aqueous phase,

$$\text{thus} \quad D_{ABA} = \frac{\text{radioactivity (o)}}{\text{radioactivity (aq)}} \quad \text{and } D_{DW} = \frac{\text{dry weight (o)}}{\text{dry weight (aq)}}$$

The efficiency of extraction of solutes from the aqueous phase to the organic was given by

$$\text{efficiency, } E = \frac{D}{(D + V_{aq} V_o^{-1})} \quad ,$$

where V represents the volume of the relevant phase. Since $V_{aq} V_o^{-1} = 1$ in these experiments, $E = \frac{D}{D+1}$. The relative separation of ABA from the bulk of the extract was calculated using the D values for ABA and dry weight distribution. Thus,

$$\text{separation factor } \beta = \frac{D_{ABA}}{D_{D.W}}$$

DE 52 columns: Whatman's DE 52 powder was prepared by acid/base precycling in the manner advised by the manufacturer. 30 mm was layered

over 5 mm glass beads (40 mesh) placed on the sinter of a 8 x 10^{-6} m^3 Pyrex mercury filter tube (sinter porosity 1, height 100 mm, I.D. 10 mm). The extract in water was passed through the column and followed with 25 x 10^{-6} m^3 water. This eluate was discarded. The ABA-containing fraction was eluted with 35 x 10^{-6} m^3 500 mol m^{-3} $NaSO_4$. All the eluate was retained. Columns were used once only.

To test column efficiency and elution characteristics, a sample of 2- ^{14}C - ABA in 5 x 10^{-6} m^3 water was passed through the column and followed by 25 x 10^{-6} m^3 water and 50 x 10^{-6} m^3 500 mol m^{-3} $NaSO_4$. 3-5 x 10^{-6} m^3 samples of eluate were collected for radioassay.

Methylation: Methyl derivatives of standards and extracts were prepared using etherial diazomethane employing a small-scale method similar to that of Schlenk and Gellerman (1960).

Equal parts of Nitrosan powder (bis- methyl, *n*-nitrosoterephthalamide, Du Pont (UK) Ltd., London, U.K.) ethylene glycol and diethyl ether were placed in a 250 x 10^{-6} m^3 reactant flask fitted with a 5 mm I.D. delivery tube. A small amount of strong sodium hydroxide solution was added and the bottom of the flask plunged into a beaker of hot water. The diazomethane gas produced was collected over iced diethyl ether to give a yellow solution. The reaction was stopped by the addition of acetone to the reaction mixture. Latterly, Diazald (*n*-methyl-*n*-nitroso-*p*-toluene sulphonamide, Aldrich Chemical Co. Ltd., Gillingham, U.K.) was used in the place of Nitrosan.

Aliquots of etherial diazomethane were added to each sample. The vials were stoppered and allowed to stand for over 1 h before evaporating the solvent under a stream of nitrogen. This was repeated once; if the yellow colour of the solution remained after 1 h it was assumed that diazomethane was precent in excess, and no more was added.

TLC: The TLC performed was used for both preparative and analytical purposes. Extracts were streaked 20 mm from the base of 200 x 200 mm glass-backed 0.25 mm silica gel plates (Camlab Ltd., Cambridge, U.K.) and run through 150 mm using *n*-hexane:ethyl acetate (1:1. v/v) as solvent. Otherwise, conditions were as elsewhere described.

To test the radiochemical purity of the purification standard, plates were examined on a TLC plate scanner (Model RTLS-1A; Panax

Equipment Ltd., Redhill, U.K.). The support and stationary phase at the Rf corresponding to the methylated standard (Rf = *ca.* 0·4–0.5) was scraped off and the extract eluted with *n*-hexane-ethyl acetate (1:1, v/v) which was subsequently evaporated.

GLC-ECD: Analyses of MeABA levels in methylated samples were carried out using a Perkin-Elmer F17 gas chromatograph (Perkin-Elmer, Ltd., Beaconsfield, U.K.) fitted with FID and ECD detectors. GLC was performed isothermally at 230 to 240 C on a 1 m ($\frac{1}{8}$ inch I.D.) stainless steel column packed with $2\frac{1}{2}$% XE-60 (2-cyanoethylmethyl silicone) on Chromasorb G AW DMCS 80-100 mesh (1596 plates min^{-1}). The column eluate was split 9:1 between the FID and ECD detectors and gas flow rates were kept constant (583 mm^3 s^{-1}) at the following pressures:

- A. Carrier gas (oxygen-free nitrogen) 278 KPa,
- B. Air (FID) 165 KPa,
- C. Hydrogen (FID) 97 KPa,
- D. Make up gas – oxygen-free nitrogen (ECD) 165 KPa.

The ECD detector incorporated a Ni^{63} β-source and was operated at 275 C in pulse 6 mode.

The responses of both detectors were recorded on 200 mm chart paper moving at 600 mm h^{-1}. Peak areas were estimated by a triangulation method (Weyers, 1975).

Estimation of the ABA content of tissues was made by comparison of the peak areas obtained with standards and samples, adjustment for dilution before injection, adjustment for sample purification efficiency, and consideration of tissue weight.

Attenuation linearity was tested by injecting constant amounts of MeABA and altering the attenuation setting, whilst detector response linearity was tested by injecting different amounts of MeABA of Me- ^{14}C-ABA and expressing peak areas relative to a specific attenuation setting.

Details of attenuation setting and other parameters differing from the above are given in the examples considered.

Mass Spectrometry

An AEl MS-30 with a WF-055 attachment (Multi-peak Monitor) was used to detect ABA in extracts of *Ricinus* phloem exudate.

10×10^{-6} m^3 sap was acidified to pH 2 with HCl and partitioned against ethyl acetate ($3 \times 2 \times 10^{-6}$ m^3). The combined organic layers were dried and taken up in diethyl ether. The residue was removed by filtration and the extract methylated.

Aliquots of this extract were subjected to GLC-MS analysis under the following conditions.

GLC: 2.74 m glass column ($\frac{1}{8}$ inch I.D.) containing 2% OV-210 on Gas Chrom Q 100-120 mesh support. Operating temperature 230 C isothermal. Flow rate 666 mm^3 s^{-1} (helium).

MS: Source temperature 250 C, electron current 4, resolving power 3000, separator temperature 235 C.

Hexadeutero MeABA (prepared by V. Math, Glasgow University) was incorporated as internal standard. Base peaks of MeABA(m/e 190) and hexadeutero MeABA (m/e 194) were used for detection purposes and full spectrum scans were also carried out.

(ix) Microscopy

Sample preparation: Plant material which required sectioning prior to light microscopy, and tissue for electron microscopy, were prepared by a method common for both techniques.

Segments of tissue were fixed for 12-24 h in 3% gluteraldehyde (w/v) in 50 mol m^{-3} sodium cacodylate buffer, pH 7-4, and then rinsed in buffer alone. The material was post-fixed in 2% osmic acid in buffer (w/v) for 3-12 h, followed by dehydration through a series of acetone solutions (25-100%, v/v). The tissue was then infiltrated with a 1:1 (v/v) acetone:resin solution overnight and the acetone allowed to evaporate. At this stage the resin mixture consisted of: Epon (Epikote) resin, Araldite (CY212) epoxy resin, and DDSA (dodecenyl succinic anhydride) hardener (all supplied by TAAB Laboratories Ltd., Reading, U.K.), in the approximate proportions 3:2:5 (w/w). Tissue was then placed in 100% resin for 4 h and subsequently embedded in resin to which 1.5% w/w of the accelerator DMP30 (2,4,6, tridimethyl-aminomethylphenol, TAAB Labs. Ltd.) had been added. Polymerisation was then carried out for 3 d at 60 C.

Sectioning and Staining

Light microscopy; 2 μm sections were cut using an LKB 11800 Pyramitome (LKB-Produkter AB, Stockholm, Sweden), and stained with toluidine blue.

Electron microscopy: Sections were cut with glass knives on an LKB Ultrotome III type 8801A. The thickness of sections was estimated at between 60-90 nm using the Interference Colour Index ('Silver' sections). Sections were stained for 0.5 h in uranyl acetate solution and the same period in lead citrate solution.

Photomicroscopy

Light microscopy: Slide-mounted sections were photographed using a Zeiss Photomicroscope II (Carl Zeiss, Oberkochen, West Germany) or a: Leitz Orthoplan microscope with Orthomat camera (E. Leitz Ltd., London, U.K.). These microscopes were also used for photography of microautoradiographs. The films used were Ilford Pan-F and Kodachrome 64.

Electron microscopy: Sections were mounted on copper grids coated with 2% collodin solution (w/v) in amyl acetate. Electron microscopy was carried out on AEI EM6B Electron Microscope (GEC-AEI Electronics Ltd., Harlow, U.K.) by Mrs. Denise Leake.

COMPUTATION

(i) HP67 Calculator (Hewlett Packard Ltd., Corvallis, U.S.A.).

Calculation such as least squares curve fitting and statistical analysis of data was performed using the manufacturer's programme cards. Other repeated calculations were carried out using specially written programmes. The following equations were used for statistical calculations.

$$\text{mean} \quad \bar{x} = \frac{1}{n} \Sigma x_i$$

$$\text{standard error (S.E.)} = \sqrt{\frac{1}{n}} \sqrt{\frac{\Sigma x^2 - \frac{(\Sigma x)^2}{n}}{n-1}}$$

t – tests (a) $$t = \frac{\bar{x} - \bar{y} - d}{\sqrt{\frac{1}{n_x} + \frac{1}{n_y}}\sqrt{\frac{\Sigma x_i^2 - n_x\bar{x}^2 + \Sigma y_i^2 - n_y\bar{y}^2}{n_x + n_y - 2}}}$$

with $n_x + n_y - 2$ degrees of freedom

(b) $$t = \frac{r}{\sqrt{\frac{1 - r^2}{n - 2}}}$$

with n – 2 degrees of freedom

where x_i, y_i = observations

n(x) = no. of observations (in group x)

d = hypothesised difference between means

Σ = sum over i = 1 – n

r = coefficient of correlation (see Table 5)

Curve-fitting, see Table 5.

(ii) NUMAC computer

Analyses of data from tracer efflux experiments was carried out by a computer-assisted least squares fitting process (Hipkins, 1978) on the Newcastle University-Glasgow University IBM computer. The programme used was produced by Dr. P. Rosenberg of Glasgow University Computing Service. This fitted data points (x_i, y_i) by least squares to combinations of exponential functions of the form

$$y = ae^{-bx}$$

In this case: y = Ln efflux rate (μBq mm^{-2} s^{-1}), x = efflux time(s). The maximum number of functions was 3 and the nominal upper limit of iterations for each fit 200. A weighting equivalent to x^{-1} was given to each y value.

Table 5. Equations used for curve fitting

Type of curve & equation	a	b	r
Linear Regression $y = a + bx$	$\frac{\Sigma y_i}{n} - b\,\frac{\Sigma x_i}{n}$	$\dfrac{\Sigma x_i y_i - \frac{\Sigma x_i\ \Sigma y_i}{n}}{\Sigma x_i - \frac{(\Sigma x_i)^2}{n}}$	$\dfrac{\left\{\Sigma x_i y_i - \frac{\Sigma x_i\ \Sigma y_i^2}{n}\right\}}{\left\{\Sigma x_i^2 - \frac{(\Sigma\ x_i)^2}{n}\right\}\left\{\Sigma y_i^2 - \frac{\Sigma y_i)^2}{n}\right\}}$
Exponential curve fit $y = ae^{bx}$	$\exp\left(\frac{\Sigma \ln y_i}{n} - \frac{b\Sigma x_i}{n}\right)$	$\dfrac{\Sigma x_i\ \ln_y - \frac{1}{n}(\Sigma x_i)(\Sigma \ln y_i)}{\Sigma x_i - \frac{1}{n}\Sigma(x_i)^2}$	$\dfrac{(\Sigma x_i\ \ln y_i - \frac{1}{n}\Sigma x_i\ \Sigma \ln y_i)^2}{\left\{\Sigma x_i^2 - \frac{(\Sigma x_i)^2}{n}\right\}\left\{\Sigma(\ln y_i)^2 - \frac{(\Sigma \ln y_i)^2}{n}\right\}}$
Logarithmic curve fit $y = a + b\ln x$	$\frac{1}{n}(\Sigma y_i - b\Sigma \ln x_i)$	$\dfrac{\Sigma y_i\ \ln x_i - \frac{1}{n}(\Sigma x_i)(\Sigma \ln y_i)}{\Sigma(\ln x_i)^2 - \frac{1}{n}(\Sigma \ln x_i)^2}$	$\dfrac{(\Sigma y_i\ \ln x_i - \frac{1}{n}\Sigma \ln x_i\ \Sigma y_i)^2}{\left\{\Sigma(\ln x_i)^2 - \frac{1}{n}(\Sigma \ln x_i)^2\right\}\left\{\Sigma y_i^2 - \frac{1}{n}(\Sigma y_i)^2\right\}}$
Power curve fit $y = ax^b$	$\exp\left(\frac{\Sigma \ln y_i}{n} - \frac{\Sigma \ln x_i}{n}\right)$	$\dfrac{\Sigma(\ln x_i)(\ln\ y_i) - \frac{(\Sigma \ln x_i)(\Sigma \ln y_i)}{n}}{\Sigma(\ln x_i)^2 - \frac{(\Sigma \ln x_i)^2}{n}}$	$\dfrac{\left\{\Sigma(\ln x_i)(\ln y_i) - \frac{(\Sigma \ln x_i)(\Sigma \ln y_i)}{n}\right\}^2}{\left\{\Sigma(\ln x_i^2) - \frac{(\Sigma \ln x_i)^2}{n}\right\}\left\{\Sigma(\ln y_i)^2 \frac{(\Sigma \ln y_i)^2}{n}\right\}}$

RESULTS

PRESENTATION OF RESULTS

This thesis presents the results of an investigation on the hormonal role of ABA in the regulation of stomatal aperture. Several approaches have been attempted using specific physiological properties of the plant species *Commelina communis* and *Ricinus communis*. For this reason the results are divided into seven sections, each with a separate Introduction and Discussion.

Section 1. Experiments on Epidermal Segments I. Development and characterisation of the experimental systems.

Section 2. Experiments on Epidermal Segments II. Uptake, distribution and Metabolism of ABA.

Section 3. Experiments on Epidermal Segments III. Use of $^{86}Rb^+$ as tracer for K^+.

Section 4. Experiments on whole leaves.

Section 5. Endogenous ABA in *Commelina*.

Section 6. Transport of ABA in *Ricinus*.

Section 7. Electron microscope studies of *Commelina*.

GRAPHS AND TABLES

The axes of each graph illustrated are accompanied with the quantity measured, a solidus (/), and the units used in the graph (e.g. M.S.A./ μm = Mean stomatal aperture in μm). The same convention (Morris, 1974) was used in table headings.

Bars passing through symbols on graphs represent 2 standard errors. These were omitted when the radius of the graph symbol exceeded the size of the standard error. Rather than making assumptions about the expected trend of the data, graph points were joined with straight lines, except where calculated regression lines indicated otherwise.

STATISTICS

Probabilities ($0 < P < 1$) given with t or r values represent the probability that the statistic would be obtained due to chance variation. For the purpose of these tests the data were assumed to have a Normal distribution, and in the case of t-tests between means, the sample variances were assumed to be equal.

SECTION 1.

EXPERIMENTS ON EPIDERMAL SEGMENTS I. DEVELOPMENT AND CHARACTERISATION OF EXPERIMENTAL SYSTEMS

INTRODUCTION

This section reports the results of experiments designed to establish systems for the study of the action of ABA on stomata. The methods utilised involved the isolation of epidermis material and the subsequent incubation of segments of abaxial epidermis in buffer solutions. This approach has been used in many studies of stomatal action (see Meidner and Willmer, 1975; Raschke, 1975a; Hsiao, 1976), but there has been little discussion concerning the materials and methods commonly employed. The introduction to this section therefore brings together information about the use of epidermal segments dispersed throughout several papers and reviews, with regard to the methods later investigated.

One of the first problems encountered was the choice of plant material. A large number of species yield epidermal strips, but not all sources give material which is suitable for specific experiments. Factors of importance are summarised in Table 6. Examination of these characteristics for several species led to the choice of three potential experimental plants:- *Commelina communis, Tulipa gesnariana* and *Vicia faba*. Epidermis could be removed relatively easily from leaves of these species and their stomatal pores were large when open.

Although tulip epidermis had good experimental characteristics, this species was rejected, primarily because it could not be obtained year-round, but also because of its leaf morphology.

Vicia epidermis has been used in many studies. Willmer *et al.* (1973) stated that it had higher densities of adhering mesophyll cells and trichomes than either *Commelina* or tulip. Willmer and Mansfield (1969) also reported that the responses of *Vicia* stomata on epidermal strips were less predictable than those of two *Commelina* species under their experimental conditions. Furthermore, large areas of epidermis were found to be difficult to manipulate (personal observation).

Table 6: Important Factors (not ranked) in the Choice of Plant Material for Epidermal Segment Studies.

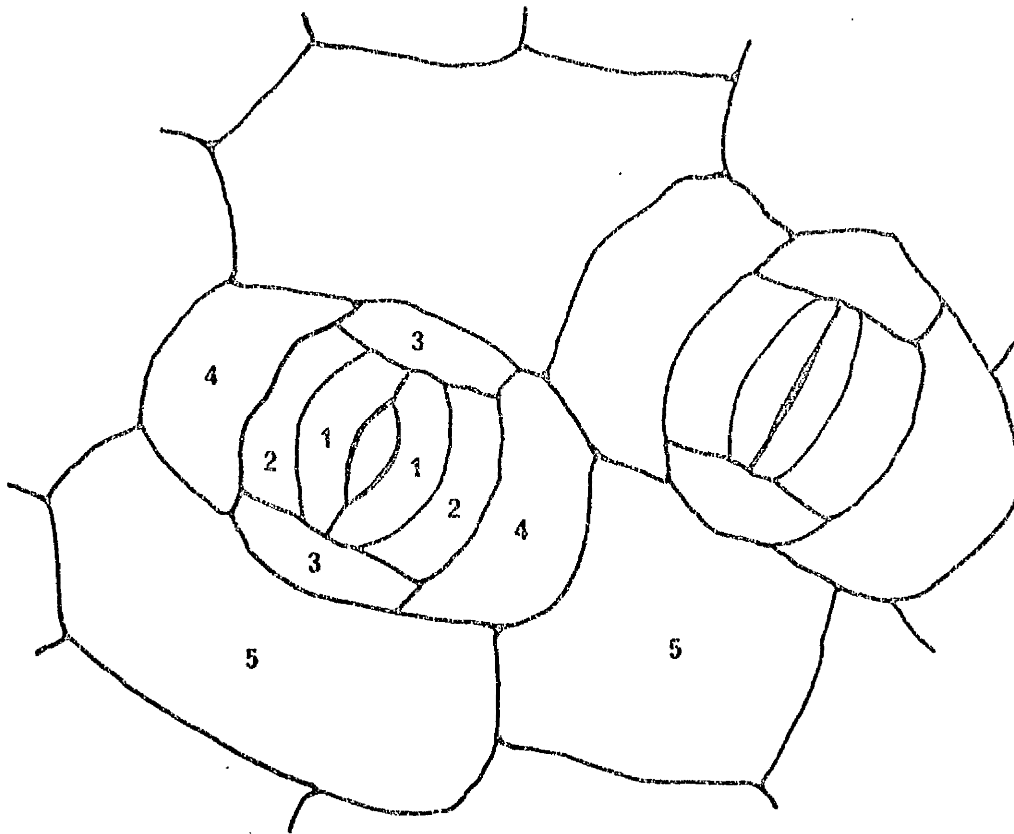
Factor	Comment/Example(s)
Established Plant Material	Few species have well characterised responses, examples are *Commelina, Vicia*.
Stomatal Behaviour	C.A.M. plants have abnormal stomatal rhythms.
Availability	Material from bulbous or deciduous species not readily available throughout the year.
Leaf Area	Affects peeling methods; controls maximum area of epidermis available per leaf.
Mode of Leaf Insertion	Important in transpiration stream donation experiments.
Stomatal Distribution	Some species amphistomatous, others hypostomatous.
Leaf Cuticle	Pronounced cuticle may aid segment flotation.
Glands and Trichomes	May not be desirable in metabolism experiments as metabolically active. May make some observations difficult.
Epidermal Cell Survival and Mesophyll Contamination after removal	Both factors apparently species dependent.
Ease of Peeling	Species dependent; may even vary with *var.* and *cv.*
Ease of Handling	Includes factors such as 'rigidity' of segments, ease of flotation.
Type of Stomatal Complex	Presence of morphological subsidiary cells may be important.
Size of Stomatal Pore	Should be large to minimise measurement inaccuracies.
Guard Cell Biochemistry	May vary, e.g. onion guard cells do not store starch (Schnabl and Zeigler, 1977).

For these reasons, *Vicia* was also rejected. *Commelina communis* plants could be grown all year from seed and they displayed leaf growth from extended stems, which meant that leaves of similar physiological ages could be obtained from branches of a single plant. Sections of stem could be removed with a leaf to provide convenient means of solution uptake. *Commelina* was therefore chosen as the experimental plant for these studies. The stomatal complex of this species (Fig.9) involves the guard cells and six subsidiary cells (Willmer and Mansfield, 1969) which are apparently important for guard cell function (Squire and Mansfield, 1972; Penny and Bowling, 1974; Penny *et al.*, 1975; Penny *et al.*, 1976). *Commelina* epidermal segments have been used in many investigations of stomatal physiology (Willmer and Mansfield, 1969) and formed the basis of a sensitive bioassay for ABA (Ogunkanmi *et al.*, 1973).

Secondary problems occurred with the choice of peeling method and incubation system. Removal of epidermis is an obvious point of control of the quality of experimental material used. The method employed in this thesis was adopted from that of Travis and Mansfield (pers. comm.). Investigations were carried out to characterise the effects of peeling Several post-peeling techniques to modify the isolated epidermis tissue have been described: Raschke and Fellows (1971) reduced the extent of mesophyll contamination in *Zea* by a series of rinses; Allaway and Hsiao,(1973) prepared *Vicia* epidermis by crushing it ('rolling') so that guard cells were the only viable cells; Squire and Mansfield (1972) described a low pH buffer treatment to achieve the same effect with *Commelina*. None of these methods was used routinely in these experiments, although the latter technique was employed in one particular case .

Isolated epidermal segments are usually floated on solutions, cuticle up, for experimentation. The nature of the medium on which segments are incubated defines to a great extent the reaction of the stomata to various stimuli (Humble and Hsiao, 1969; Willmer and Mansfield, 1969). The advent and use of zwitterionic buffers having low membrane permeability (Good *et al.*, 1966) was a significant advance since their use prevents the chance of buffer effects on guard and epidermal cell metabolism. This possibility is most marked in the case of citrate and phosphate based buffer systems: citrate is a

Fig.9. Diagram of *Commelina* Stomatal Complex.



1. Guard cell
2. Inner lateral subsidiary cell
3. Terminal subsidiary cell
4. Outer lateral subsidiary cell
5. Epidermal cell

The cells numbered 1-4 in the figure constitute one stomatal complex.

close metabolite of malate, an important anion in stomatal turgor increases, and may itself be implicated (Outlaw and Lowry, 1977), whilst phosphate is known to be involved in metabolic interconversions which may be fundamental to stomatal action (Fuijino, 1967).

The effects of the ionic content of the incubation medium have been examined thoroughly due to the discovery of the involvement of alkali metal cations in stomatal opening (Humble and Hsiao, 1969; Willmer and Mansfield, 1969; Fischer 1972; Allaway and Hsiao, 1973). The observation that inclusion of KCl in the medium led to stomatal opening gave rise to the discovery that K^+ is taken up by guard cells during opening. In *Vicia* Rb^+ and, K^+ were the only monovalent cations which allowed opening at low concentrations (0-20 mol m^{-3}) in the system used by Humble and Hsiao (1969). At higher concentrations (*ca.* 200 mol m^{-3}) most alkali metal ions resulted in opening. In *Commelina*, K^+ and Na^+ allowed opening at 67 mol m^{-3} (Willmer and Mansfield, 1969). Divalent cations seem to inhibit stomatal opening in epidermal segment systems, as does NH_4^+ (Humble and Hsiao, 1969; Willmer and Mansfield, 1969). This has led to some confusion about the use of Ca^{++} in media to maintain membrane integrity and ion selectivity.

The pH of the medium is an important factor since guard cell sap proton concentrations alter during stomatal movements. It is, however, very difficult to separate pH and ionic effects (Williams and Shipton, 1950). The buffering capacity of the solutions used may prevent or delay guard cell pH changes. There is a school of thought which proposes that they should not be used for this reason. On the other hand, in plant hormone studies, the acidic nature of these compounds may give a spurious pH effect if buffers are not used. Also of relevance here is the fact that weak acid dissociation is lowered at low pH values, which would favour acid uptake by cellular membranes or hydrophobic protein sites (Ogunkanmi *et al.*, 1973), possibly leading to enhanced stomatal sensitivity to ABA at low pH.

In general, CO_2 - free air has been bubbled through the incubation medium. This encourages stomatal opening, removes respiratory CO_2 and produces mixing of the solution (Willmer and Mansfield, 1969; Ogunkanmi

et al., 1973). Illumination of the epidermis tissue tends to favour stomatal opening, whilst darkening often causes partial closure (e.g. Willmer and Mansfield, 1969). Both responses appear to depend on the medium used. There has been little investigation of the effect of temperature on aperture in epidermis strip systems. On whole plants, the optimum appears to be from 35-45 C (see Meidner and Mansfield, 1968). Most experiments with epidermal strips have been carried out in the range 25-35 C.

Considering the above factors, an incubation system based on the 'Good' buffer PIPES was chosen. This buffer was used by Ogunkanmi *et al.*, (1973), as the basis of an improved antitranspirant bioassay. Although they found that citrate buffer (pH 5.5) resulted in greater stomatal sensitivity, in these studies PIPES (pH 6.8) was favoured, firstly for its inertness and impermeability (Good *et al.*, 1966), and secondly, because greater solubility of ABA would be achieved at higher pH - a property which would allow the use of high concentrations of radioactive ABA. Experiments were carried out to determine the optimum KCl concentration for both stomatal opening and subsequent closure by ABA to be achieved. Two incubation methods (see Materials and Methods) were developed to suit the types of experiment later carried out. These involved aeration with CO_2 - free air and no aeration. The responses of stomata in these systems were characterised under various conditions.

Estimation of stomatal aperture on epidermal segments has generally been by microscopy using an eyepiece graticule and this method was normally used in this thesis. Various methods have been described to 'fix' apertures: Fischer (1968) mounted the segments in immersion oil, which was said to maintain apertures for at least 30 min. A method of fixing aperture by freeze-drying was developed to provide a permanent record. This was used in experiments where timing did not allow measurements from live tissue.

The possibility that uncontrolled factors might influence experiments was minimised as far as possible; specific experiments were also carried out to identify parameters of importance. Thus, the effects of stomatal rhythms were reduced by carrying out all experiments at a particular time of day. Effects of the water relations of plants

were investigated by analysing stomatal opening of epidermal strips after water stresses to the whole plant. For all other experiments care was taken to give the same watering treatment to all plants used. Watering regimes and equilibration treatments were strictly adhered to. The effects of leaf age on responses of stomata on epidermis were also inspected, but otherwise the leaves used were of uniform physiological age. The chief purpose of the following series of experiments was therefore to develop an optimal system for analysis of the effects of ABA on *Commelina* epidermis, based on the above summary of critical factors.

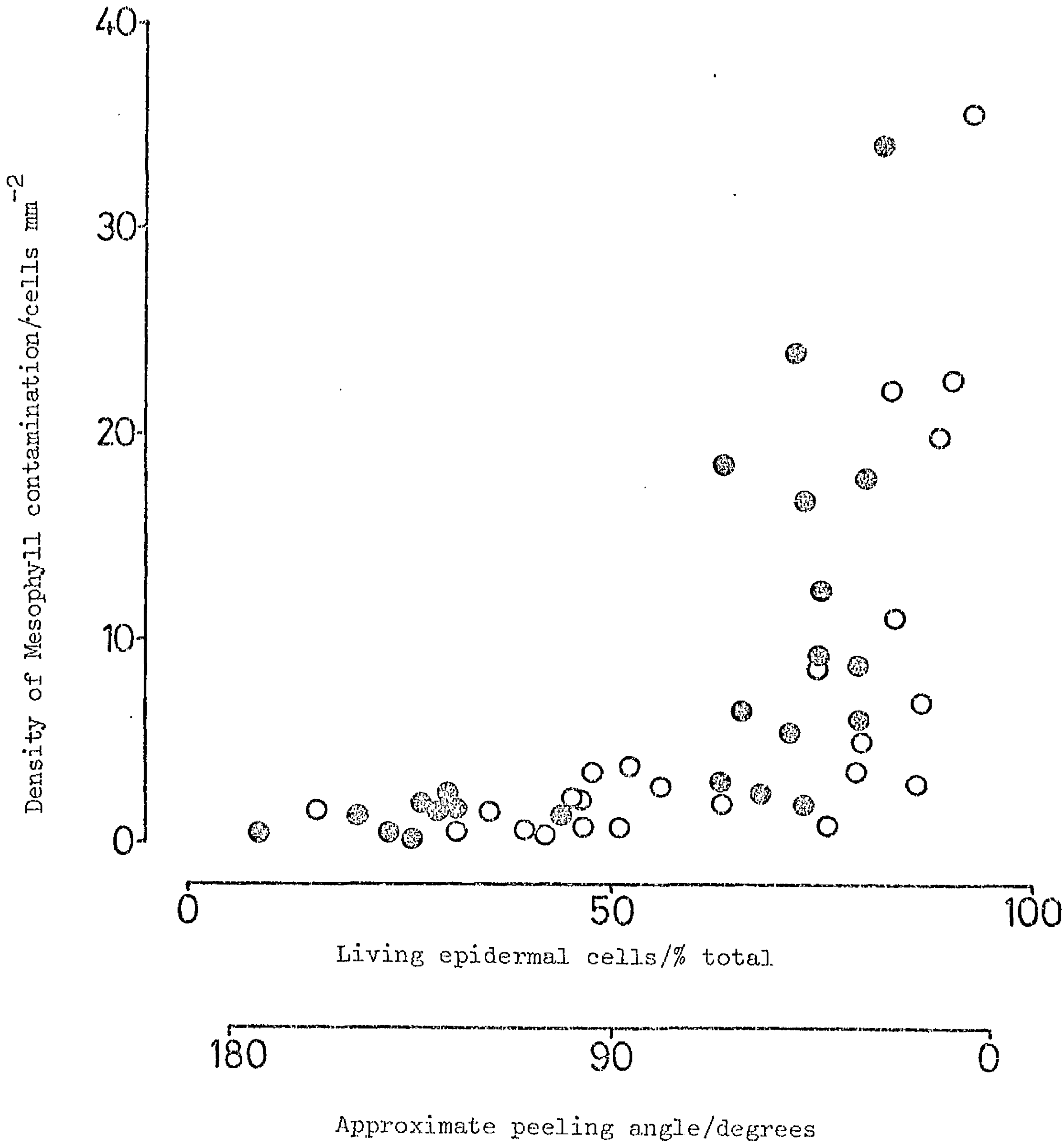
RESULTS AND DISCUSSION

Experiment 1.1 Effects of Peeling Technique on Epidermal Cell Survival and Mesophyll Contamination.

This experiment was designed to investigate the influence of the method of peeling on parameters connected with the isolated epidermis. The basic peeling procedure (Fig.4) was used, but the angle of peeling was varied between strips. For each strip a constant angle of 0·1 - 3·05 rad (5 - 175°) was used. The strips were cut into segments and epidermal cell survival assayed using either neutral red or Evan's blue. Mesophyll contamination was also estimated. The results are shown in Fig.10. Since the peeling angle was not precisely measured, the two variables are plotted against each other. As a rough guide to peeling angles, approximately 90% epidermal cell survival was obtained at 0 rad, 10% at 3 rad and 50 - 60% with a vertical peeling angle.

It is clear from Fig.10 that there is a point at which mesophyll contamination dramatically increases. This is presumably related to the stress required to break mesophyll-epidermis cellular connections. In terms of an'optimum peeling angle' it seemed that vertical peeling (1·6 rad) gave the best epidermal cell survival for the minimum of contamination. In view of these results, the procedure for stripping epidermis was normally to peel vertically. In cases where mesophyll contamination was specifically to be avoided (uptake and metabolism

Fig.10. Epidermal Cell Survival and Mesophyll Contamination on Epidermis Tissue Peeled at Different Angles.



Open circles: Evan's blue
Closed circles: Neutral red

Points based on 5 fields of view (1·46 mm^2).
Total for living epidermal cell determination was *ca.*300. The graph is the combination of results from two repeats of the same experiment. One value obtained is not included since it was off the scale used (x = 85·4%, y = 79 cells mm^{-2}).

studies), a peeling angle of about 1.75 rad (100°) was used. These results were obtained with plant material similar to that used in other experiments (namely, the fully expanded leaf closest to the apex on branches of healthy turgid, plants), but it was observed that unhealthy or senescent leaves frequently yielded epidermis with a high mesophyll density. On some segments, contamination occurred where veins crossed the epidermal strip. In practice, therefore, each epidermal segment was visually observed before uptake and metabolism studies and those with obvious contamination rejected.

The criterion of dye uptake as an indicator of cell activity is questionable, but neutral red uptake was *always* correlated with microscopic observations of cytoplasmic streaming and protoplast integrity when these were made. Difficulty was experienced in assaying for subsidiary and guard cell survival with Evan's blue dye, and neutral red was consequently used for such determinations.

It should be emphasised that although epidermal cell survival was found to vary between 8·2 - 92·9% the proportion of living guard and subsidiary cells (see Fig.9) was always close to 100%. This fact may be a critical difference between *Commelina* epidermis material and that from species without a stomatal complex. The relative importance of epidermal and subsidiary cell survival in stomatal opening was examined in experiment 1·2.

Experiment 1.2 Effects of PIPES (pH 6·8) and MES (pH 4·5) Buffers on Stomatal Opening and Survival of Cells on Epidermal Segments.

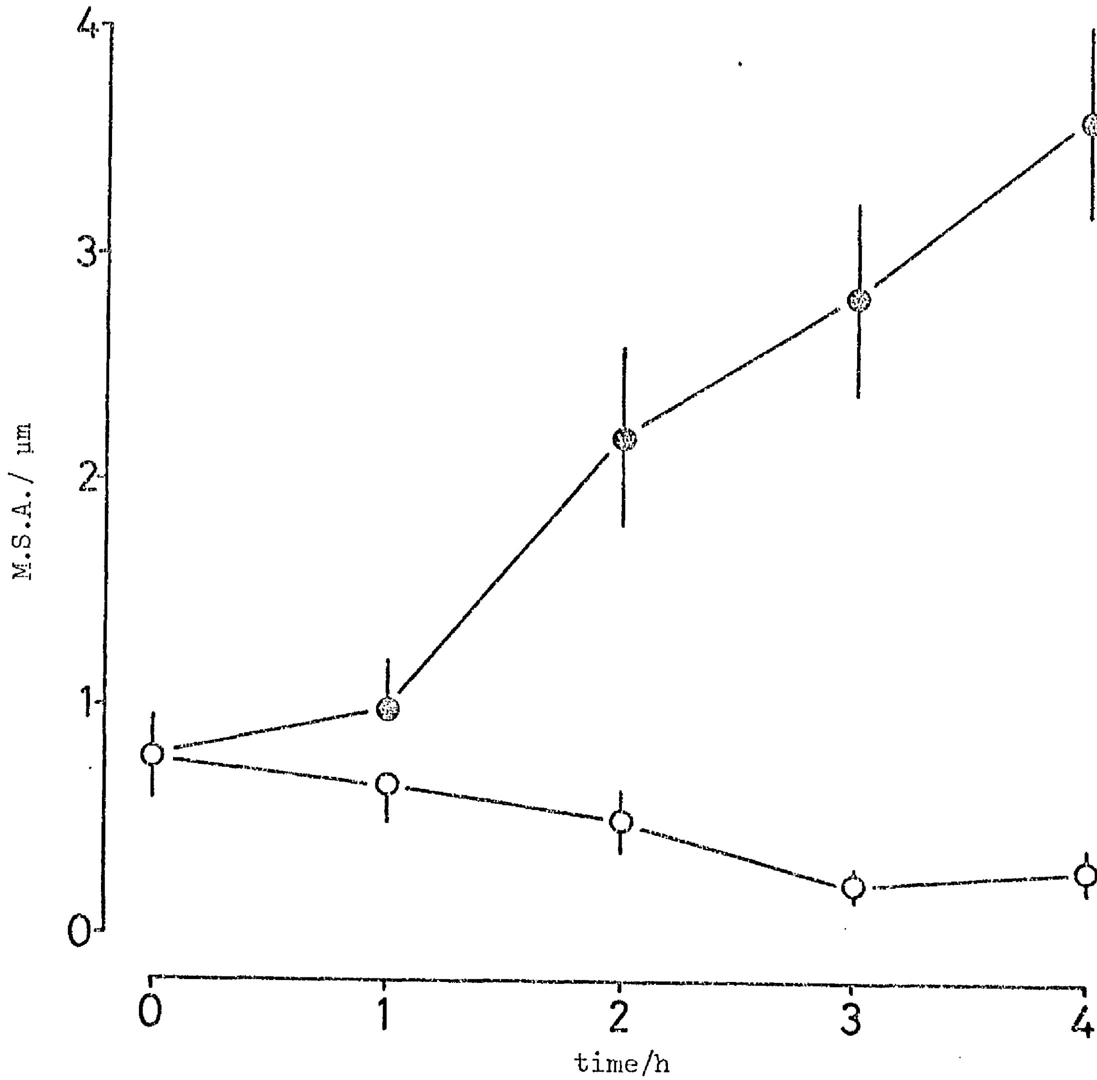
Squire and Mansfield (1972) demonstrated the effects of subsidiary cell death on stomatal aperture. After low pH treatments which killed all the cells of the epidermis except the guard cells, the stomata were found to be open. Itai and Meidner (1978b) showed that stomatal closure on epidermal segments in this state did not occur when ABA was applied. In view of the importance of subsidiary cell presence for stomatal reaction, the viability of cells of *Commelina* epidermis was

investigated during incubation in the chosen medium (10 mol m^{-3} PIPES pH 6·8).

Fig.11 illustrates the effects of incubation in this buffer and in 10 mol m^{-3} MES pH 4·5 on stomatal aperture over 4 h. The low pH treatment resulted in stomatal opening, confirming the observations of Squire and Mansfield (1972). Apertures in PIPES narrowed slightly during incubation. The effects of the two buffer treatments on cell viability is presented in Fig.12. The proportion of living guard cells (Fig.12c) was unaffected by either treatment and remained close to 100%. The small numbers of dead guard cells found appeared to be those injured during manipulation of the segments with tweezers. Both epidermal cell survival (Fig.12a) and the number of stomatal complexes with one or more viable subsidiary cell (Fig.12b) declined during MES treatment but remained relatively constant during PIPES treatment, indicating that the PIPES incubation had no injurious effects on the epidermal segments. It is of interest to compare the timing of epidermal and subsidiary cell death in MES buffer as indicated by Figs. 12a and b with the increase in stomatal aperture found in this medium (Fig.11). Subsidiary cell death as estimated by the '% living complexes' bore greater relation to the kinetics of opening than did epidermal cell death. Thus, although considerable epidermal cell death had occurred after 1 h in MES, the mean stomatal apertures on segments incubated in the two media were not significantly different ($t = 1{\cdot}24$, $P > 0{\cdot}1$). This contention was supported by observations of individual stomata surrounded by either dead or living subsidiary cells (Fig.13) where it was found that stomata were almost totally closed if surrounded by living cells. Fig.12b shows that in this experiment, total subsidiary cell death was not obtained even after 4 h MES treatment. This may have been due to the plant material or method of peeling. Variability in the effects of some buffer treatments was also noted by Squire and Mansfield (1972).

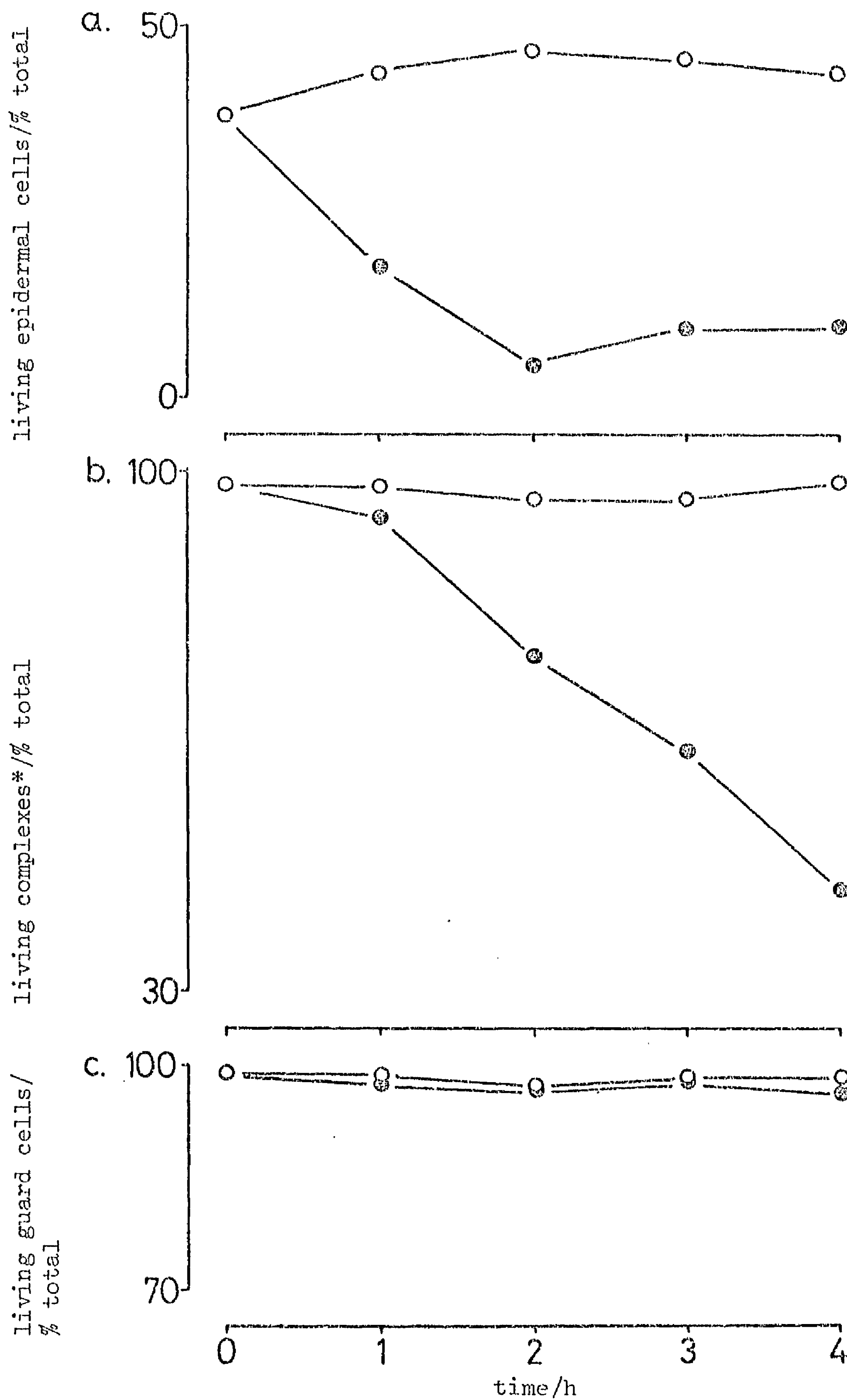
Degrees of opening in MES-treated segments were found, depending on the number of living cells in the complex. A small effect of absence of living epidermal cells was noted in PIPES - treated segments to result in a slight stomatal opening although this was not quantified. If the mean aperture obtained on epidermal strips exposed to MES for

Fig. 11. Effect of Incubation in PIPES and MES Buffers on Stomatal Aperture of Epidermal Segments.



Open circles: PIPES pH 6·8 treatment.
Closed circles: MES pH 4·5 treatment.
System 1, R, N = 50, s = 3.

Fig.12. Effect of Incubation in PIPES and MES Buffers on Viability of Cells of Epidermal Segments.



Open circles: PIPES pH 6·8 treatment;

Closed circles: MES pH 4·5 treatment.

System 1; a. R, n = 140, s = 3
b. R, n = 300, s = 3
c. R, n = 300, s = 3

n values approximate.

* ≡ % complexes with one or more subsidiary cell alive.

Fig.13. Effect of Viability of Guard Cells on Stomatal Aperture

Neutral-red - treated epidermis (240 x)

A. Stomatal complex where all subsidiary cells have taken up the stain: pore closed.
B. Stomatal complex where no subsidiary cells have taken up the stain: pore open (*ca.* 8·3 μm).
C. Stomatal complex where only inner lateral subsidiary cells have taken up the stain: pore partially open (*ca.* 3·1 μm).

different periods was compared to the '% living complexes', a linear relationship was found (Fig.14). Squire and Mansfield (1972) considered this effect to be due to altered turgor relationships in the epidermis and also described an enhancement of guard cell solute uptake after subsidiary cell death. They suggested that the subsidiary cells might fulfil a regulatory function in the supply of ions to the guard cells, and also that the guard cell plasmalemma adjacent to the inner lateral subsidiary cell (see Fig.9) might take up most of the ions which accumulated in guard cells.

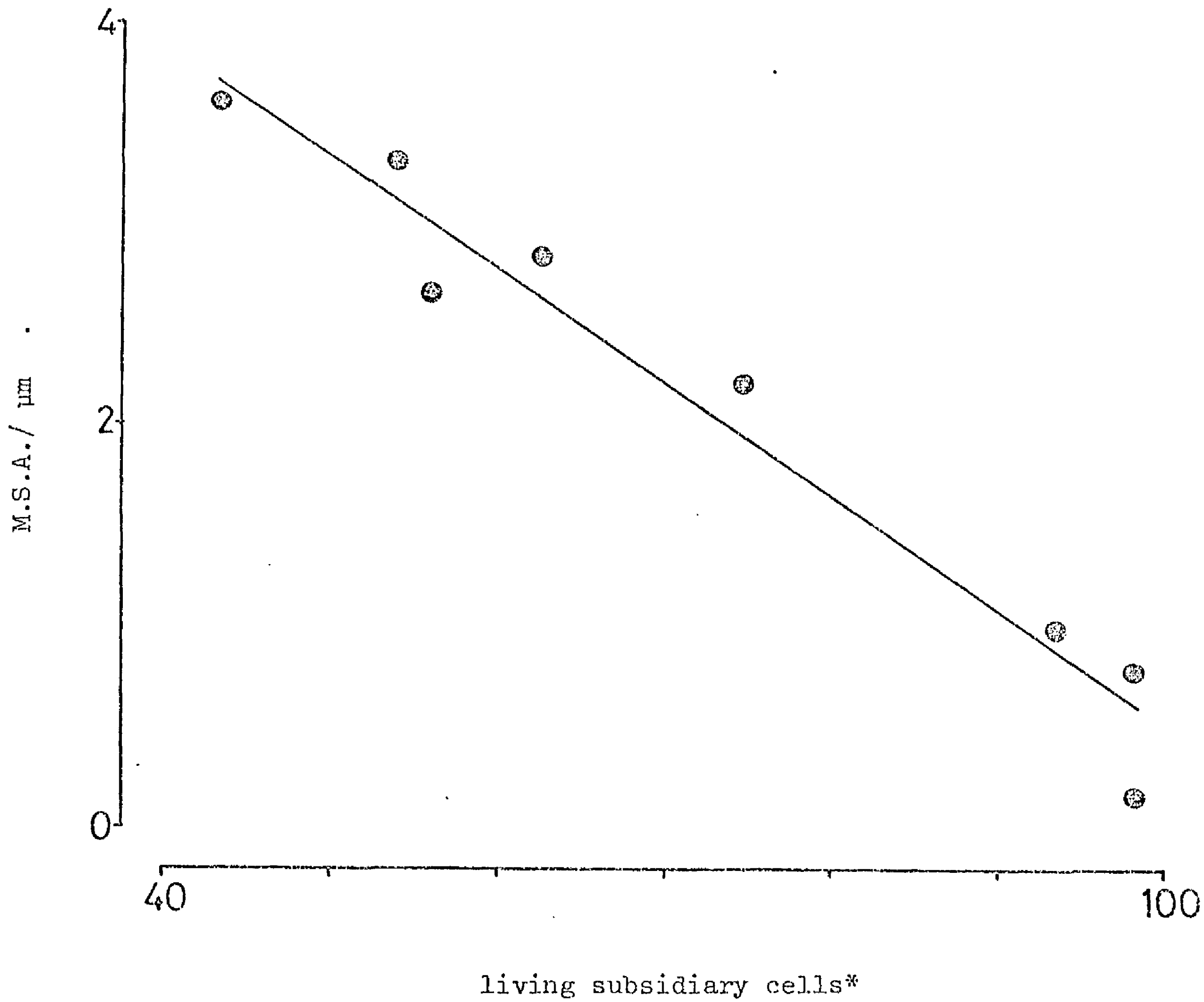
The results of this experiment confirm and extend the observations of Squire and Mansfield (1972). No deliterious effects of the PIPES medium on cell survival were observed.

Experiment 1·3 Effect of KCl Concentration on Stomatal Opening.

In order to examine the effects of ABA on stomatal closure, a treatment was required which resulted in maintained stomatal *opening* during the experimental period. This clearly was not provided by 10 mol m^{-3} PIPES alone (Fig.11). Many authors had reported that the presence of monovalent cations in the incubation medium enhanced stomatal opening in epidermal segments (see Hsaio, 1976). Since this effect was specific to some degree for K^+ (Humble and Hsaio, 1969; Willmer and Mansfield, 1969), and in view of the apparent importance of this cation in normal stomatal opening (Humble and Raschke, 1971), the effects of addition of KCl to the 10 mol m^{-3} PIPES buffer were investigated. Fig.15 illustrates the results of a 3 h incubation in PIPES solutions containing 25 - 300 mol m^{-3} KCl on stomatal aperture. A 3 h incubation period was chosen because other studies indicated that apertures attained from initially closed stomata reached a maximum between 2-4 h (Willmer and Mansfield, 1969; Squire and Mansfield, 1972).

Apertures between 18 and 21 µm represented the widest possible openings; at such apertures the stomatal orifice was circular in appearance. Squire and Mansfield (1972) suggested that wide openings found at high salt concentrations might, at least in part, be due to plasmolysis of subsidiary cells. This effect was observed in these

Fig.14. Relationship Between Subsidiary Cell Survival and Stomatal Aperture after MES Treatment.



Points were obtained from two separate experiments. The line of least-squares fit is shown, having the equation:

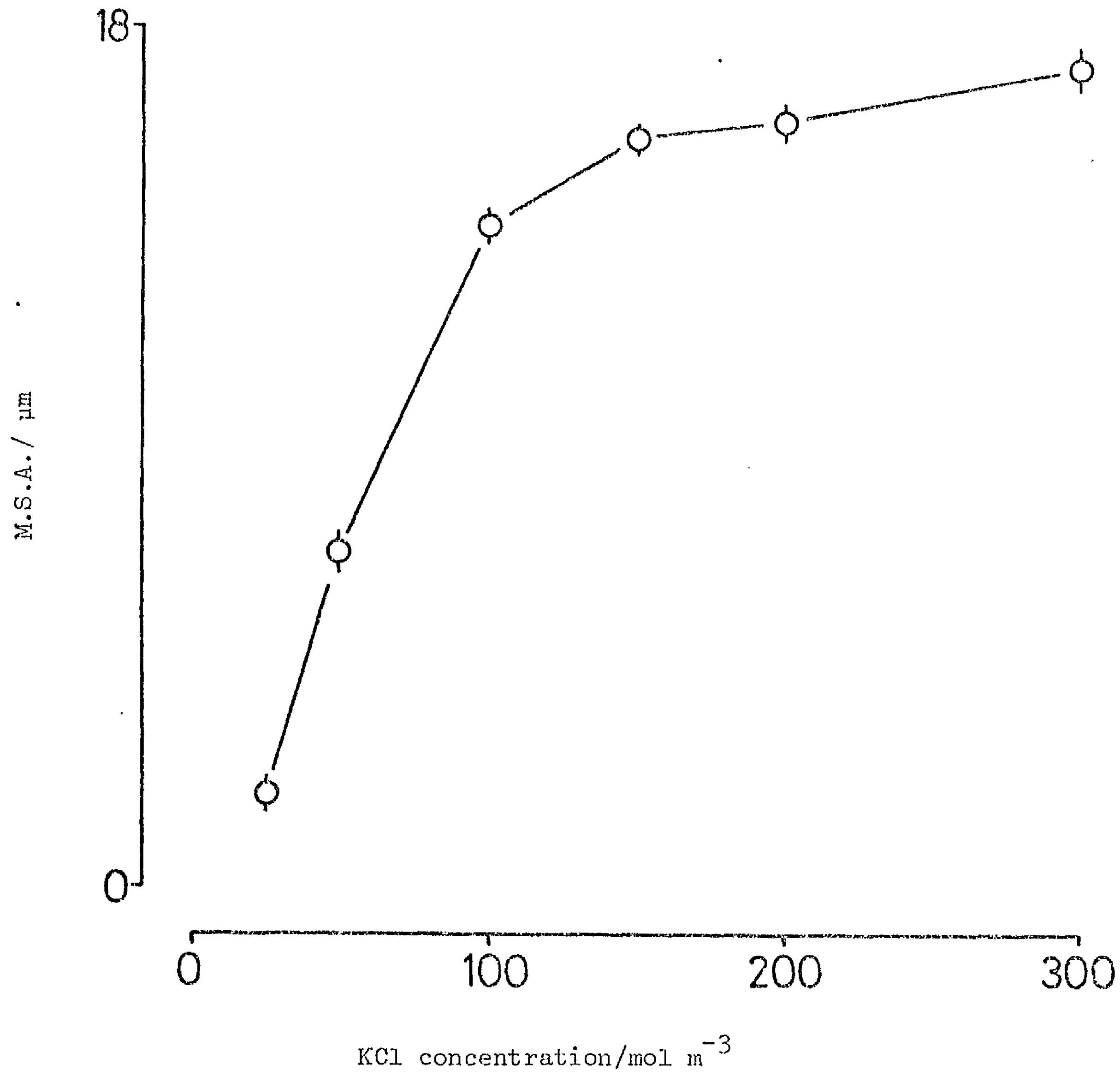
$$y = -0{\cdot}0565\,x + 6{\cdot}1598;$$

$$r = 0{\cdot}9771 \; (p < 0{\cdot}001)$$

Details of measurement same as Fig.12b.

* see Fig.12.

Fig.15. Effect of KCl Concentration on Stomatal Opening in PIPES Buffer.



System 1, NR, n = 50, s = 2.

studies in the short term (0 - 30 min) with segments stained with neutral red after incubation in KCl concentrations above 100 mol m^{-3}. A thorough investigation of this effect was not made.

Experiment 1.4 Effects of 0·1 mol m^{-3} ABA on Stomatal Aperture at Different KCl Concentrations.

Investigations concerning the effect of ^{3}H-ABA on stomatal aperture using the PIPES/100 mol m^{-3} $NaNO_3$ buffer system of Ogunkanmi *et al* . (1973) revealed that although ABA caused stomatal closure if applied *ab ovo usque ad mala* in the medium, the hormone did not cause closure when applied after opening had occurred (Table 7).

Table 7: *Effects of ^{3}H-ABA on Stomatal Aperture When Applied in PIPES/100 mol m^{-3} $NaNO_3$.*

Treatment	M.S.A. (S.E.)/ μm	
	Series 1	Series 2
Control	5·39 (0·31)	5·60 (0·28)
^{3}H-ABA-treated (0·1 mol m^{-3})	0·83 (0·16)	5·70 (0·45)

Series 1: Continuous application for 3 h; System 1, NR, n = 75, s = 3.

Series 2: 1 h application after 3 h pre-opening treatment floating leaves on water through which CO_2- reduced air was bubbled (Itai *et al*, 1978); System 1, NR, n = 25, s = 1.

For this reason, the effects of ABA on 0·1 mol m^{-3} were investigated at the different KCl concentrations which had been used in Experiment 1·3. Two treatments were used, the first involving constant incubation with or without ABA for 3 h, and the second involving transfer to ABA-containing or control solutions for 1 h after 3 h in buffer (Table 8). The percentage closures caused by ABA in both treatments (series 1 and 2) were similar: the hormone had virtually no effect at 200 and 300 mol m^{-3}

Table 8. *Effect of ABA on Stomatal Aperture of Different KCl Concentrations*

KCl Concentration/ mol m^{-3}	M.S.A. (S.E.)/ µm					
	Series 1			Series 2		
	A	B	$^{B}/_{A}$ × 100	C	D	$^{D}/_{C}$ × 100
25	1·93(0·21)	0·03(0·02)	1·55	2·20(0·39)	0·03(0·03)	1·36
50	6·68(0·24)	0·20(0·07)	2·99	8·31(0·39)	0·60(0·20)	7·22
100	7·72(0·33)	3·75(0·21)	48·58	13·70(0·50)	6·67(0·27)	48·69
150	11·61(0·30)	9·38(0·48)	80·79	12·93(0·44)	10·63(0·11)	82·21
200	14·73(0·35)	14·57(0·28)	98·91	13·14(0·43)	11·90(0·34)	90·56
300	16·99(0·42)	15·78(0·22)	92·98	15·35(0·75)	15·25[a](1·06)	99·35

a Based on n = 25, s = 1

Series 1 A = 3 h in buffer control, B = 3 h in buffer plus 0·1 mol m^{-3} ABA. System 1, NR, n=100, s=4.

Series 2 C = 3 + 1 h in buffer control (equivalent disturbance), D = 3 h in buffer + 1 h in buffer plus 0·1 mol m^{-3} ABA. System 1, NR, n = 50, s = 2.

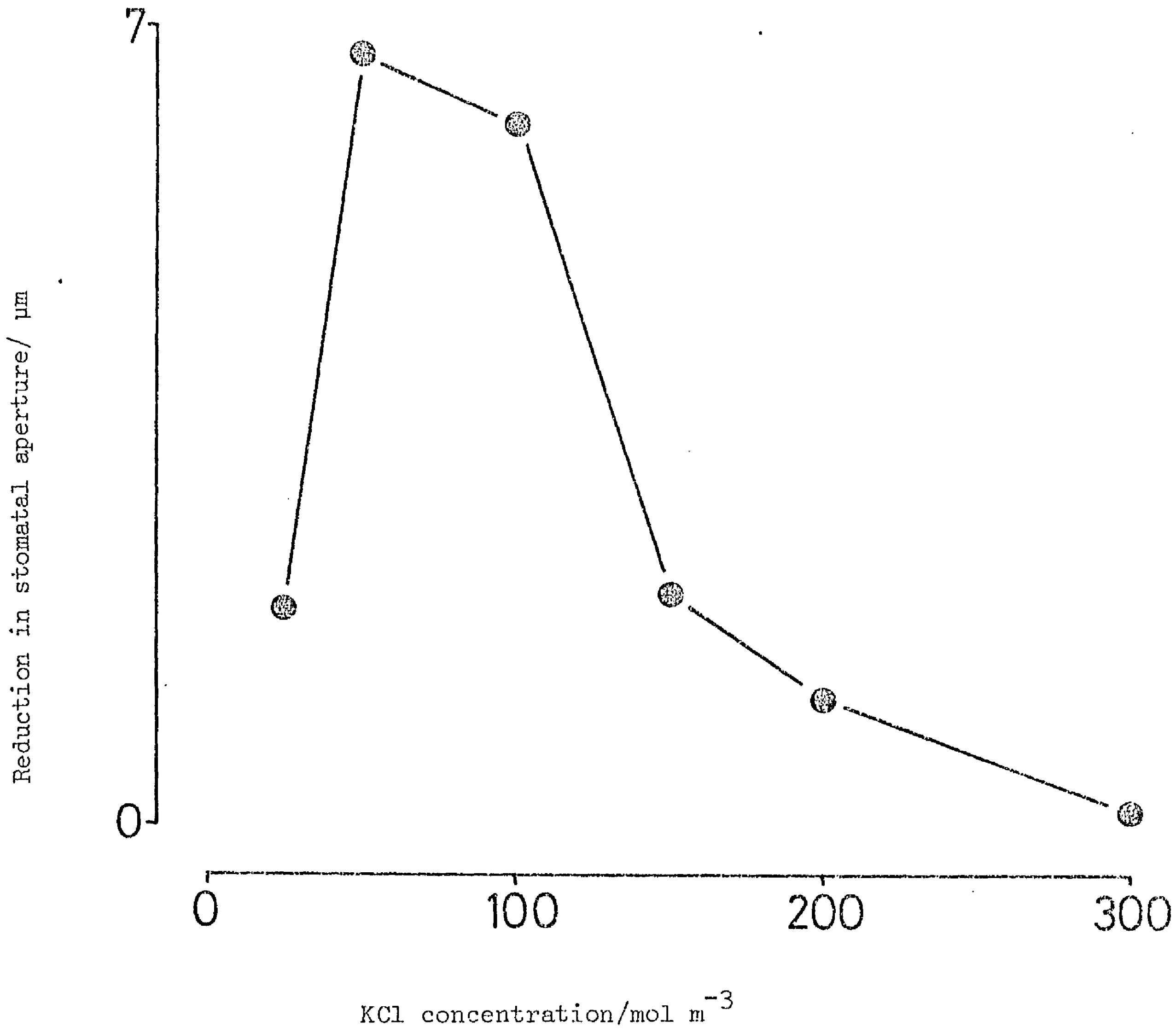
KCl and had its greatest percentage reduction at 25 and 50 mol m^{-3}. When expressed as net aperture reduction due to ABA treatment (Fig.16), the results of series 2 illustrate that the effect of ABA was greatest at 50 and 100 mol m^{-3} KCl. The effect at 25 mol m^{-3} was not large because the initial opening was small at this concentration (Fig.15). Buffer containing 50 mol m^{-3} KCl was selected to investigate further the effects of ABA; this concentration of the salt allowed adequate and reproducible opening whilst ensuring sensitivity in terms of closure.

Certain correlations between ABA effects and salt concentrations were also noted by Willmer *et al.*, (1978), who showed that stomatal closure did not always occur when the hormone was included in media containing high KCL concentrations (300 mol m^{-3}). This seemed to be dependent both on the buffer used and the initial state of the stomata. In the light of these results, the failure to obtain stomatal closure with ABA on epidermal strips of *Lupinus luteus* reported by Lancaster *et al.*, (1977), may have been due to the high KCl concentrations (300 mol m^{-3}) that they used and not necessarily to insensitivity of the species to ABA.

Experiment 1·5 Effects of ABA at 0·1 mol m^{-3} on Stomata of Epidermal Segments incubated in 50 mol m^{-3} KCl/PIPES.

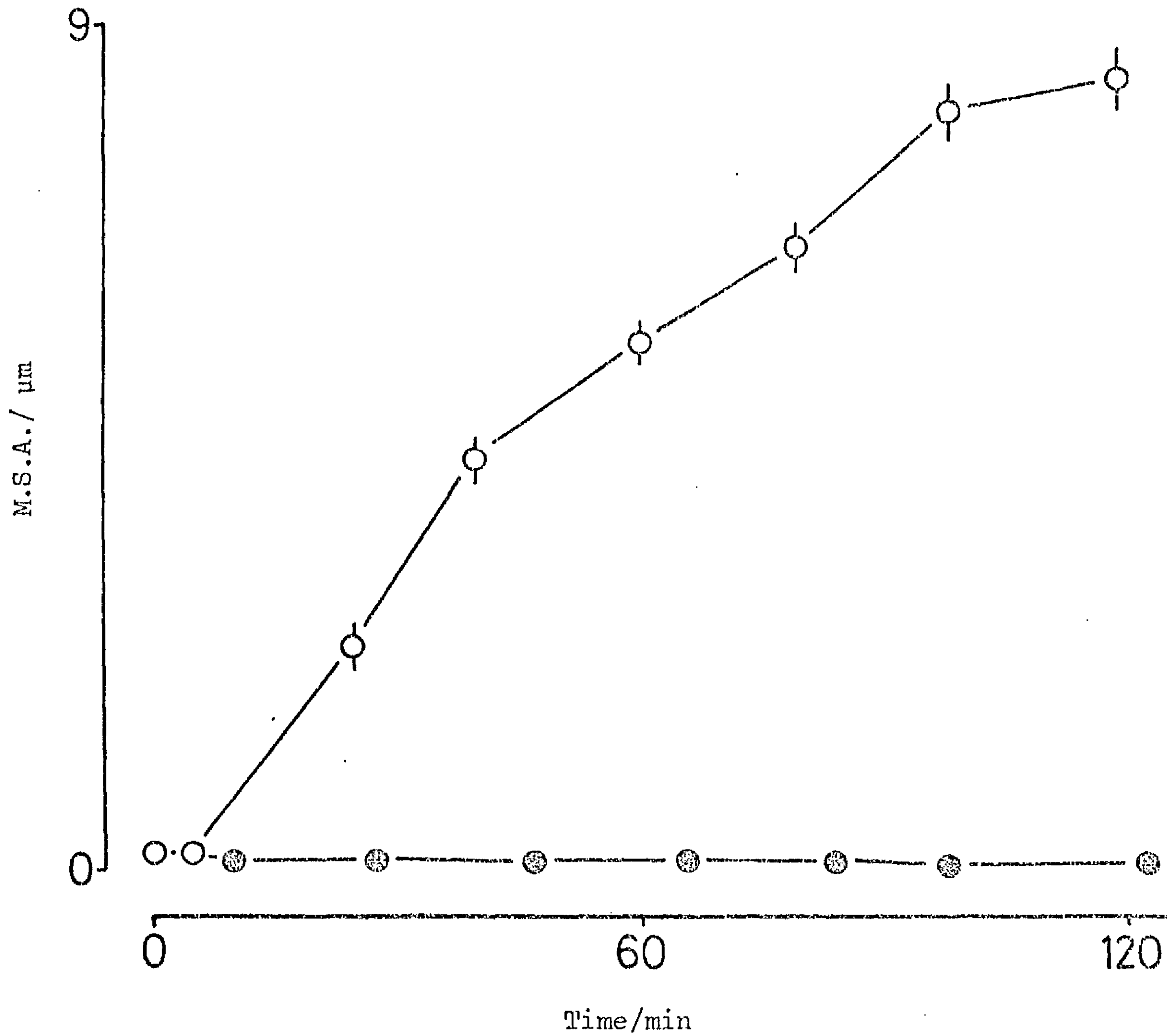
Having chosen buffer containing 50 mol m^{-3} KCl, it was necessary to characterise further the responses of stomata and the effects of ABA. Fig.17 demonstrates the results of a 2 h incubation of segments in this medium with and without 0·1 mol m^{-3} ABA. Stomata on segments incubated in buffer alone opened to *ca* 8 μm after 2 h. This was found to be repeatable, and such openings were maintained for at least a further hour. Thereafter, a slight decline in apertures was often observed, an effect also shown in other studies (e.g. Willmer and Mansfield, 1969). This may have been due to buffer effects (e.g. toxicity) or to natural stomatal rhythms. In order to reduce the latter possibility, all experiments were carried out at the same time of on plants equilibrated in a similar manner. A 2 h incubation in 50 mol m^{-3} KCl/PIPES was adopted as a standard procedure for obtaining epidermal

Fig.16. Reduction in Stomatal Aperture Caused by 0·1 mol m^{-3} ABA at Different KCl Concentrations.



Details as in Table 8, Series 2.

Fig. 17. Stomatal Responses in 50 mol m^{-3} KCl/PIPES Buffer.



Open circles: buffer alone
Closed circles: buffer plus 0·1 mol m^{-3} ABA.
System 1, R, n = 50, s = 2.

segments with open stomata.

Stomata remained closed during 2 h when ABA treated (Fig.17), an effect predictable from the results of Table 8. The series 2 results of this Table also suggested that stomatal closure would occur within 1 h in this system. The precise kinetics of this closure are illustrated in Fig.18. Closure commenced within 5 min and was complete by 15 min. This speed of reaction to ABA is compatible with reported results obtained by feeding the compound via the transpiration stream to whole leaves (e.g.Kriedemann *et al.*, 1972; Raschke, 1975b; Raschke *et al* ., 1975). Stomatal re-opening did not occur within 50 min.

Experiment 1·6 Effect of Incubation Method on Stomatal Apertures Attained in 50 mol m^{-3} KCl/PIPES.

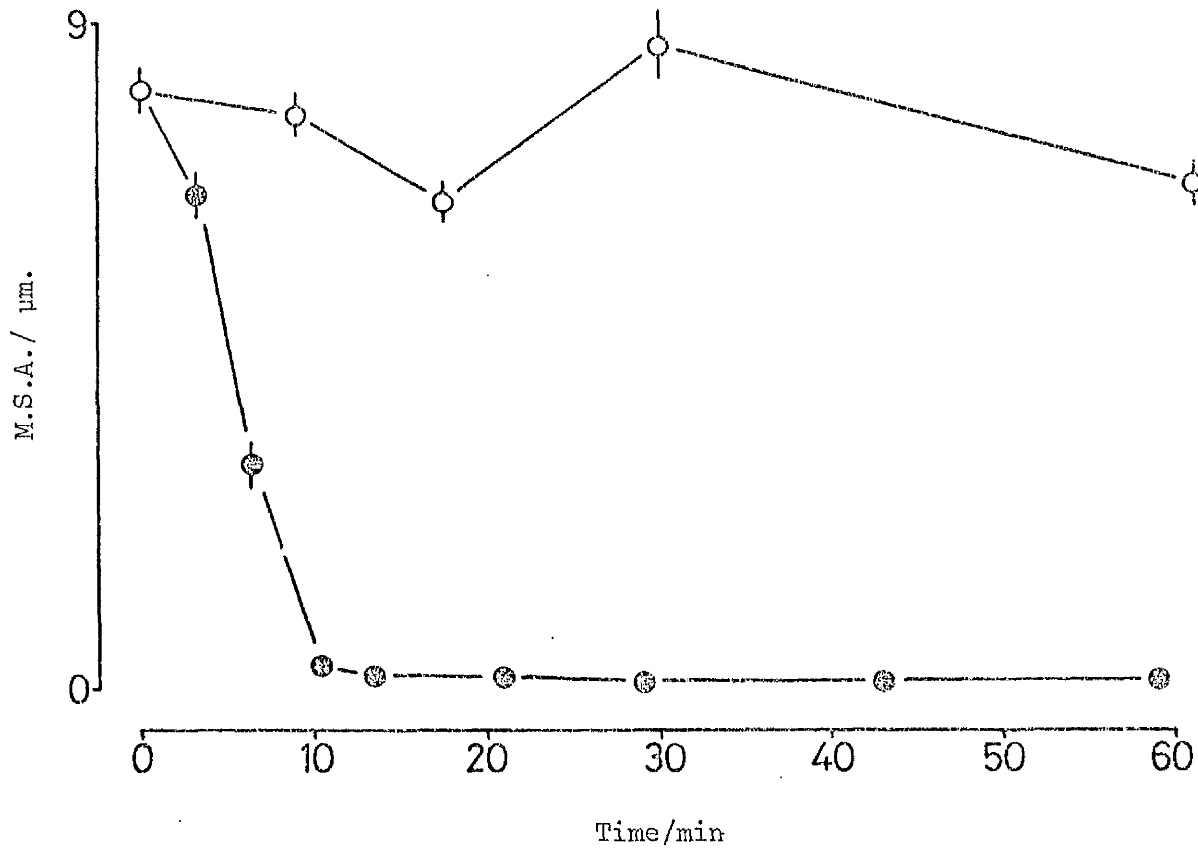
All the results described in Experiments 1·2 - 1·5 were obtained using Incubation System 1, which involved illumination, and aeration of the medium with CO_2- free air. In order to carry out certain experiments with ^{86}Rb (see Results Section 3), it was thought desirable to minimise splashing and vaporisation of isotope - containing solutions. For this reason, the effect of non-aeration (System 2) on stomatal apertures and reaction to ABA was investigated. The effect of placing the incubation dishes under cardboard boxes ('darkness') was also examined.

Fig.19a shows that stomata attained wider apertures in System 1 than in System 2. In the particular experiment illustrated in Fig.19a (1·6·1) the difference between mean apertures at 2 h was not statistically significant (Table 9). However, in a repeat (1·6·2) the deviation was significant.

Table 9. *Comparison of M.S.A. Attained in Two Incubation Systems*

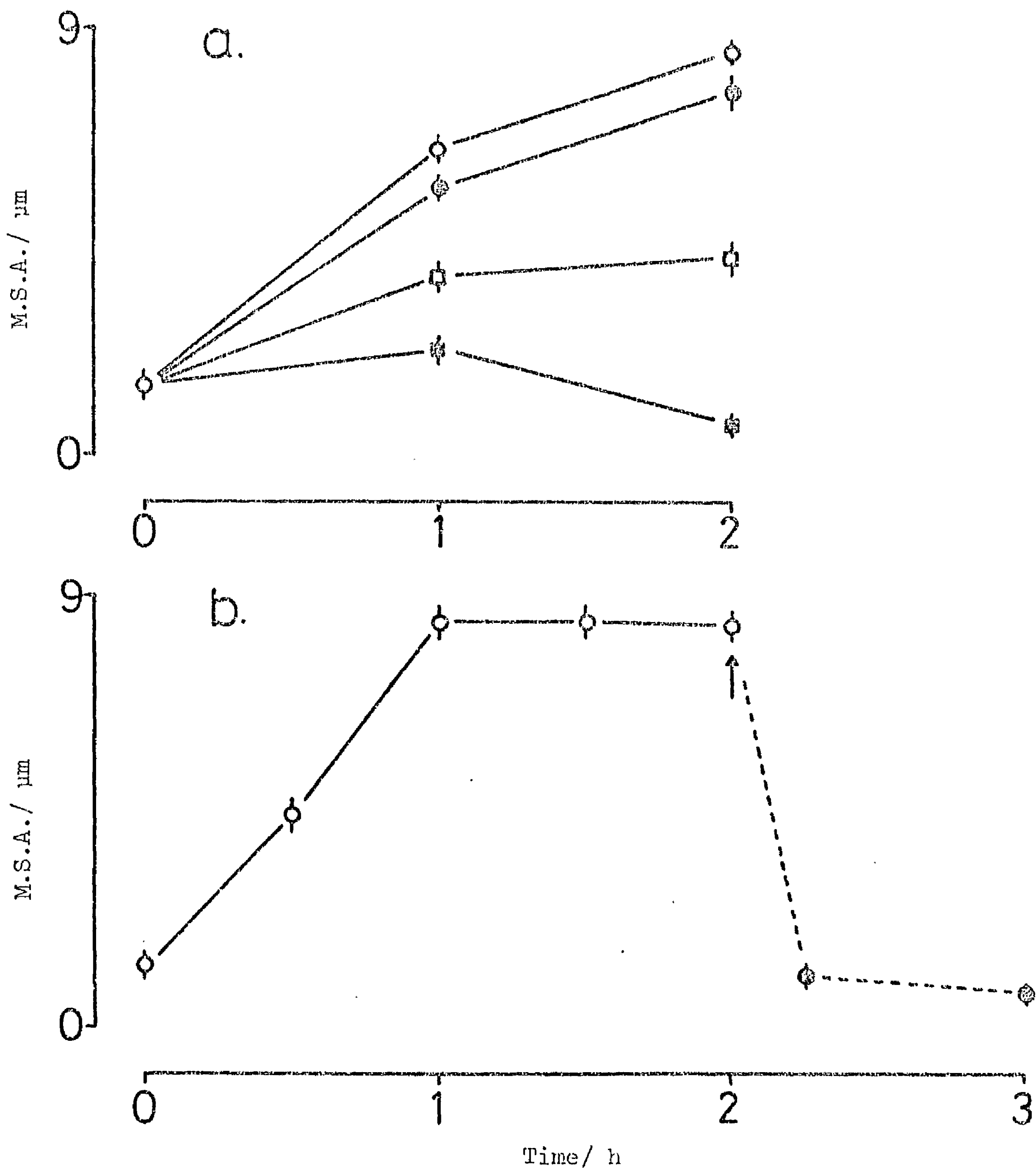
	M.S.A. (S.E.)/ µm.			
	System 1	System 2	t	P
Experiment 1·6·1	8·53(0·34)	7·69(0·38)	1·66	>0·05
Experiment 1·6·2	7·16(0·27)	5·49(0·26)	4·37	<0·001

Fig. 18. Effect of 0·1 mol m^{-3} ABA on Stomatal Aperture in 50 mol m^{-3} KCl/PIPES Buffer as a Function of Time.



Stomata were pre-opened in 50 mol m^{-3} KCl/PIPES buffer for 2 h before transfer of segments to buffer alone (open circles) or buffer plus 0·1 mol m^{-3} ABA (closed circles). System 1, R, n=50. s=2.

Fig.19. Effects of Aeration, Darkness, and ABA on Stomatal Apertures Attained on 50 mol m^{-3} KCl/PIPES



a. Open symbols: system 1.
Closed symbols: system 2.
Circles: normal illumination.
Squares: not illuminated.

b. Open circles: buffer alone.
Closed circles: buffer plus 0.1 mol m^{-3} ABA
Arrow: point of transfer to ABA solution.

a and b R, n = 50, s = 1.

The trend of these results therefore confirmed the observations of Willmer and Mansfield (1969) concerning the effects of CO_2- free air. The effects of darkness in both systems was a reduction in stomatal aperture which was highly significant (for Experiment 1·6·1 at 2 h: System 1 $t = 8{\cdot}86$; System 2 $t = 17{\cdot}40$. For both, $P < 0{\cdot}01$) This again corroborated results of Willmer and Mansfield, and also indicated that the 50 mol m^{-3} KCl/PIPES medium allowed stomatal closure to stimuli other than ABA. The possibility that this closure too, was dependent on KCl concentration was not examined.

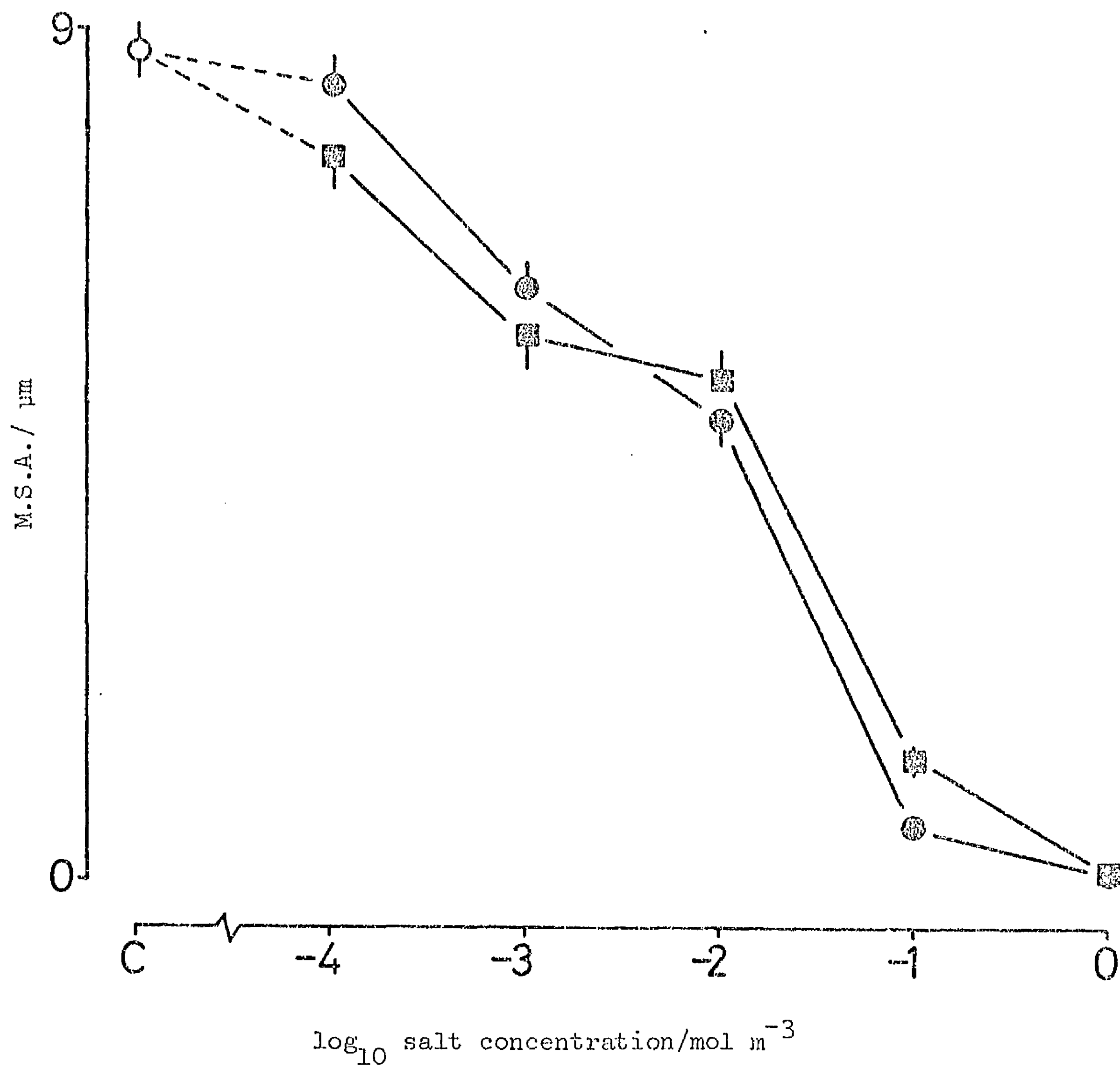
The ABA-induced closure shown in Fig.19b obtained with System 2 appeared to be as rapid and complete as that obtained with System 1 (Fig.18), so, despite the differences in apertures obtained using the two systems, System 2 was adopted for further experiments when required.

Experiment 1·7 Effects of Calcium and Strontium Chlorides on Stomatal Aperture.

Divalent cations are known to be inhibitory to stomatal opening (see commentary in Section Introduction), but this effect is both species (Willmer and Mansfield, 1969), and medium (Fischer, 1972) dependent. Since the use of calcium in the incubation medium has been advocated to preserve membrane function (Hsaio, 1976) the effects of this ion on stomatal apertures in 50 mol m^{-3} KCL/Pipes buffer was examined (Fig.20). Effects of strontium were also of interest because this cation is the decay product of ^{86}Rb disintegration. It was thus necessary to see whether strontium caused stomatal closure, and if so, whether the effective concentrations were close to those calculated to be present in $^{86}Rb^+$ solutions.

1 mol m^{-3} Ca^{++} is commonly added to tissue incubation media (Epstein, 1972), but from Fig.20 it would be impossible to carry out investigations of ABA effects in buffer containing this concentration since the stomata would already be closed. Furthermore, cell activity seemed to be unaffected by lack of calcium, both in terms of viability (Fig.12), and ability to function (Fig.18), in the time periods used. Calcium was therefore not incorporated in the medium for these reasons.

Fig.20 Effects of Calcium and Strontium Chlorides on Stomatal Aperture.



Closed circles: $Ca\ Cl_2$.

Closed squares: $Sr\ Cl_2$.

Open circle: normal buffer (c).

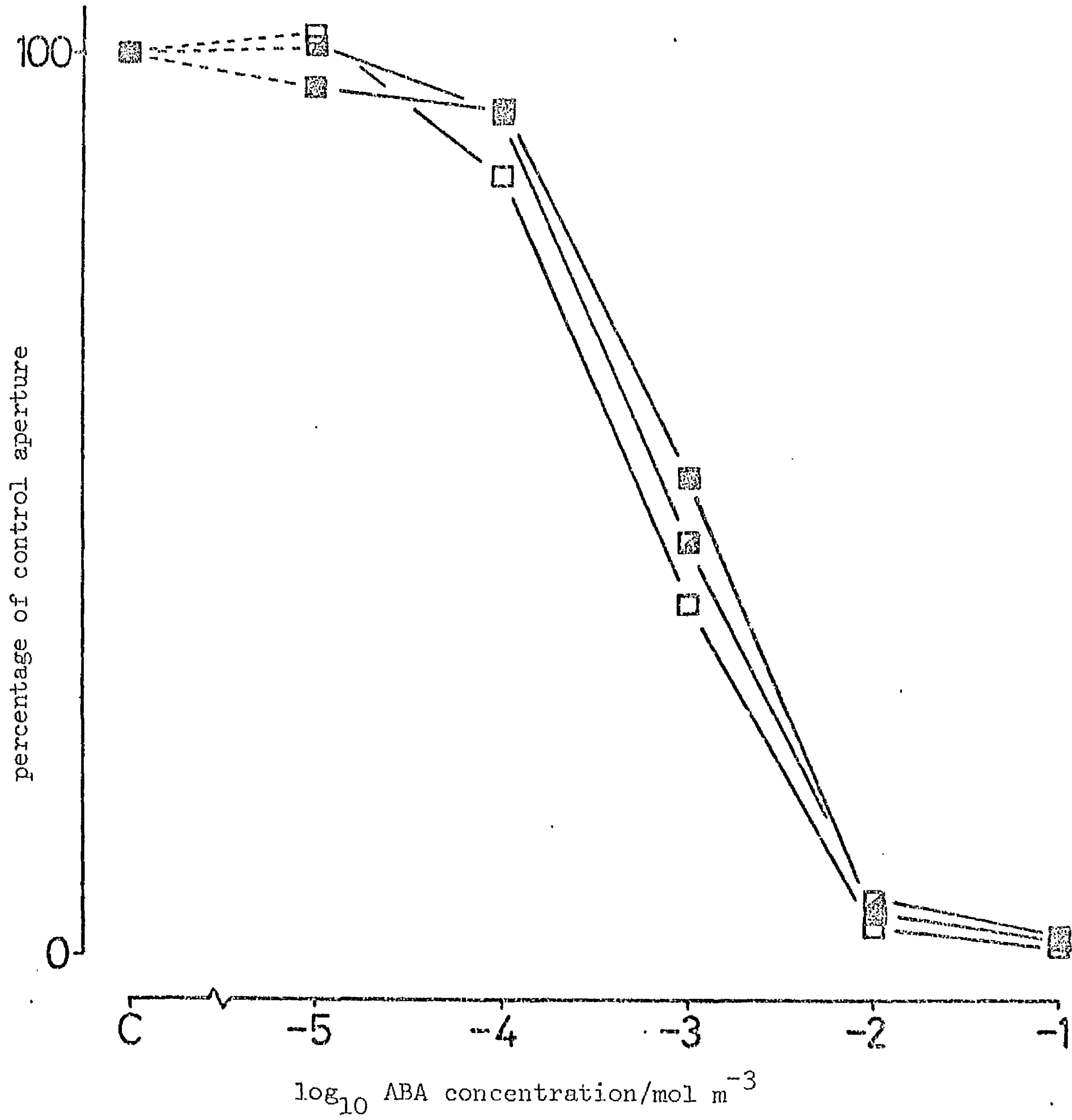
System 1, R, n = 50, s = 2.

The fact that strontium had effects resembling those of calcium was perhaps unsurprising in view of their chemical similarity. Given that the Radiochemical Centre $^{86}RbCl$ source solution was free of Sr^{++} from sources other than ^{86}Rb decay, the calculated strontium concentration in typical working solutions was of the order of 0·1 - 1 μmol m^{-3} after one ^{86}Rb half-life. Since even 0·1 mmol m^{-3} $SrCl$ caused virtually no aperture reduction (Fig.20), it was thought unlikely that Sr formed by decay would have physiological effects.

Experiment 1·8 Effects of the Physiological State of the Leaf on Responses of Stomata I. Senescence.

Experiments 1·8 and 1·9 were carried out to evaluate physiological factors in the responses of stomata from epidermal segments. For Experiment 1·8 a *Commelina* plant having suitable 'green', 'yellow-green' and 'yellow' leaves on the same branch was selected. At the time of peeling, three samples of lamina were taken from each leaf for chlorophyll a + b assay and two samples of epidermis for incubation in buffer. The buffer system chosen for this experiment was that of Ogunkanmi *et al.*, (1973), because their method gave partial stomatal openings at certain ABA concentrations. Fig. 21 shows that the stomatal responses of the first expanded leaf (that used in all other studies) at the ABA concentrations used were similar to those obtained by Ogunkanmi *et al.*(1973). However, the response curve was rather more sigmoidal in nature: Ogunkanmi *et al.* achieved linearity spanning four decades of ABA concentration (10^{-1} - 10^{-5} mol m^{-3}), whilst in this study responses were only linear between 10^{-2} - 10^{-4} mol m^{-3}. Differences in amount of closure between leaves were greatest at around 50% closure, which was obtained with 1 mmol m^{-3} ABA. The senescent leaves responded to a lesser degree to ABA treatment (Fig.21; Table 10).

Fig.21: Stomatal Responses of Epidermal Segments from Leaves at Different Stages of Senescence.



Open squares: 1st expanded leaf
Half-closed squares: 4th expanded leaf
Closed squares: 5th expanded leaf
System 1, R, n = 50, s = 2, Incubation period 2 h.

Table 10. *Chlorophyll Contents and Stomatal Responses in 1 mmol m^{-3} ABA.*

leaf	colouration	mean chlorophyll a+b content (S.E.)/ng mm^{-2}	M.S.A. (S.E.) at 1 mmol m^{-3} ABA/μm
1st expanded	'green'	395(5·8)	5·05(0·26)
4th expanded	'yellow-green'	159(2·3)	6·42(0·34)
5th expanded	'yellow'	33(6·0)	6·17(0·32)

The difference between the mean stomatal responses to ABA of the 'green' and 'yellow' leaves was statistically significant ($t = 2\cdot70$, $P < 0\cdot01$).

The above experiment was not repeated in the same form. However, responses of leaves having different chlorophyll contents were compared using 1 mmol m^{-3} ABA alone and similar results were obtained. In several cases the responses of 'very sensecent' leaves to ABA at this concentration were extremely small. It thus appears that the sensitivity of stomata on *Commelina* epidermis to ABA alters during leaf senescence. This vindicated to some degree the choice of young, healthy leaf material for all other experiments.

Raschke and Zeevaart (1976) examined ABA contents and stomatal conductance of *Xanthium* leaves as related to ageing. They found that stomatal sensitivity to ABA was similar in all ages of leaves, in contrast to this investigation. The precise reasons for sensitivity alterations found in *Commelina* are debatable. Measurements of whole plant transpiration rates, normal ABA contents of leaves and epidermal turgor relationships are required before conclusions can be made. A further possibility which must be entertained is that the observed effects are an artifact of the peeling process. In experiment 1·1 it was noted that epidermis from senescent leaves was found to be heavily contaminated with mesophyll. The results of Fig.14 indicate that survival of epidermal cells after peeling may influence subsequent aperture measurements. The viability of cells of epidermis from

senescent leaves was not assayed. This may thus be an important factor for future study.

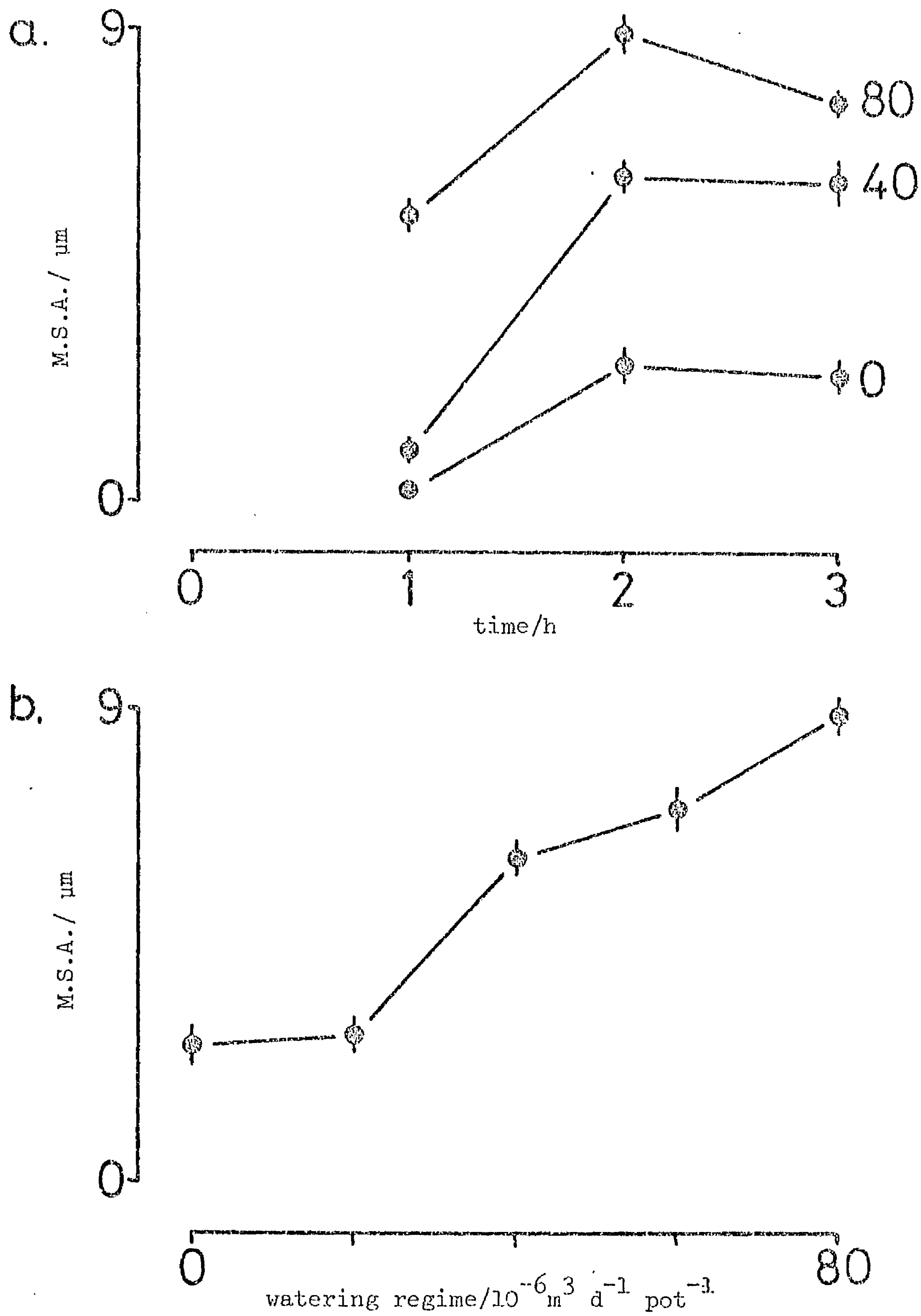
Experiment 1·9 Effects of the Physiological State of the Leaf on Responses of Stomata II. Water Status.

Many investigators have demonstrated the effects of water stress on stomatal behaviour of whole plants, and Fischer (1970) noted results of stressing the donor plant on stomatal aperture of epidermal strips subsequently obtained. Despite the acknowledged importance of prior stress as a factor in experiments with epidermis incubation systems (e.g. Orton and Mansfield, 1974), there appear to be no reported evaluations of its effects on segment stomatal behaviour.

The procedures normally adopted for this thesis to avoid undue water stress were to equilibrate the plants in the growth cabinet for at least 1 d, and to water to soil capacity twice daily throughout their lifespan. Stresses due to root cooling and overwatering were avoided by using water at room temperature and ensuring adequate drainage. In this experiment, stresses were induced by altering the watering regime and by wilting detached leaves.

Long-term stresses were applied by transferring plants from the greenhouse, watering to soil capacity, and then giving each plant a daily dose (midday) of 0, 20, 40, 60 or 80 $\times 10^{-6}$ m^3 tap water for 6 d. The positions of the five plants were altered daily using a random number table. The highest dose given was approximately equal to soil capacity and the plant watered with this amount was turgid and healthy, whilst the plant given no water exhibited leaf curling and flaccidity by about day 3. At the time of peeling, leaf conductance was measured. Conductance (mm sec^{-1} (S.E.), n = 3) fell from 17·5 (1·68) at 80 $\times 10^{-6}$ m^3 d^{-1} pot^{-1} and 0·36(0·06) at 60 10^{-6} m^3 d^{-1} pot^{-1} to below 0·28 at all other regimes. The stress thus had a great effect on stomatal conductivity. This reduction corresponded to a fall in apertures attained by stomata on epidermal segments from leaves of the stressed plants (Fig.22). Fig.22a shows that epidermis from the plant given 80 $\times 10^{-6}$ m^3 daily gave responses similar to those found with epidermis

Fig. 22. Effect of 6-day Watering Regime on Apertures Attained by Stomata on Epidermal Segments.



a. Figures at right of 3 h points correspond to watering regime (10^{-6} m^3 d^{-1} pot^{-1}). Two sets of figures are omitted for clarity. System 1, R, n = 50, s = 2.

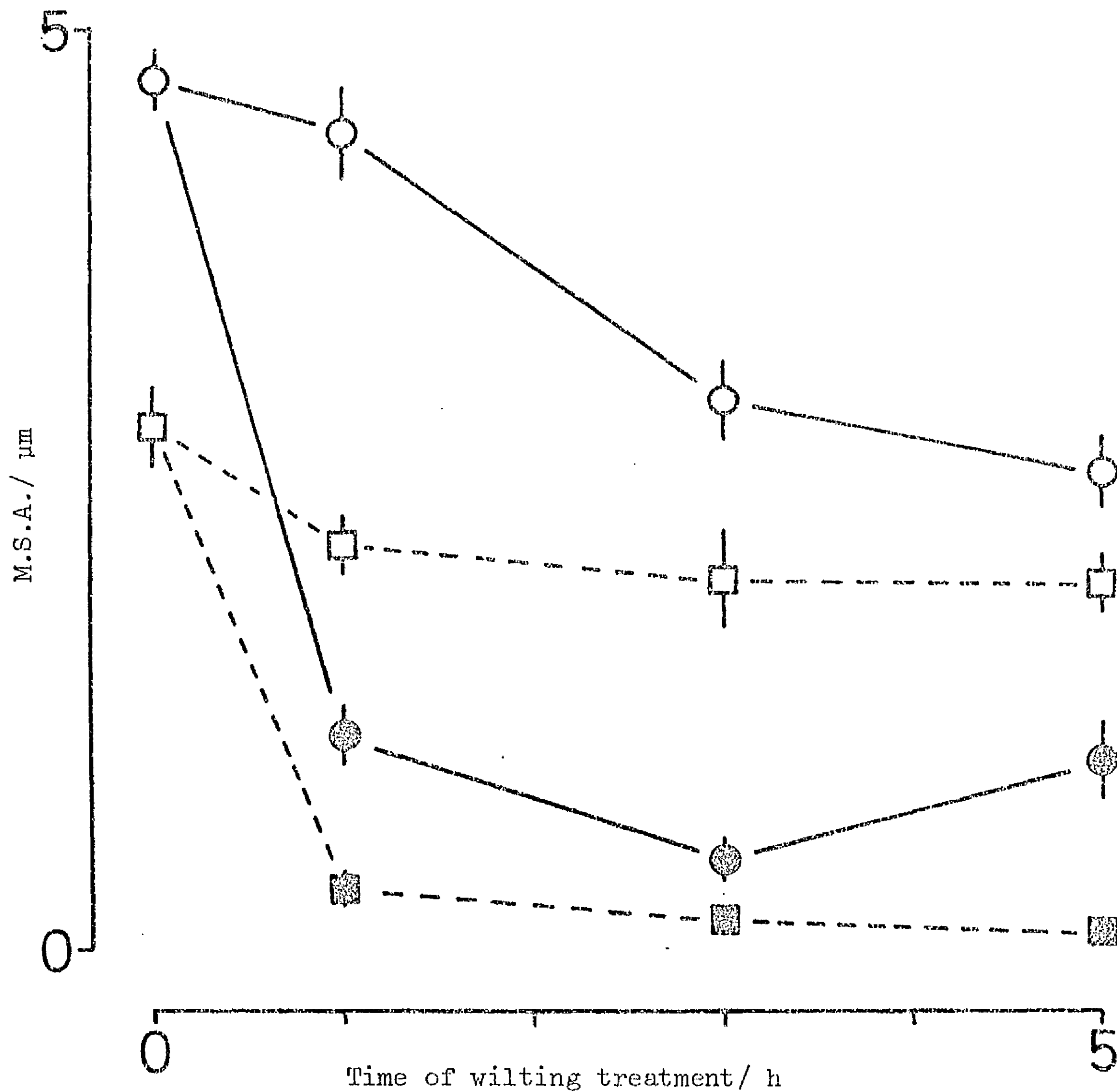
b. Aperture attained after 2 h incubation. System 1, R, n = 50, s = 2, incubation. Medium 50 mol m^{-3} KCl/PIPES.

from other unstressed plants (e.g. Figs. 17 and 19), whilst the other treatments resulted in lower apertures being attained. Calculation of the correlation coefficient for a least squares linear regression fit of the mean responses after 2 h incubation (the data shown in Fig.22b) indicated that the effect of the stress was significant ($y = 2{\cdot}11 + 0{\cdot}0844\ x$, $r = 0{\cdot}972$, $P < 0{\cdot}01$). However, a number of factors might have contributed to this effect. Long-term osmotic and other changes may have occurred, as well as alterations in hormone status and other possibilities. In order to restrict the chance of the first factor influencing results a further method of treatment was given in the short-term. This involved various wilting treatments applied to leaves from turgid plants.

The details of the short-term wilting stress are given in the Materials and Methods section. At the end of the treatment incubation three leaves were selected at random and epidermis removed. Segments were placed in 50 mol m^{-3} KCl/PIPES and aperture measurements made after 1 and 2 h (Fig.23). Data for both assay incubation times were of similar form: epidermis from wilted leaves gave lower stomatal apertures than that from 'control' leaves. For the 2 h assay, even the means which were closest (5 h treatment incubation) were significantly different ($t = 5{\cdot}19$, $P < 0{\cdot}001$). The length of the treatment incubation did not seem to affect apertures of epidermis from wilted leaves; these were uniformly low. It did seem to have an effect on the control values obtained after 2 h incubation, however. Calculation of the correlation coefficient for the 0, 1, 3 and 5 h control mean apertures (2 h) revealed a significant downward trend with time of treatment incubation ($y = 4{\cdot}88 - 0{\cdot}68\ x$, $r = 0{\cdot}976$, $P < 0{\cdot}02$). This effect was thought to be produced by turgor loss occurring in control leaves during the treatment incubation period, which was exacerbated with increasing time. This was slight when compared with the effect of wilting.

It must be emphasised that these results alone do not necessarily indicate that a hormonal influence on stomatal apertures occurred. They do show how important the water status of experimental plants must be to the results of epidermal strip bioassays. The prior stress factor may thus explain some of the variation in results found between experiments. The above experiments were not repeated. Further

Fig.23. Effect of 0-5 hour Wilting Treatment on Aperture Attained by Stomata on Epidermal Segments



Circles: apertures attained after 2 h assay incubation
Squares: apertures attained after 1 h assay incubation
Open Symbols: no fresh weight loss before treatment incubation.
Closed symbols: 10% fresh weight loss before treatment incubation
System 1, R, N = 75, s = 3.

investigations, possibly involving epidermis-mesophyll transfer operations combined with appropriate stress treatments, in experiments similar to those of Fischer (1970), are required before conclusions about the nature of the influence of stress on subsequent stomatal opening can be made.

Experiment 1·10 Comparison of Methods of Stomatal Aperture Measurement.

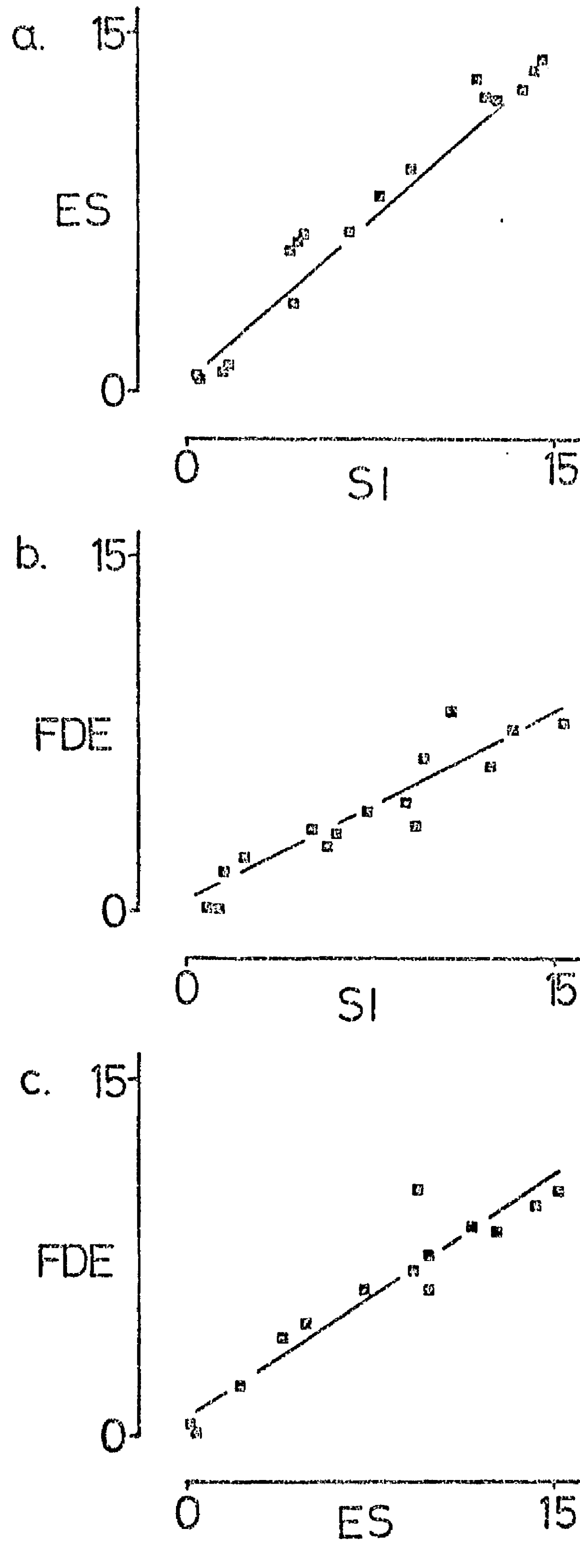
In view of the correlations between stress, leaf conductance, and epidermal segment aperture attainment shown in Experiment 1·9, it was of interest to examine the relationship between apertures found on isolated epidermis to those measured on the whole plant prior to peeling. It was also required that the freeze-drying aperture fixation method was assayed against aperture before freezing. Accordingly, an experiment was set up to compare apertures measured on whole leaves (silicone impression method - SI), those obtained immediately after peeling (epidermal segment -ES), and those obtained from freeze-dried epidermis (FDE). Rather than carry out a complex 3-way comparison, the individual methods were compared in pairs, with measurements from both being made from the same leaf on adjacent pieces of epidermis. A variety of apertures was obtained by treating leaves with CO_2-free air or darkness. The three graphs obtained are shown in Fig.24. The curves shown were fitted by least squares linear regression, and in all cases gave significant correlation coefficients ($P < 0{\cdot}001$)

The comparison between stomatal aperture on leaves immediately before peeling with apertures on freshly peeled epidermis (< 4 min) shown in Fig.24a revealed an almost one-to-one relationship:

$$ES = 0{\cdot}974\ SI + 0{\cdot}330, \quad r = 0{\cdot}987.$$

The slope of this curve was not significantly different from unity ($t = -\ 0{\cdot}623$, $P > 0{\cdot}25$), indicating that the peeling process had little effect on stomatal apertures. In contrast, freeze-drying epidermis

Fig. 24. Comparison of Three Different Methods of Aperture Estimation on Stomata from *Commelina* Leaves.



SI = Silicone impression
ES = Epidermal segment
FDE = Freeze-dried epidermis
} M.S.A./μm

n = 25, s = 1, from adjacent areas of lamina of individual leaves.

appeared to cause a reduction in stomatal aperture: in both Figs. 24a and 24c the slope of the regression line was significantly less than one. Thus:

$$FDE = 0{\cdot}542\ SI + 0{\cdot}224 \quad r = 0{\cdot}931$$

$$(\text{for } b = 1, \quad t = -\ 7{\cdot}77, \quad P < 0{\cdot}001)$$

and

$$FDE = 0{\cdot}675\ ES + 0{\cdot}876 \quad r = 0{\cdot}951$$

$$(\text{for } b = 1, \quad t = -5{\cdot}15, \quad P < 0{\cdot}001)$$

The reason for this reduction in apertures upon freezing may have been related to distortion or creeping during the drying process. Because of the high correlation coefficients, however, it was concluded that apertures estimated from freeze-dried material gave a reliable and fixed indication of the state of opening before freezing. Maintenance of stomatal aperture in open stomata after drying was also noted by Humble and Rashke (1971) and Itai and Meidner (1978b), but no quantitative relaitonship has been described before this study.

SECTION 2.

EXPERIMENTS ON EPIDERMAL SEGMENTS II. UPTAKE, DISTRIBUTION, AND METABOLISM OF ABA

INTRODUCTION

Several authors have concluded that the site of ABA action is the guard cell (e.g. Horton and Moran, 1972; Meidner and Willmer, 1975; Raschke, 1975a; Meidner and Sherriff, 1976), an inference which was primarily based on two sets of evidence. Firstly, although several plant hormones affected transpiration when applied to whole plants or leaves (Vaadia, 1976), only ABA affected stomata on isolated epidermis tissue (Horton, 1971; Tucker and Mansfield, 1971; Ogunkanmi *et al.*, 1973). Secondly, more detailed studies indicated a range of guard cell properties and functions affected by ABA which paralleled the effects of stomatal closure (see Section IV of General Introduction). Other investigators stressed the importance of subsidiary cells for closing movements (Squire and Mansfield, 1972; Itai and Meidner, 1978a; 1978b) but it was not clear from the results of Itai and Meidner whether ABA affected subsidiary cells directly.

The important experiments of Loveys (1977) established an endogenous movement of ABA between mesophyll and epidermal tissues during stress: it thus appeared that the guard cells (or stomatal complex as a whole) could be viewed as 'target cells' for ABA under certain environmental conditions. Despite Loveys' contribution, however, little information was available about distribution or movement of ABA *within* the epidermis. There appeared to be two approaches available to investigate this problem. In the first, endogenous hormone measurements would be required for individual types of cell within the epidermis. This was rejected as being outwith the scope of current methodology. A second method involved the study of uptake and movement of exogenous (radioactive) ABA within epidermis tissue, and this was adopted.

The experiments of this section investigated ABA uptake by *Commelina* epidermal segments, and the method used was simply to present the radioactive hormone in the buffer system developed in Section 1. Further studies on the intercellular distribution of the hormone were made using

microautoradiography, and the physiological relationships between uptake and closure were assessed.

RESULTS AND DISCUSSION

Experiment 2.1 Efflux Characteristics of Radioactivity from ^{14}C-ABA Applied to Epidermal Segments.

The intended study of ABA uptake by epidermal tissue floating in buffer solution containing radioactive ABA (0·1 mol m^{-3} 2-^{14}C-ABA in 50 mol m^{-3} KCl/PIPES) gave rise to an immediate problem, which was the removal of radioactive solution adhering to the tissue in the free space. Simple 'blotting' of epidermal segments was not satisfactory, owing to the high specific activity of the ^{14}C-ABA solution (22·6 Bq mm^{-3}). It was decided that a rinsing procedure was required to remove excess solution from the strips. Two procedures provided information on free space efflux. In the first, the uptake period was short, as was the efflux time, resulting in a low amount of information. In the second, uptake was allowed for 1 h (see Experiment 2.2) and efflux was studied over a 2 h period. This yielded further information on the uptake process as well as data on rapid free space efflux.

In order to study efflux after uptake for a short period, an epidermal segment was placed in the ^{14}C-ABA solution for 10 s and then given 12 sequential 15 s washes in 2 × 10^{-6} m^{-3} 50 mol m^{-3} KCl/PIPES buffer. Radioactivity present in each wash and in the epidermal segment was then analysed. This was repeated once and the results obtained are shown in Table 11.

It was clear that a major portion of the radioactivity was removed in the first few 15 s rinses. Characterisation of efflux of label from tissue incubated in ^{14}C-ABA medium for 1 h was carried out in a similar manner. Four segments (total area 44·8 mm^2) were placed in the solution for 1 h, removed, and then transferred through a series of 2 × 10^{-6} m^3 rinse solutions containing non-radioactive ABA (0.1 mol m^{-3} ABA in 50 mol m^{-3} KCl/PIPES). Transfer took place after the following efflux times:- 15 s 60 s(×3), 300 s (×7), 600 s (×2), 1200 s (×3). Radioactivity

Table 11: *Efflux of Radioactivity from Epidermal Segments*

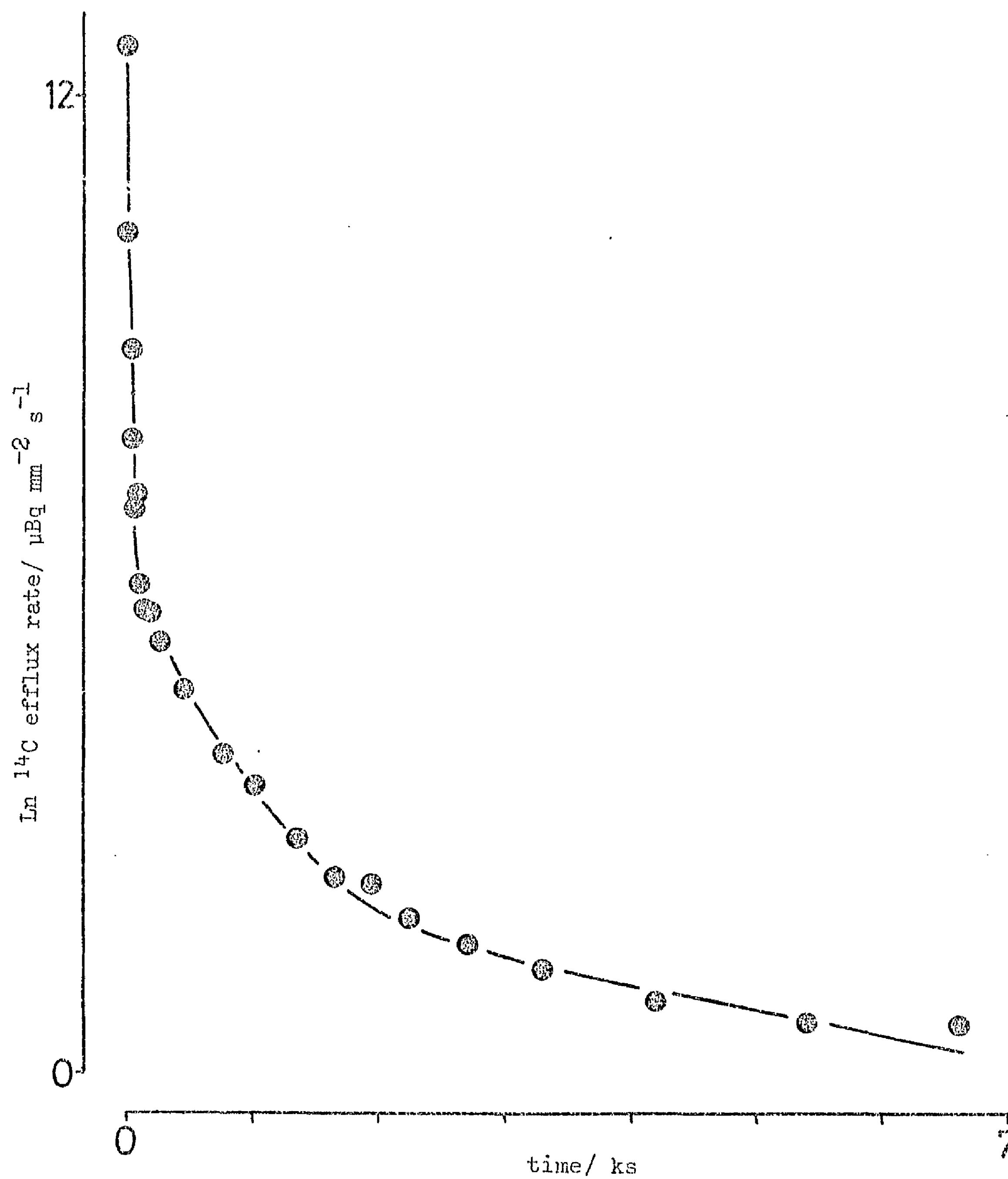
	% total ^{14}C recovered	
Wash No.	Segment 1	Segment 2
1	93.8	94.2
2	5.7	3.5
3	0.0	0.2
4	0.0	0.0
5	0.0	0.2
6	0.0	0.6
7	0.0	0.0
8	0.0	0.1
9	0.0	0.0
10	0.0	0.0
11	0.0	0.0
12	0.0	0.2
Tissue	0.5	0.9
Total ^{14}C/ Bq	18.1	15.8

present in the solutions was estimated and the natural logarithm of the efflux rate plotted against the mid-time of the relevant rinse (Fig.25). The rate of efflux of solutes in simple model systems follows an exponential decay pattern (see Baker and Hall, 1975) of the form

$$y = ae^{-bx}, \quad \text{where} \quad a = \text{original concentration}, \; b = \text{decay constant}.$$

A semi-log plot of such data should therefore be linear. The points shown in Fig.25 clearly did not fall in a straight line, although there were linear portions. It was thus assumed that there was more than one

Fig. 25. Rate of Efflux of ^{14}C from Epidermal Segments.



Segments were pretreated as stated in text. System 2, R, s = 4.
Efflux medium included 0·1 mol m^{-3} ABA.

phase to the efflux (Baker and Hall, 1975), so the data were subjected to a computer-assisted least-squares curve-fitting process, as used by Hipkins (1978). This described the decay in efflux rate as the sum of up to three first-order phases of the above equation. The programme was run to compute a and b values for both two- and three-phase curves, but the latter had a lower sum of squared residuals (1·0201 *versus* 5·5201) and was accepted as the best fit. The experiment was also carried out without ABA in the efflux medium and the data (which were of the same form) subjected to a similar analysis. Again, the three-phase sum of squared residuals was lower (1·2946 *versus* 4·1258).

The best fit was therefore of the form:

$$y = a_1 e^{-b_1 x} + a_2 e^{-b_2 x} + a_3 e^{-b_3 x} \quad .$$

The values of a_i and b_i with standard deviations for efflux medium with and without ABA and the relevant phase half-lives are given in Table 12.

Table 12: *Least-squares Fit Parameters for ^{14}C-ABA Efflux Data*

Efflux Medium	Phase	a_i/Bq mm^{2}	S.D.	b_i/s^{-1}	S.D.	$t_{\frac{1}{2}}$/s
with ABA	i = 1	284,420	97,752	0.0958	0.0092	7.2
	i = 2	449	71	0.00287	0.00032	241
	i = 3	12.0	4.1	0.000329	0.00008	2109
without ABA	i = 1	36,225	15,729	0.0514	0.00909	13.5
	i = 2	491.1	131.9	0.00327	0.000609	212
	i = 3	22.4	6.9	0.000402	0.000075	1724

These data were used to obtain the fitted curve shown in Fig.25.

The fastest efflux phase (i=1) was assumed to represent the exchange of free-space label, which in both cases appeared to be complete after about 90 s. Using a_1 = 100 and x = 90, it was calculated that over 99.98% of the label removed in this phase was exchanged after 90 s with ABA in the efflux medium (99.02% without ABA). Consequently, a standard efflux procedure involving six 15 s rinses was adopted for all

other experiments in order to remove free space ^{14}C-ABA. The difference in half-lives for the fast phase with and without ABA in the efflux medium would be predicted if a certain proportion of the label was loosely bound to wall sites in the epidermal tissue: use of a 'cold rinse' should facilitate exchange from such sites.

The significance of the slower phases of efflux will be discussed with the results of Experiment 2.2.

Experiment 2.2 Uptake of ^{14}C from 2-^{14}C-ABA by Epidermal Segments I. Quantitative Radioassay by Scintillation Spectrometry.

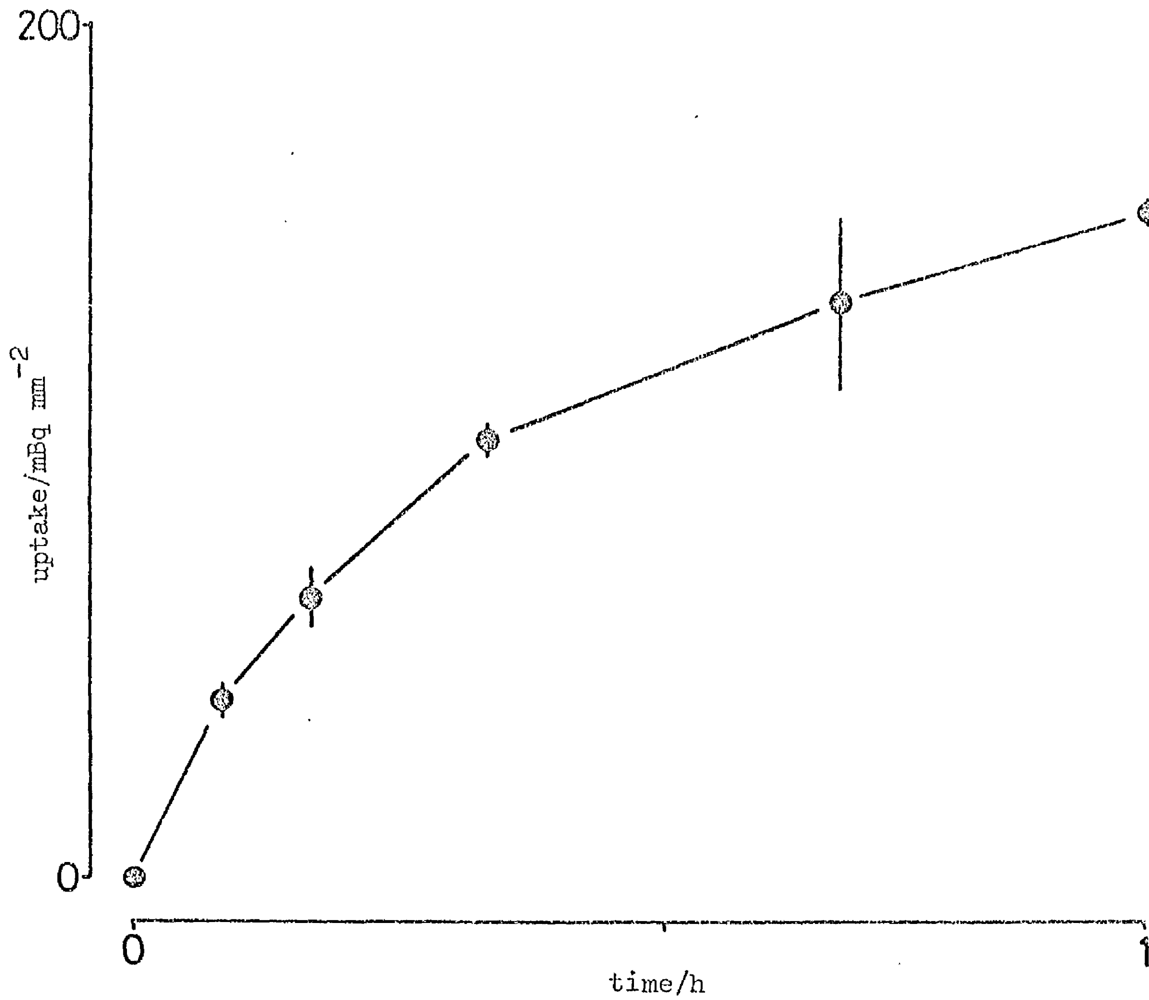
Uptake of label from 2-^{14}C-ABA by epidermal segments was studied by transferring segments incubated for 2 h in 50 mol m^{-3} KCl/PIPES buffer to radioactive ABA solution. Six segments were removed at '0', 5, 10, 20, and 40 min (mean area 13.4 mm) and 8 at 60 min. Those at 60 min were subjected to efflux analysis in a repeat of Experiment 2.1 but uptake was back-calculated from the total efflux and the amount remaining in the tissue at the end of efflux. The segments were rinsed using the standard procedure and radioactivity present assayed by liquid scintillation spectrometry. The results are shown in Fig.26. Uptake appeared to reach a maximum after 1 h. Such an asymptotic curve is predicted by simple model systems (Baker and Hall, 1975). In order to test whether this uptake phenomenon was a property of living epidermal cells, ^{14}C present in normal segments and those killed by the freeze-thaw method was compared after 1 h incubation. The efficiency of this method for killing cells was also tested by comparing the uptake of neutral red by both types of tissue. The results are shown in Table 13.

Table 13: *Uptake of ^{14}C from 2-^{14}C-ABA by Untreated and Freeze-thawed Epidermal Segments.*

Treatment	Mean uptake (S.E.)/in Bq mm^{-2}	n	% living cells in epidermis*	Nos. of cells examined		
				epidermal	subsidiary	guard
None	81.5(4.7)	6	97.0	237	2694	898
freeze-thawed	11.9(2.2)	6	0.0	220	2670	890

*Ascertained on tissue which was not incubated.

Fig. 26. Uptake of ^{14}C from 2-^{14}C-ABA by Epidermal Segments.



System 1, R, s = 6 (except at 1 h, where s = 2)

The difference between uptake of living and dead epidermis was highly significant ($t = 13.5$, $p < 0.001$). It was not clear whether the ^{14}C uptake by freeze-thawed tissue (11.9 mBq mm^{-2}) was physiologically significant: the rinsing procedure used was tested on live tissue and may not have been applicable to dead tissue. This uptake may therefore represent unrinsed free space label. On the other hand, a proportion of normal uptake may have been into sites not destroyed by the freeze-thaw treatment. Certainly, at least 87% of the uptake of Fig.25 appeared to be conditional on the presence of living cells. It thus seems that the second and third efflux phases of Fig.25 may be due to discrete (living) compartments within the epidermal tissue which had taken up ^{14}C from ABA. In simple model systems (see Baker and Hall, 1975) such an efflux pattern may relate to cytoplasmic and vacuolar compartments. However, in a complex tissue such as *Commelina* epidermis the situation is confounded: different types of cell may represent compartments as well as different intercellular areas. It was therefore concluded that uptake was largely a property of living epidermal tissue, but that clear-cut designation of efflux phases could not realistically be made.

The data of Fig.26 may be combined with those of Fig.18 to give a sensitivity estimate for stomatal closure in response to ABA application. Thus, stomatal closure was complete after 10 min in 0.1 mol m^{-3} ABA (Fig.18), and at this time the uptake of ^{14}C from 2-^{14}C-ABA was 66 mBq mm^{-2} (Fig.26). From an estimate of 50 stomata mm^{-2}, which was justified by observation, and knowing the specific activity of the ^{14}C-ABA (0.226 TBq mol^{-1}), it was possible to calculate that no more than 5.9 fmol ABA was present per stomatal complex at the time of closure.

A study of G-^{3}H-ABA uptake was also made in which the hormone was incorporated at 0.1 mol m^{-3} in 100 mol m^{-3} $NaNO_3$/PIPES buffer, and the characteristics of uptake of label were similar to those of Fig.25. When uptake by epidermis with 'pre-opened' (detached leaves placed on tap water under fluorescent lights and treated with CO_2-reduced air for 2 h) and 'pre-closed' (detached leaves placed on tap water in darkness) stomata was compared in this system, there was no significant difference between ^{3}H uptake in the two treatments (mean uptake 'pre-opened' = 11.6 Bq mm^{-2}, S.E. = 0.64, $n = 3$; mean uptake 'pre-closed' = 13.5 Bq mm^{-2}, S.E. = 1.28, $n = 3$; $t = 1.31$, $P > 0.25$). Using the same treatments,

but incubating epidermis on ^{14}C-ABA in distilled water, Itai *et al* . (1978) obtained a similar result. It therefore appears that the state of aperture of the stomata did not affect uptake of ^{14}C from ABA.

Experiment 2.3 Uptake of ^{14}C from 2-^{14}C-ABA by Epidermal Segments II. Qualitative Radioassay by TLC and Scintillation Spectrometry.

This experiment was devised to investigate the molecular state of the ^{14}C-label present in epidermal segments after 1 h incubation in 0.1 mol m^{-3} 2-^{14}C-ABA in buffer. Segments were incubated for 2 h in 50 mol m^{-3} KCl/PIPES, transferred to the radioactive solution for 1 h and then rinsed by the standard procedure. Radioactivity present was extracted with methanol for 24 h at 4 C and analysed by TLC. Standards were applied using method A. The results are illustrated in Fig.27.

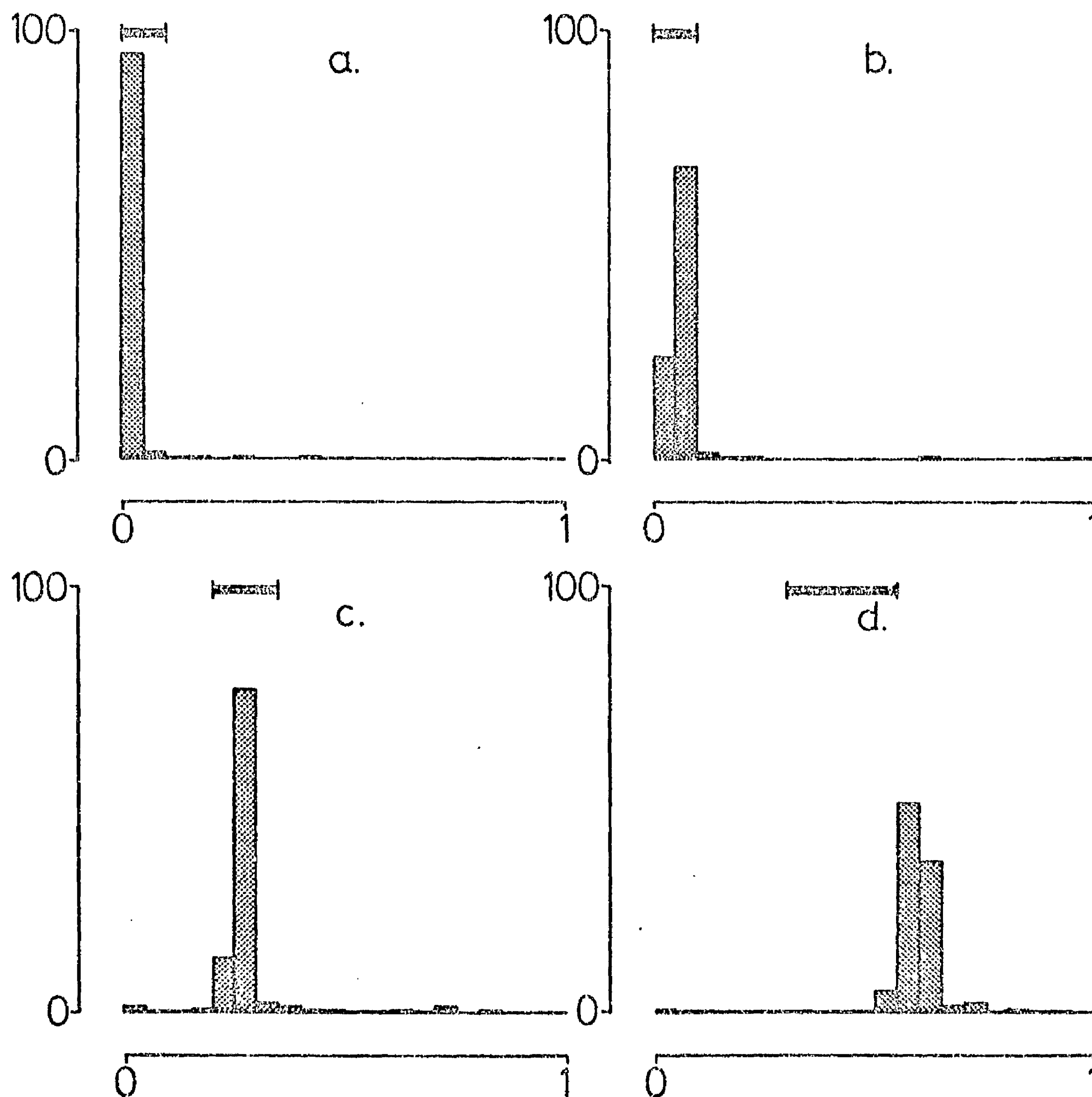
For each solvent system used there is one major peak of activity present. This corresponds closely to the position of the standard except in the case of solvent system d. Chloroform:methanol, however, is a system known to give variable Rf values between runs (Colquhoun, 1974). In this case it is suspected that there were edge effects in the chromatography which led to a difference in Rf values for the standard and extract.

It is not possible, using TLC, to conclude that the extract was entirely 2-^{14}C-ABA. On the other hand, it *is* possible to state that metabolism to products having markedly different chromatographic properties to ABA had not occurred. It therefore seemed that extensive metabolism of ABA by epidermis tissue had not occurred by 1 h.

Experiment 2.4 Uptake of ^{14}C from 2-^{14}C-ABA by Epidermal Segments III. Qualitative Radioassay by Microautoradiography.

A priori, it was thought that autoradiography with ABA would require the use of soluble-compound techniques, since the hormone is relatively soluble in water. The efflux evidence of Experiment 2.1 confirmed this

Fig.27. TLC of Epidermis Extracts.



x-axes; Rf value; y-axes: Radioactivity recovered in Rf zone/% total. Bars represent position of 2-^{14}C-ABA standard.

Solvent Systems, all v/v (with total recovered ^{14}C)

a. n-hexane:ethyl acetate; 1:1 (18·9Bq)

b. Ethyl acetate: toluene: acetic acid; 1:10:2 (20·2 Bq)

c. Benzene: acetone: acetic acid; 70:30:1 (20·0 Bq)

d. Chloroform: methanol; 1:1 (21·0Bq)

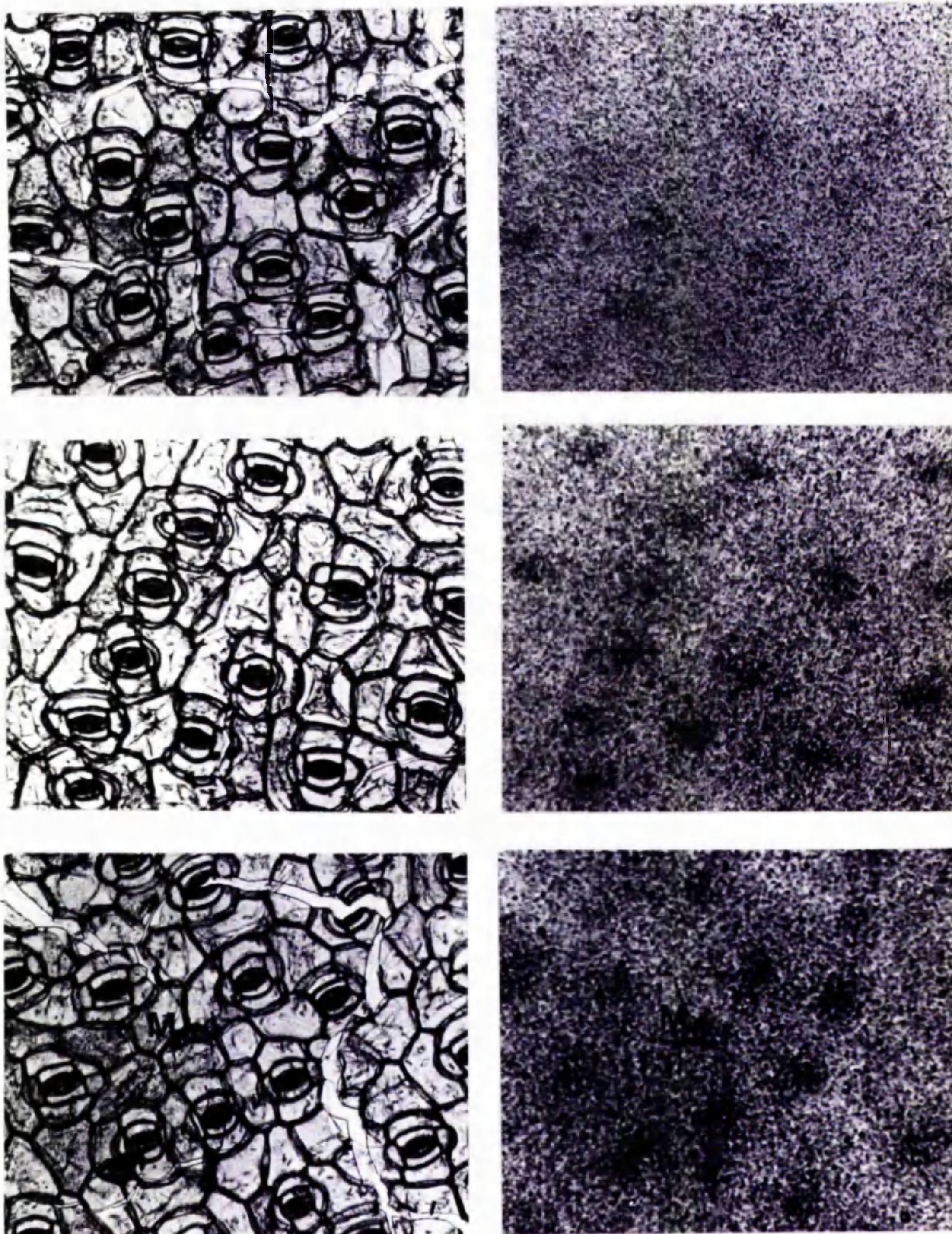
assumed mobility of the label. Normally, soluble-compound microautoradiography requires methods involving the freezing of the tissue of interest followed by sectioning in a cryostat (Rogers, 1973). The tissue is maintained in the frozen state (thus 'fixing' soluble moieties in place) whilst a photographic emulsion sensitive to charged particles is brought into contact with it (in darkness) for exposure. Fitzgerald *et al.* (1961) pioneered a method whereby dry film was applied to sections which had been freeze-dried to obviate solute migration. Experiments with epidermis tissue has the advantage that no sectioning is required for work in the plane parallel to the lamina, since the specimen is effectively a monolayer of cells. There are a number of reports of the location of various substances in epidermal strips using microautoradiography (e.g. Shaw and MacLachlan, 1954; Nishida, 1963; Willmer *et al.*, 1973; Dittrich and Raschke, 1977a, 1977b). Most of these involved the location of non-water-soluble compounds such as starch and did not utilise soluble-compound methods. Willmer *et al.*, however, studied the location of soluble products of CO_2 fixation. The autoradiographic method was derived from one of their techniques.

Epidermal tissue was incubated as in Experiments 2.2 and 2.3, and removed from the ^{14}C-ABA solution after 20, 40 and 60 min for rinsing. The tissue was placed on subbed slides, freeze-dried and exposed for 98 d. Representative areas of microautoradiographs and the associated tissues are shown in Fig. 28.

There was a considerable degree of correspondence between the positions of guard cells in the epidermis and the aggregation of silver grains in the film emulsion. This was not always found to be a one-to-one relationship, however, as stomatal complexes for which there was no corresponding silver grain accumulation, and also areas of grain aggregation which did not correspond to stomata, were found. These could be explained respectively by lack of tissue-film contact and the presence of mesophyll contamination. Although the diameters of the areas of grain aggregation appeared to be larger than those of the guard cell pairs, this does not necessarily imply that other subsidiary cells accumulated the hormone. A sphere of radius 20 μm in photographic emulsion (gelatin) contains all silver grains formed in 90% of the β-tracks originating from a point source of ^{14}C at its centre (Rogers, 1973).

Fig.28. Microautoradiographs of Epidermal Segments Supplied with 2-^{14}C-ABA.

Column A Column B



Column A: appearance of freeze-dried tissue under light miscroscopy.
Column B: corresponding distribution of silver grains in the photographic emulsion.
Rows: microautoradiographs from tissue incubated in 2-^{14}C-ABA for 20 min (top), 40 min (middle) and 60 min (bottom).
M: contaminating mesophyll cell which has also taken up radioactivity (122 x)

Thus a β-particle from a ^{14}C source at the edge of a guard cell might reasonably form a latent image within the subsidiary cell outlines (see Fig.28 for scale). This does not exclude the possibility that both epidermal and subsidiary cells also took up radioactivity, but indicates that most of the uptake was by the guard cells.

A particularily important aspect of autoradiography is the elimination of the possibility of artifacts by the use of the correct controls (Rogers, 1973). The controls used for positive and negative chemography (chemicals from the tissue causing or removing latent images) described in the Materials and Methods section were always without artifact. Pressure artifacts would also have been revealed by the positive chemography control.

No specific controls were employed to test for solute migration during the freezing process. However, three observations indicated that it was not of importance: firstly, when tissue was prestained with neutral red and freeze-dried, the dye remained within the cell outlines in the dry state, secondly, when epidermal tissue having a proportion of pigmented cells (from *Cyclamen persicum*) was freeze-dried, the pigment remained in the cell outline, and thirdly, crystals (assumed to be of calcium oxalate) in some *Commelina* epidermal cells remained in position after freeze-drying.

Artifacts due to spatial effects might have occurred if the freeze-dried epidermis tissue had non-planar geometry. Thus, the existence of raised areas around each stoma could have brought the guard cells into closer contact with the film, leading to the possibility of greater *pro rata* latent image formation. This was investigated by the study of transverse sections of dried epidermis. Such sections indicated that there were no raised or sunken regions.

It was therefore concluded that the accumulation of silver grains in the photographic emulsion represented a corresponding accumulation of radioactivity from 2-^{14}C-ABA at or near the guard cells. From Fig.28 it was apparent that this accumulation increased over the 1 h treatment period.

Experiment 2.5 Efflux of ABA and Stomatal Re-opening.

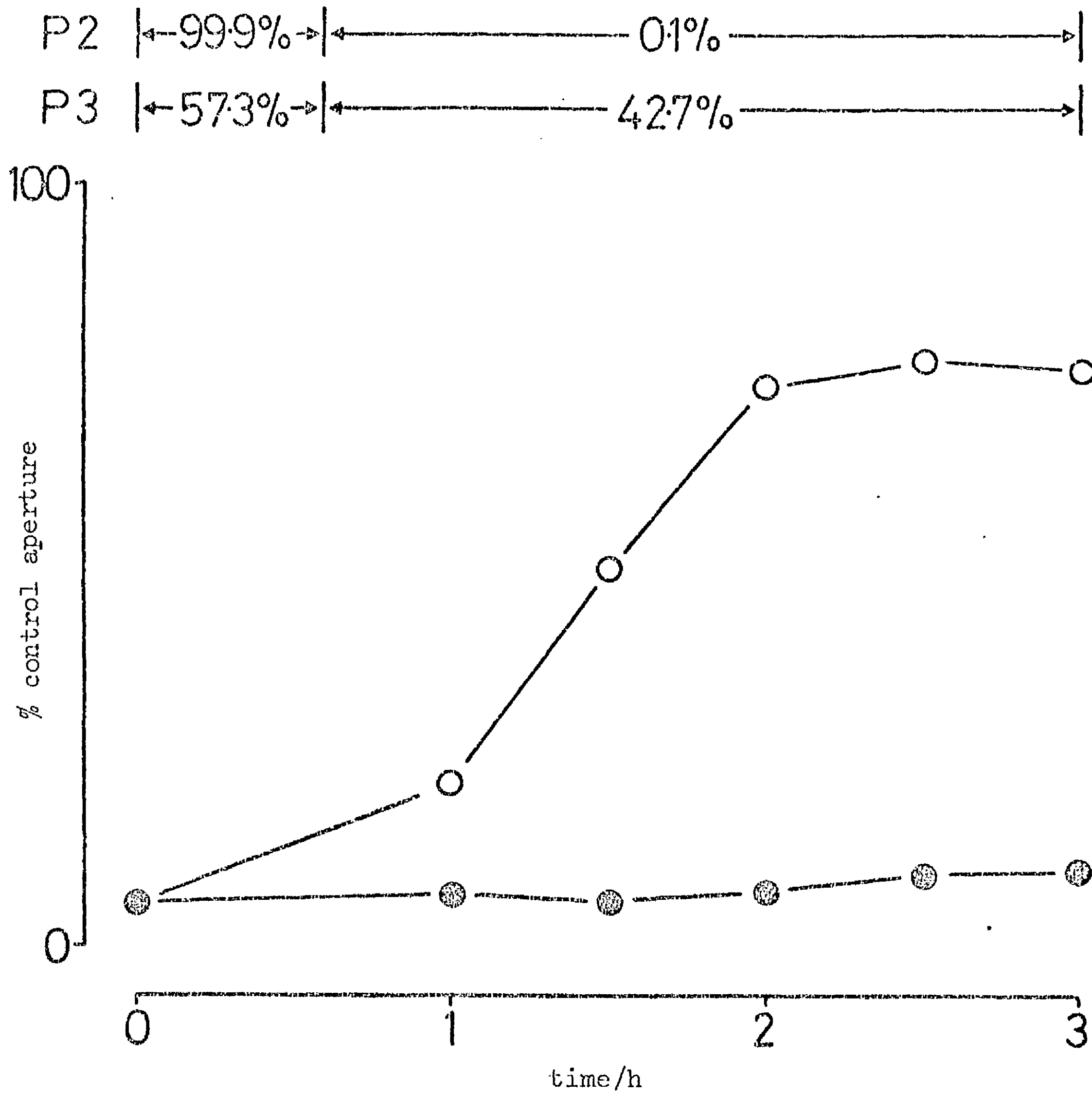
In Experiment 1.5 it was observed that stomatal re-opening did not occur when ABA was supplied continuously to epidermal segments. However re-opening has previously been observed after short, pulsed, application of the hormone (Cummins *et al* ., 1971) and also after transfer of epidermal segments from medium with ABA to that without (Horton, 1971). The efflux data of Experiment 2.1 indicated that the hormone would leak out of the epidermis tissue rapidly when transferred to medium without ABA. This experiment was carried out to correlate efflux with any stomatal re-opening which might occur.

Segments were incubated on buffer for 2 h to open stomata as before, transferred to 0.1 mol m^{-3} ABA for 1 h to close the stomata, rinsed for 90 s, and then transferred to either 0.1 mol m^{-3} ABA or buffer alone for 3 h. 50 mol m^{-3} KCl/PIPES was used throughout. At hourly intervals, the buffer was changed to avoid effects of ABA which may have leaked to the medium. As mentioned in Experiment 1.5, periods of incubation in excess of 3 h tended to result in stomatal closure. For this reason, results were expressed as a percentage of control segments not treated with ABA at all, but given equivalent disturbance at the relevant times. The mean stomatal apertures on these controls declined to 5.12 μm after 6 h incubation.

The losses from 'compartments' corresponding to phases 2 and 3 (inclusive of the rinse time) were calculated in order to compare the re-opening data with the characteristics of efflux (Experiment 2.1, Table 12, figures for efflux medium without ABA). These are illustrated in such a way that the time taken for 99.9% of the predicted efflux from phase 2 to occur could be seen and compared with the predicted efflux from phase 3 at this time (Fig.29).

Stomatal re-opening occurred if net efflux was allowed, but not if ABA was continuously supplied. The difference between mean stomatal apertures for the two treatments was significant after 1 h ($t = 5.76$, $P < 0.01$). Re-opening was correlated to some degree with efflux, but the exact relationship between phases 2 and 3 and stomatal movement was unclear. Opening may have commenced only as 'compartment' 2 became empty or may have been influenced primarily by efflux from 'compartment' 3.

Fig.29. Stomatal Re-opening after ABA Treatment.



Open circles: transferred to 50 mol m^{-3} KCl/PIPES
Closed circles: transferred to 0·1 mol m^{-3} ABA in 50 mol m^{-3} KCl/PIPES
P2 = phase 2, P3 = phase 3 of Table 12. %s refer the calculated amount of efflux from the relevant a value.
System 1, R, n = 50, s = 2.

The shape of the re-opening graph bears a striking similarity to those obtained with pre-stressed leaves (Experiment 1.9). It is possible that reduced opening in stressed leaves was the result of increased epidermal ABA levels, and that incubation in buffer allowed efflux of the endogenous hormone pool to give comparative results. An interesting facet of both sets of results is the fact that re-opening did not occur to 100% of the control value within 3 h.

Although re-opening occurred when net efflux of ABA was allowed, it is not possible to conclude that the *uptake* of ABA is necessarily directly related to the events of stomatal closure. It is, however, reasonable to say that the ABA content of the epidermal segment appeared to affect the ability of stomata to open or close.

SECTION 3.

EXPERIMENTS ON EPIDERMAL SEGMENTS III. USE OF $^{86}Rb^+$ AS TRACER FOR K^+

INTRODUCTION

The importance of ion fluxes in stomatal opening was discussed in Part IV of the General Introduction. Several methods have been employed to evaluate ion movements between cells of the epidermis, all of which appear to have advantages and disadvantages: stains may not be specific and are difficult to quantify (see Fischer, 1972); there are problems with quantification of results obtained with the otherwise definitive electron microprobe (Humble and Raschke, 1971); methods used in micro-electrode studies have been criticised (Moody and Zeigler, 1978). In the case of tracer uptake, there are problems of exogenous application and the relationship between the movement of the tracer and the ion of interest. However, the latter method has considerable advantage in simplicity and expense.

The relatively short-lived isotope ^{86}Rb ($t_{\frac{1}{2}}$ = 18.66 d) has been used extensively in studies of ion uptake and transport in plant physiology. It is generally employed as an analogue of potassium, for which only radioisotopes of very short half-life are commercially available (e.g. $t_{\frac{1}{2}}$ of ^{42}K = 0.52 d). Rubidium occupies a position in the periodic table immediately below potassium in the same column. Although it has eighteen more protons and electrons, the physical properties of rubidium and its salts are remarkably similar to those of potassium. Some representative values for the two alkali metals and their chlorides are shown in Table 14.

The resemblance between the two metals evinced by Table 14 has led to the assumption that fluxes of $^{86}Rb^+$ and K^+ are quantitatively comparable. This can be tested by experiments in which the features of both rubidium and potassium movement have been evaluated. The study most relevant to this work is that of Fischer (1972). He found that $^{86}Rb^+$ and $^{42}K^+$ uptake characteristics of *Vicia* epidermal segments were similar. On the other hand, Hiatt (1970) reported apparent anomalies between K^+ uptake by roots estimated by spectroscopy and $^{86}Rb^+$ movement, and Erdei and Zsoldos (1977)

demonstrated that $^{86}Rb^+$ uptake consistently overestimated $^{42}K^+$ uptake in rice roots and callus tissue.

Table 14. *Comparative Properties of Potassium and Rubidium and their Chlorides (data taken from Mahan, 1970).*

Characteristic or Property	Element or salt	
	^{39}K	^{85}Rb
Atomic Number	19	37
Atomic Weight	39.1	85.5
Electron orbital configuration	$1s^2 2s^2 2p^6 3s^2 3p^6 4s$	$1s^2 2s^2 2p^6 3s^2 3p^6 3d^{10} 4s^2 4p^6 5s$
First ionisation energy/kJ mol^{-1}	418.8	402.9
Ionic radius/pm	133	148
	^{39}KCl	$^{85}RbCl$
Molecular weight	74.55	120.97
Interionic distance/pm	314	329
Crystal lattice energy/kJ mol^{-1}	708	679
Solubility/ kmol m^{-3} (25 C)	4.6	7.5

In this study, few assertions involving precise quantitative relationships between the two ions have been made. Rather, movement of $^{86}Rb^+$ and K^+ have been assumed to be *qualitatively* similar. Accordingly, results are expressed in units of radioactivity and not in 'potassium ion equivalents'. Such values vary for similar treatments between experiments because of radioactive decay, but comparison between treatments within experiments is still valid. For each experiment the $^{86}Rb^+$ content of solutions used is given in Bq mm^{-3}; in terms of added RbCl concentration this was normally less than 50 mmol m^{-3}. The proportion of $^{86}Rb^+$ to $^{85}Rb^+$ in radioactive solutions was less than $1:2.5 \times 10^4$.

The objective behind the series of experiments was to determine the effects of ABA on stomata of *Commelina* shown in Section 2 in terms of

movement of $^{86}Rb^+$. A number of other experiments were also carried out to characterise the system used and the fluxes of tracer under different conditions.

RESULTS AND DISCUSSION.

Experiment 3.1 Effects of RbCl on Stomatal Aperture.

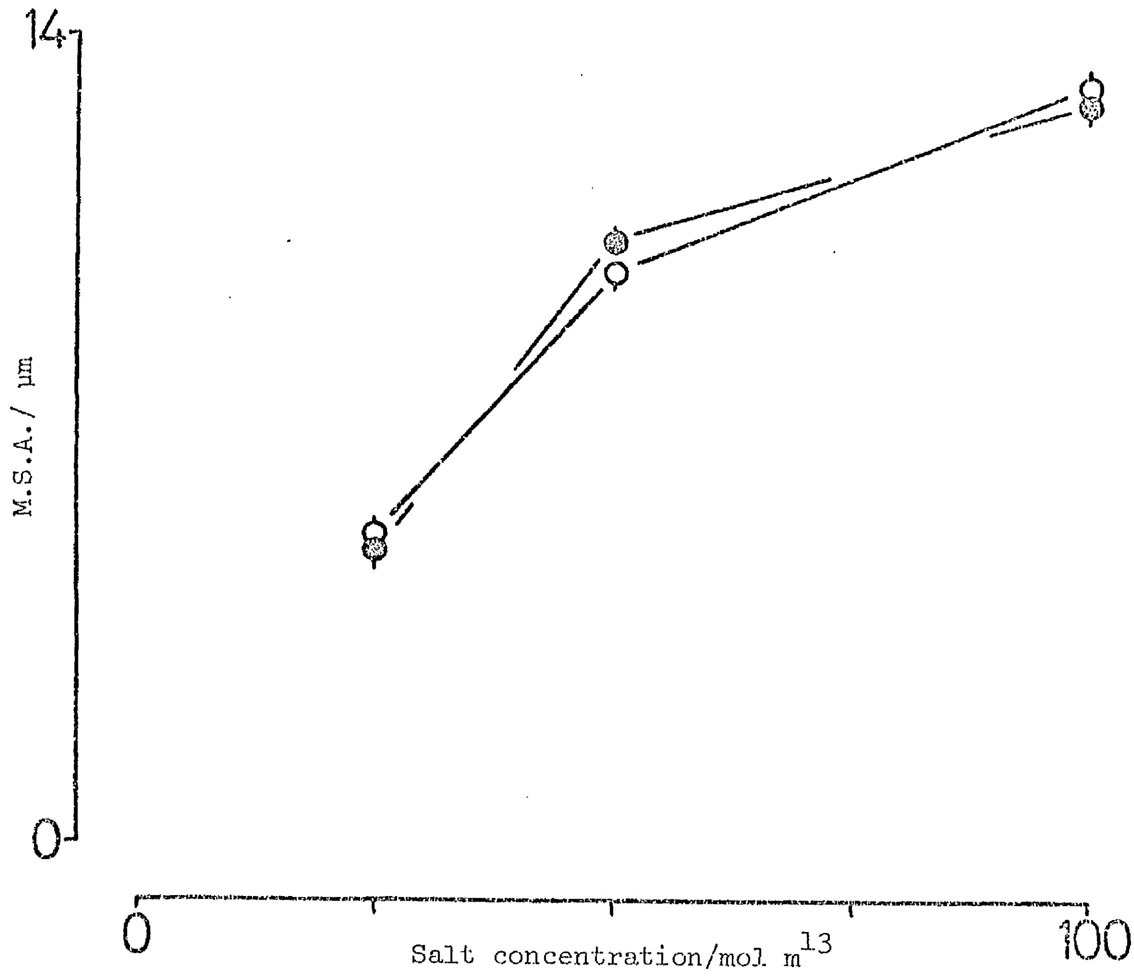
One approach to the problem of differential uptake of Rb^+ and K^+ is the use of the epidermal segment system as a 'bioassay' of ion fluxes. If it is assumed that stomatal aperture is related to guard cell ion uptake (Fischer, 1972; and later results), then differences in uptake of the ions should be reflected in the apertures attained by stomata of epidermal segments incubated in solutions containing the ions. Humble and Hsaio (1969) showed that K^+ and Rb^+ had similar effects in *Vicia,* and this experiment was carried out to examine the comparative effects of the two ions on *Commelina* stomatal movements, using the PIPES buffer system.

Segments were incubated in solutions of either RbCl or KCl in 10 mol m^{-3} PIPES pH 6.8 for 2 h and apertures measured. The results are shown in Fig.30. Stomatal openings with the two salts were clearly very similar: at the concentration where the greatest difference in apertures was obtained, 50 mol m^{-3}, the means were not significantly different ($t = 1.50$, $P > 0.1$). *Commelina* stomata therefore appear to react to the presence of Rb^+ or K^+ in a similar manner to *Vicia* . From the evidence of this 'bioassay', uptake of the two ions by guard cells appears to be equivalent.

Experiment 3.2 Uptake of $^{86}Rb^+$ by Epidermal Segments I. Correlation with Stomatal Aperture.

The method of ^{86}Rb-labelling of incubation solutions has been used to correlate K^+ uptake with stomatal aperture, particularly with *Vicia faba* epidermis (Humble and Hsaio, 1969; Fischer, 1972). The following

Fig. 30. Effects of KCl and RbCl on Stomatal Aperture.



System 1, R, n = 50, s = 2.
Open circles: KCl
Closed circles: RbCl.

experiments were designed to monitor $^{86}Rb^+$ uptake in *Commelina*.

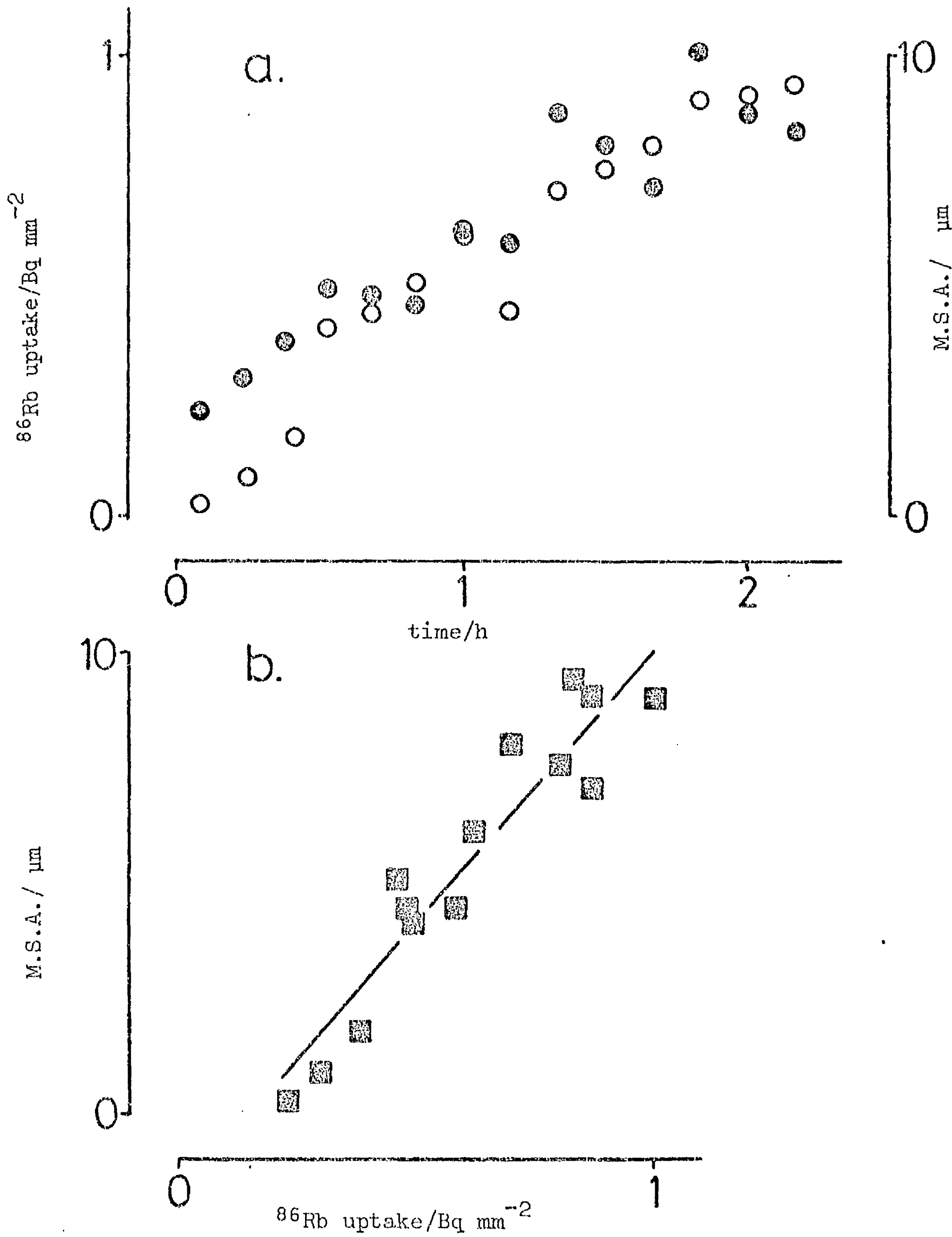
To study uptake of $^{86}Rb^+$ with time, segments were placed in $^{86}Rb^+$/ 100 mol m^{-3} KCl/PIPES buffer (*ca*. 580 Bq mm^{-3}). At various times, a segment was removed from solution and placed on a slide. After stomatal measurements and area estimation had been made, the segments were rinsed for 5 × 1 min in 100 mol m^{-3} KCl/PIPES, submerging on the last rinse. Radioactivity present was then estimated.

As shown in Fig. 31a, both uptake and aperture increased with time over 2 h 10 min, in a similar fashion to that illustrated in Fig.17. When the two parameters 'aperture' and 'uptake' were plotted together (Fig. 31b) a linear relationship with a high coefficient of correlation was found ($r = 0.941$, $P < 0.001$). It thus seemed that these parameters were closely related.

Uptake of $^{86}Rb^+$ was also investigated at different KCl concentrations, keeping the time of incubation at 2 h. The ratio of K^+ to $^{86}Rb^+$ was also maintained as a constant (roughly 3.8×10^{12}atoms per Bq). The tracer content of the solutions therefore varied from 67 - 801 Bq mm^{-3}. Radioactivity taken up would correspond to an equivalent number of K^+ atoms whatever the salt concentration if it was assumed that there was no discrimination of the ions. Ten segments of area 16 mm^2 were incubated at each concentration for 2 h, then rinsed for 5 min in solutions of comparable K^+ and Rb^+ concentrations to those used for incubation. Eight segments were used for radioassay by scintillation spectrometry and 2 were freeze-dried. Stomatal apertures were measured on the freeze-dried tissue which was later used for micro-autoradiography.

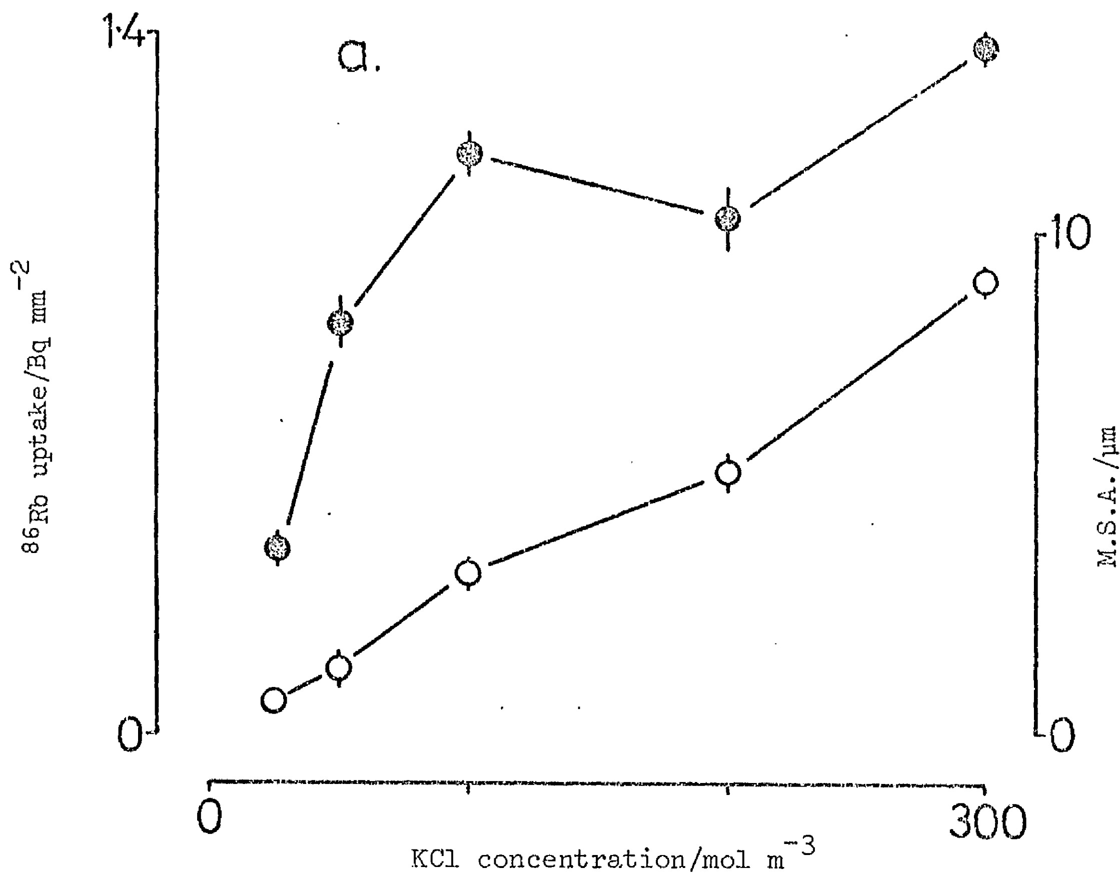
Fig. 32a illustrates the $^{86}Rb^+$ uptake as a function of KCl concentration. Uptake of tracer increased with the salt concentration, but this appeared to be asymptotic at higher levels. This correlated with an increase in stomatal aperture, but the shape of the opening curve was not similar to that of Fig. 15: values were depressed in the middle range of concentrations. When uptake was plotted against aperture (Fig. 32b), the best least-squares fit curve was exponential in form, and not a straight line as in Fig. 31b). This may have been due to an anomaly in aperture estimation. Since freeze-drying would apparently not cause this effect (Fig. 24), it was thought to be due to stomatal closure during the 5 minute rinse given to the tissue prior to freeze-drying. Were this effect taken into account, it would tend to make the best fit of Fig. 32b more linear.

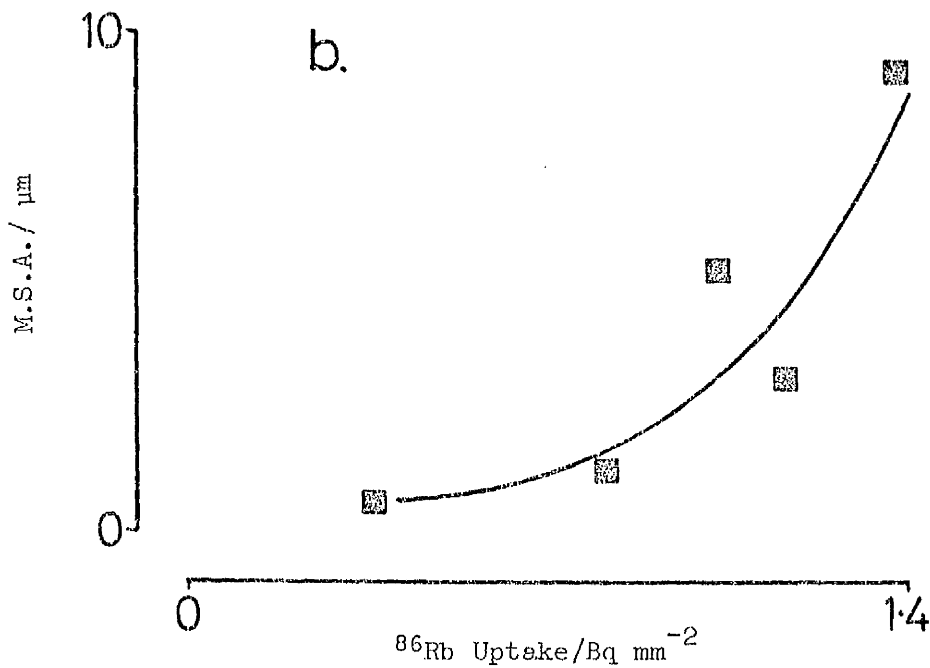
Fig.31. $^{86}Rb^{+}$ Uptake and Stomatal Aperture:
Effect of Length of Incubation



Open circles: stomatal apertures (n=25, s=1)
Closed circles: ^{86}Rb uptake
System 1, R.
Least squares curve for Fig.31b is $y = 11{\cdot}81x - 1{\cdot}82$, $r = 0{\cdot}941$

Fig.32. $^{86}Rb^{+}$ Uptake and Stomatal Aperture: Effect of KCl Concentration.





Open circles: stomatal apertures (n=100, s=4)
Closed circles: ^{86}Rb uptake (n=8)
System 2, N.R.
Least squares curve for Fig.32b is: $y = 0{\cdot}19e^{2{\cdot}74x}$, $r = 0{\cdot}944$.

The effect of killing the epidermis by freeze-thawing on $^{86}Rb^+$ uptake was tested by incubating live and dead tissues on labelled incubation medium (*ca.* 150 Bq mm^{-3}). The results are shown in Table 15.

Table 15. *Uptake of $^{86}Rb^+$ by Untreated and Freeze-thawed Epidermal Segments.*

Treatment	Mean uptake (S.E.)/ m Bq mm^{-2}	n
None	835 (64)	6
Freeze-thawed	16 (2)	6

System 2, R

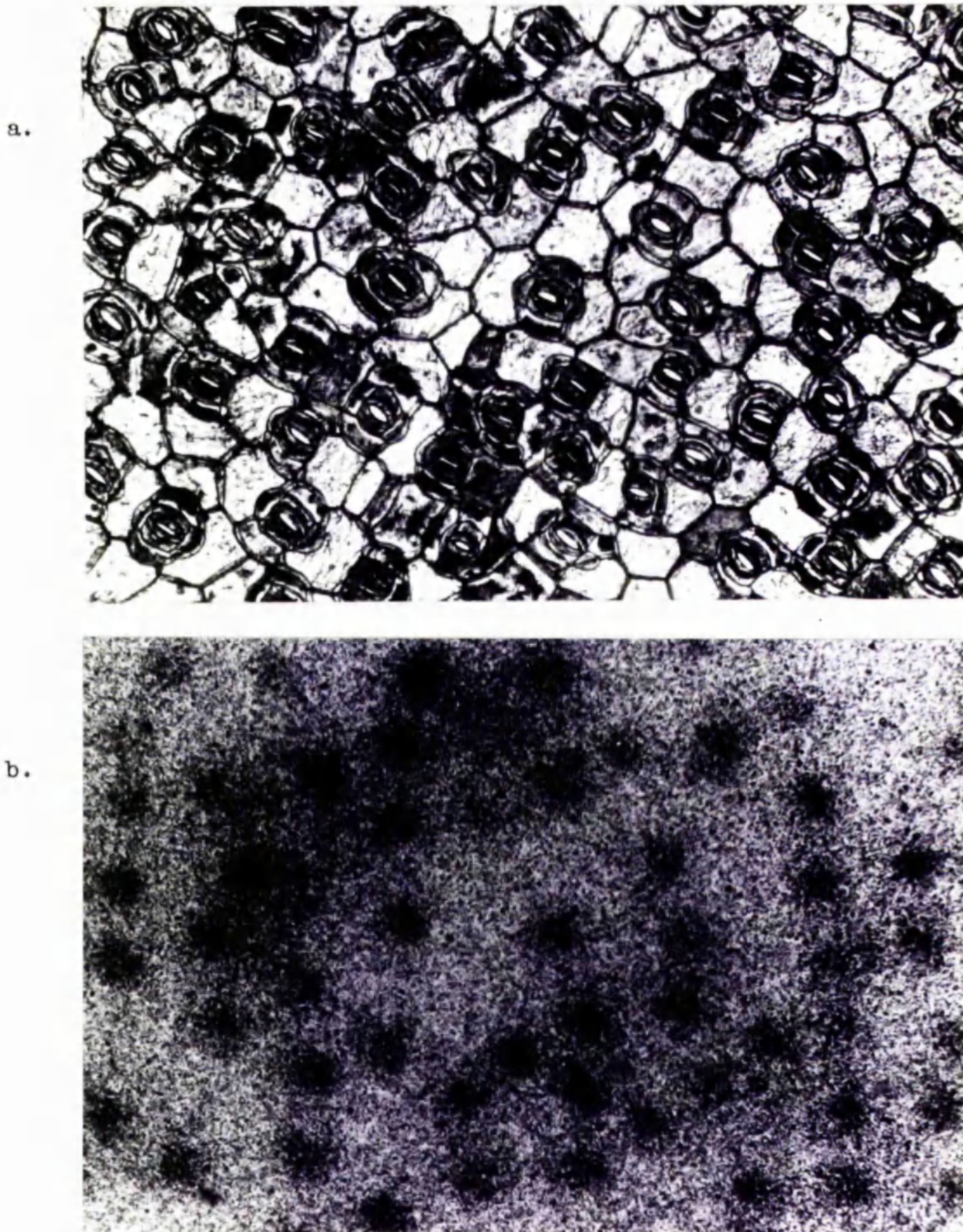
This difference was found to be highly significant ($t = 12.72$ $P < 0.001$) and it was concluded that a large proportion of uptake was a function of the presence of living cells.

Experiment 3.3 *Uptake of $^{86}Rb^+$ by Epidermal Segments II. Location of Radioactivity by Autoradiography.*

The qualitative radioassay of $^{86}Rb^+$ by microautoradiography was carried out using the same method as Experiment 2.4. Typical results are shown in Fig.33, which illustrates the location of $^{86}Rb^+$ after 2 h incubation in 300 mol m^{-3} KCl/PIPES containing *ca.* 800 Bq mm^{-3} $^{86}Rb^+$ (treatment as per Experiment 3.2). It is apparent that the major portion of uptake has been into the guard cell region. Since the β-emmissions of ^{86}Rb are more energetic on average than those of ^{14}C, the volume in which an ^{86}Rb β-particle could potentially cause latent image formation from a point source is larger than that of ^{14}C. This explains why the areas of accumulation appear more diffuse in Fig.33 compared with Fig.28.

Fig. 33. Microautoradiography of Epidermal Segment Supplied with $^{86}Rb^{+}$ in 300 mol m^{-3} KCl/PIPES

a.



b.

a. Appearance of freeze-dried tissue under light microscopy (note open stomata).

b. Corresponding distribution of silver grains in photographic emulsion. Tissue treatment as per Experiment 3. (112 x).

Similar distributions to those of Fig.33 were found at all other KCl concentrations tested (25, 50, 100, and 200 mol m^{-3}). In several examples of ^{86}Rb-autoradiography, a 'hot spot' phenomenon was observed (Fig. 34): areas of high grain density were found which did not apparently coincide with morphological features of the epidermis tissue such as hairs, contaminating mesophyll, or areas of injury due to handling. A possibility is that 'hot spots' represent areas contaminated during incubation and incompletely rinsed. On the other hand, extreme care was taken to avoid this by submerging segments completely during the rinsing procedure. If such hot spots are not artifacts of autoradiography (e.g. spatial), then quantitative estimation of $^{86}Rb^+$ activity present in epidermal tissue would appear to over-represent $^{86}Rb^+$ uptake by cells of the epidermis.

Experiment 3.4 Uptake of $^{86}Rb^+$ by Epidermal Segments III. Effects of ABA.

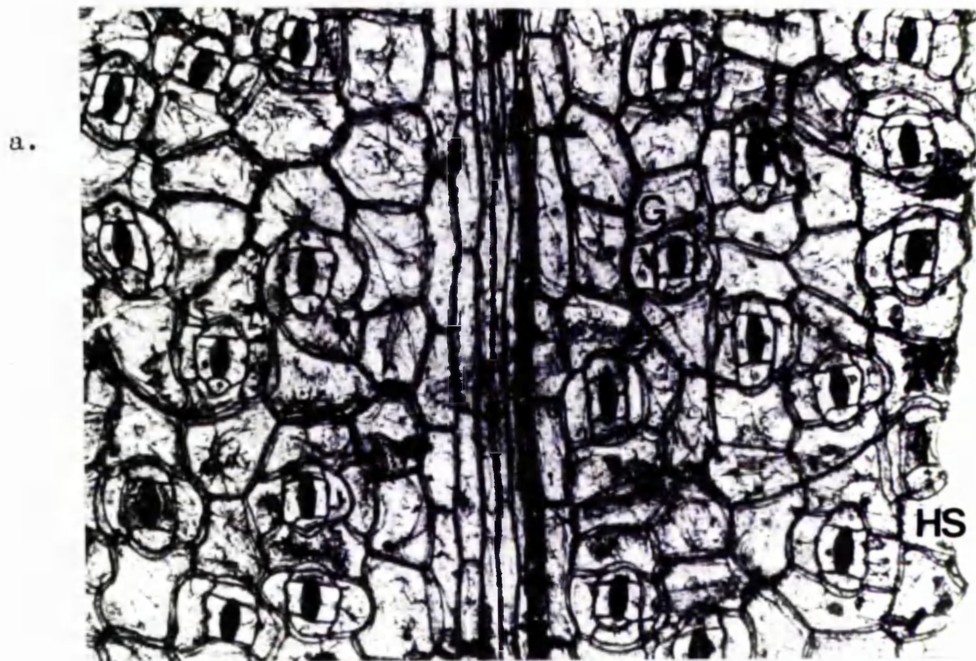
An inhibitory effect of ABA on the uptake of K^+ by guard cells has been postulated as a mechanism of action of the hormone (Hsiao, 1976). This experiment was designed to examine the effects of ABA on $^{86}Rb^+$ uptake by *Commelina* epidermal segments.

Similar methods to the previous two experiments were employed. ABA was incorporated at 0.1 mol m^{-3} in 50 mol m^{-3} KCl/PIPES containing ^{86}RbCl (*ca.* 340 Bq mm^{-3}). Fig.35 illustrates uptake of $^{86}Rb^+$ with and without ABA in the medium. In several repeats of this experiment the same general trend was found: uptake was very similar with and without hormone treatment. However, uptake of ABA-treated segments was consistently slightly lower, especially at the end of the 2 h incubation period. In Fig.35, there was no difference in mean uptake at 5, 40 and 80 min ($|t| < 1.80$, $P > 0.1$) but ABA-treated segments had significantly lower uptake after 120 min ($t = 3.7$, $P < 0.02$).

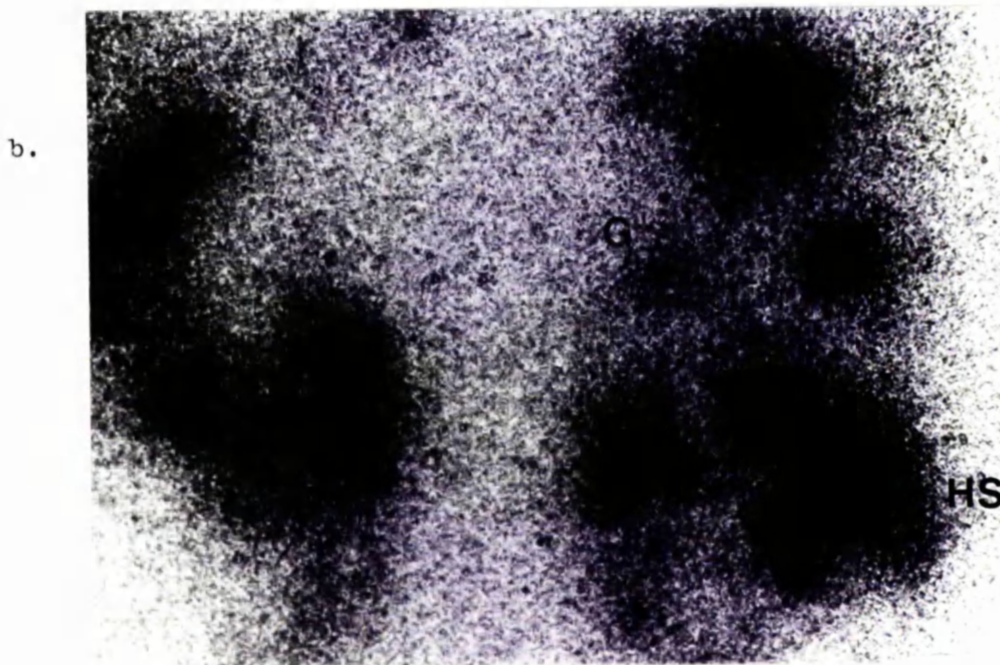
It was not possible to make conclusions about $^{86}Rb^+$ uptake by particular cells of the epidermis from the above data: in order to study differences in the pattern of uptake, the distribution of $^{86}Rb^+$ within the epidermal segment after incubation with and without ABA was examined by autoradiography. Segments were incubated for 2 h 20 min in

Fig. 34. Microautoradiography of Epidermal Segment Supplied with $^{86}Rb^{+}$ in 300 mol m^{-3} KCl/PIPES: Existence of 'Hot spots'.

a.



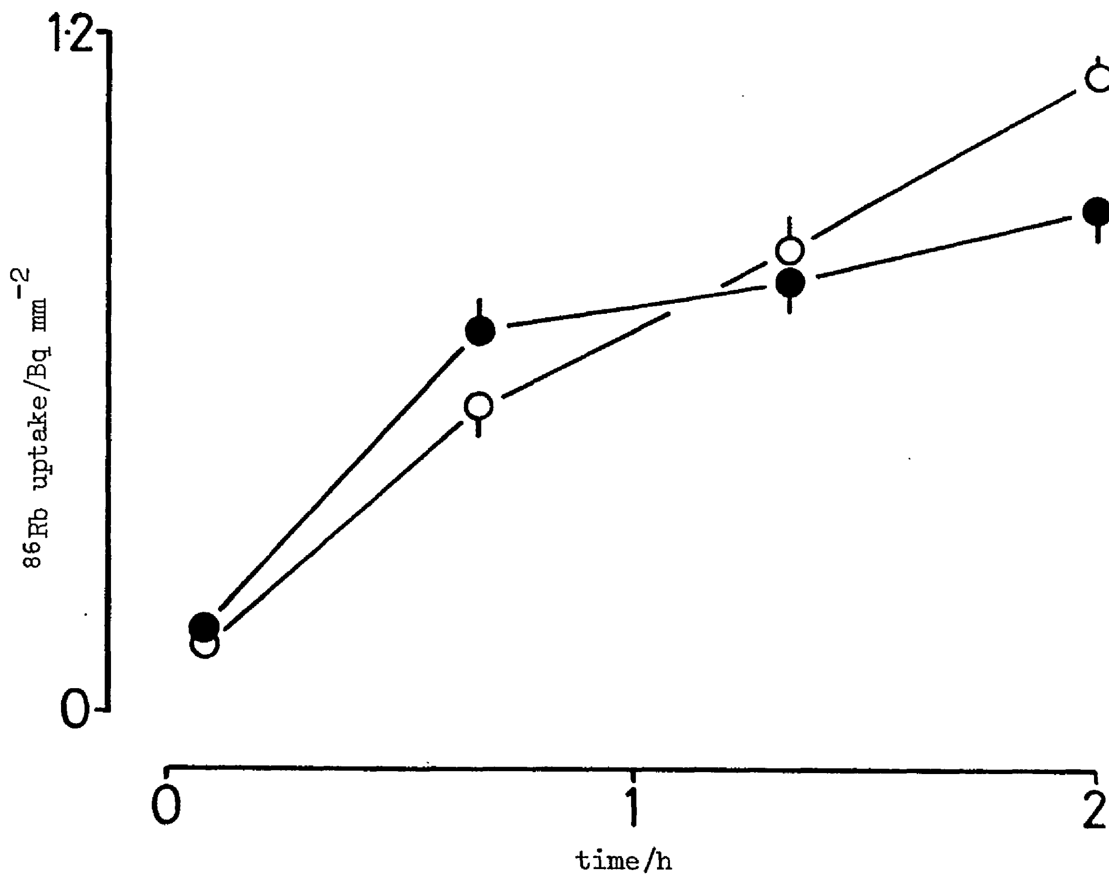
b.



a. Appearance of freeze-dried tissue under light microscopy.
b. Corresponding distribution of silver grains in photographic emulsion.

HS = 'hot spot'; G = Guard cell ^{86}Rb uptake (112 x).

Fig.35. Effect of ABA on ^{86}Rb Uptake by Epidermal Segments I. Net Uptake.



Open circles: 50 mol m^{-3} KCl/PIPES buffer +^{86}Rb Cl.

Closed circles: above buffer plus 0·1 mol m^{-3} ABA

System 1, R, n=6.

50 mol m^{-3} KCl/PIPES containing *ca.* 600 Bq mm^{-3} $^{86}Rb^{+}$. ABA was incorporated at 0.1 mol m^{-3}. Visual observations of microautoradiograms (Fig.36) suggested that accumulation by guard cells was less pronounced in the ABA-treated tissue. To test this hypothesis, silver grain density measurements were made (Table 16).

Table 16. *Grain Densities for Control and ABA-treated Epidermal Segments after $^{86}Rb^{+}$ Uptake*

Treatment	Mean grain density (S.E.) /grains $mm^{-2} \times 10^{-3}$.			mean ratio G/E (S.E.) (Accumulation Ratio)
	over guard cells(G)	over epidermal cells (E)	Total G + E	
Control	515(17)	205(14)	719(29)	2.58 (0.12)
+ ABA	377(11)	277(6)	654(14)	1.37 (0.04)

n = 10 areas of 0.03 mm^2

Silver grain density differences were assumed to represent differences in $^{86}Rb^{+}$ accumulation by the tissue. ABA treatment resulted in a lower grain density for the *total* of the two areas, as would be predicted from Fig.36. In this case, the difference found was not significant with respect to sampling errors ($t = 2.02$, $P > 0.05$). The ABA treatment caused a significant difference in the grain density immediately above guard cells ($t = 6.86$, $P < 0.001$). This was also reflected in the highly significant decrease in the accumulation ratio ($t = 9.97$, $P < 0.001$), suggesting that the hormone affected the uptake pattern for Rb^{+} in favour of the epidermal and subsidiary cells.

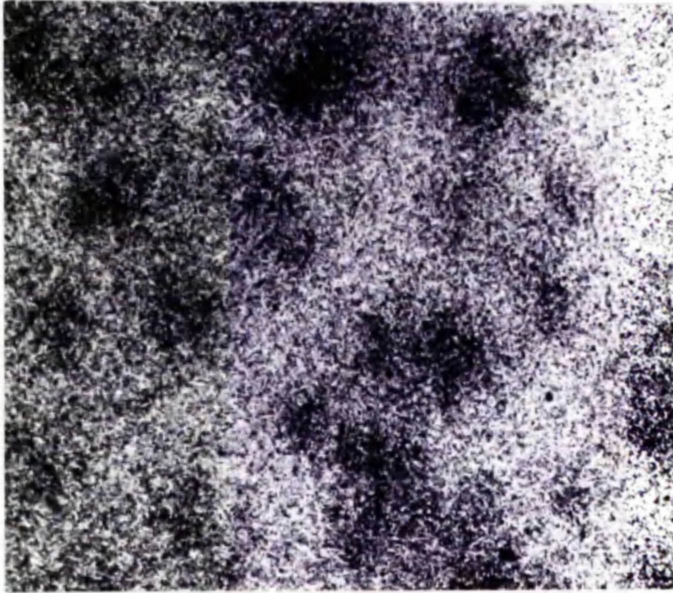
The above experiment, however, requires detailed repetition.

Experiment 3.5 Efflux of $^{86}Rb^{+}$ from Epidermal Segments I. Computer-Assisted analysis.

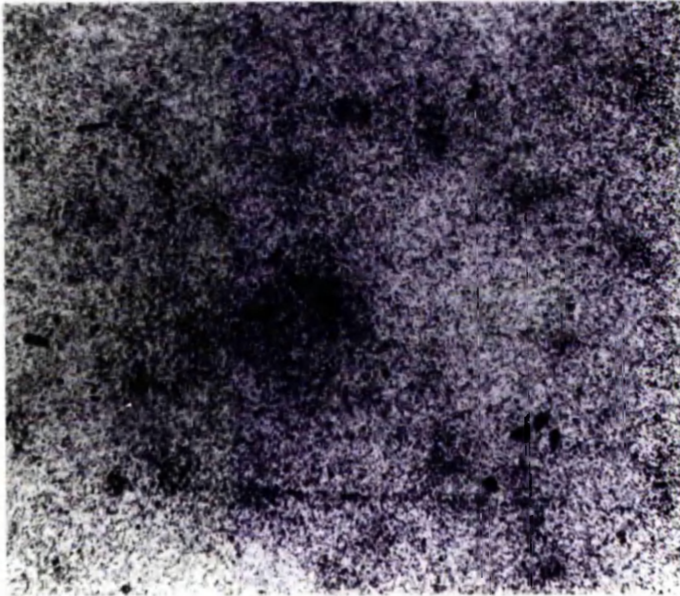
This experiment was performed in a similar manner to Experiment 2.1.

Fig.36. Effect of ABA on ^{86}Rb uptake by Epidermal Segments II. Distribution of Radioactivity.

a.



b.



a. without ABA in buffer
b. with 0·1 mol m^{-3} ABA (112 x)

four segments (total area 73.7 mm^2) were placed in 50 mol m^{-3} KCl/PIPES containing $^{86}Rb^+$ (*ca.* 190 Bq mm^{-3}) for 2 h (System 2) and then transferred through a series of 2 × 10^{-6} m^3 rinse solutions containing RbCl at a similar concentration to the incubation solution. Transfer took place after the following efflux times:- 15 s(x6), 60 s (x3), 300 s (x5), 600 s, and 1200 s. Radioactivity present in the solutions was estimated and the natural logarithm of the calculated efflux rates over the relevant time periods plotted against the mid-time of the rinses. Once again, the computer-assisted least-squares curve-fitting programme was used (see Experiment 2.1). The three-phase fit gave a lower sum of squared residuals than the two phase fit (0.354 *vs.* 2.35) and was accepted. The parameters obtained are given in Table 17.

Table 17. *Least-squares fit parameters for $^{86}Rb^+$ efflux data.*

Phase	a_i/Bq mm^{-2}	S.D.	b_i/s^{-1}	S.D.	$t_{\frac{1}{2}}$/s
i=1	3111700	595300	0.0961	0.0051	7.2
i=2	3872	904	0.00668	0.00122	103.8
i=3	251	58.3	0.000435	0.000124	1593.4

Obviously, direct comparison between efflux of ABA and $^{86}Rb^+$ is not possible. The efflux pattern of $^{86}Rb^+$ would appear to be complicated by two factors not considered in Experiment 2.1: Firstly, the Donnan free space (see Baker & Hall , 1975) will only affect the positive ion, and secondly, effects of stomatal closing reactions caused by the rinsing treatment might affect the $^{86}Rb^+$efflux pattern.

Nevertheless, the fastest efflux phase was again assumed to represent efflux from the free space. It is notable that this had a $t_{\frac{1}{2}}$ equivalent to that of ABA found in Experiment 2.1

Experiment 3.6 Efflux of $^{86}Rb^{+}$ from Epidermal Segments II. Effects of ABA.

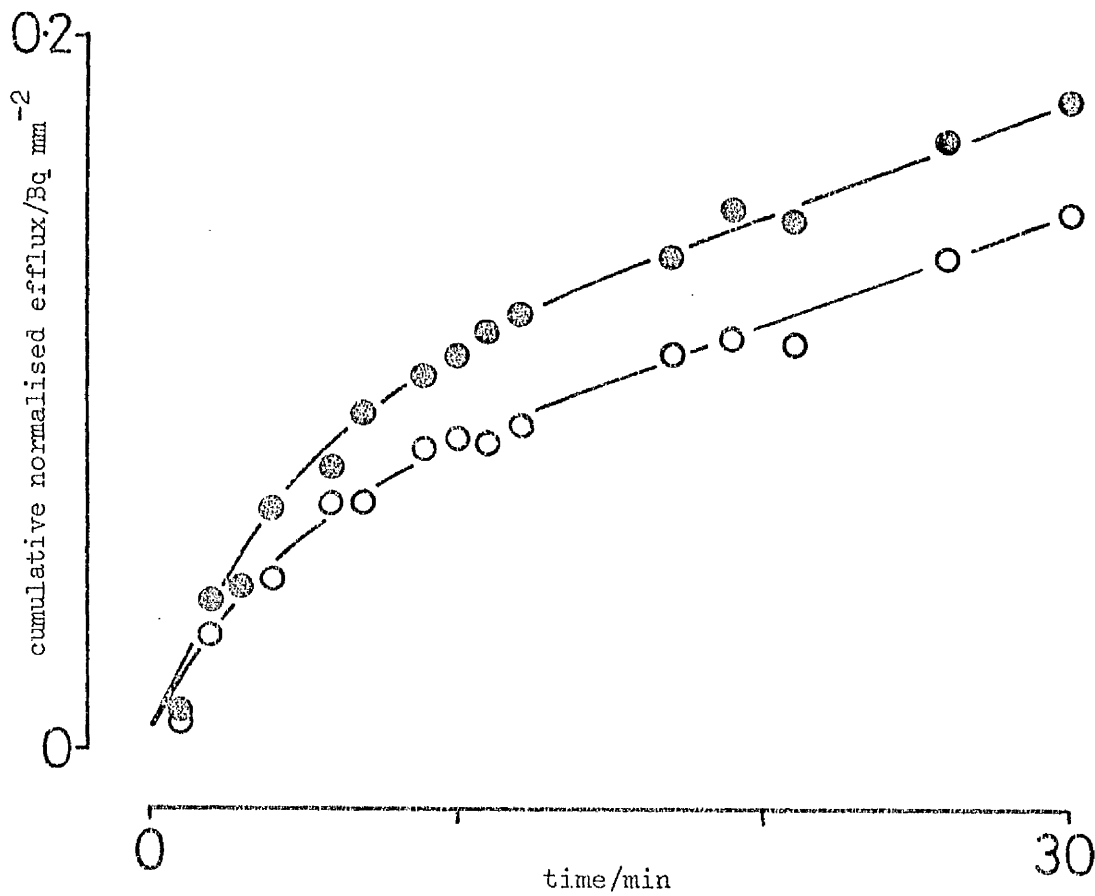
The rapid nature of ABA effects on stomatal aperture has been discussed in Section IV of the General Introduction and shown in Experiment 1.5. It may be argued that alterations in guard cell ion *uptake* phenomena alone cannot account for this speed of reaction. Furthermore, in the general nature of the postulated role of ABA in stress-induced stomatal closure, the hormone would be more likely to initiate closure than prevent opening (although, of course, prevention of stomatal *re-opening* is an important facet). It was therefore of interest to test effects of ABA on $^{86}Rb^{+}$ efflux from 'preloaded' epidermal segments having 'pre-opened' stomata.

Epidermal segments were incubated in 50 mol m^{-3} KCl/PIPES containing $^{86}Rb^{+}$ (*ca.* 460 Bq mm^{-3}) for 2 h (System 1). 15 segments (total area 240 mm^{2}) were then transferred to 10 × 10^{-6} m^{3} solutions of buffer with and without 0.1 mol m^{-3} ABA. 200 mm^{3} samples of these solutions were taken at various time intervals. Incubation System 1 was used in order to ensure mixing of the efflux solution. Cumulative efflux estimates obtained from samples of inaccurate volume due to air intake to the micropipette (a function of speed of operation) were discounted.

Cumulative efflux was calculated using the formula shown in the Materials and Methods. The results were normalised with respect to the label present in each efflux medium at 1 min; from the data of Table 17 this should have represented over 99.7% of the free-space label. The cumulative efflux plot obtained is shown in Fig.37. The best fit curves illustrated are composite: least-squares curves of logarithmic form were found to be inaccurate between 20-30 min. Since the two curves appeared to be parallel and straight from 12-30 min, linear best-fit regression lines were obtained for this portion and logarithmic curves from 1-12 min.

In a cumulative efflux graph like Fig.37, the slope of the curve represents the *rate* of efflux. It thus appears that ABA treatment caused greater rates of $^{86}Rb^{+}$ efflux for approximately the first 10 min.

Fig.37. Cumulative Normalised Efflux of $^{86}Rb^+$ from Epidermal Segments Treated with ABA.



Open circles: control treatment

Closed circles: treated with 0·1 mol m^{-3} ABA

System 1, n=15.

For example, the slope of the best fit curves at 5 min, calculated from derivative = bx^{-1}, were 6.7 mBq mm^{-2} min^{-1} for the control and 9.0 mBq mm^{-2} min^{-1} for ABA-treated segments. However, after 20 min, the respective estimated rates were both 3.2 mBq mm^{-2} min^{-1}, calculated from derivative = b. It is interesting to note that observed increases in $^{86}Rb^{+}$ efflux in the first 10 min correspond with the time taken for ABA-induced stomatal closure after similar treatments not using $^{86}Rb^{+}$, as shown in Fig.18.

Efflux results do not take into account the possibility of solute redistribution within the epidermis during closure. This possibility could be studied with autoradiographic techniques. Indeed, it is possible that the increased efflux demonstrated in this experiment represents an experimental artefact due to such redistribution, if transfer of $^{86}Rb^{+}$ between guard cells and subsidiary and epidermal cells was 'short-circuited' by the presence of the efflux medium.

This experiment was repeated once, with results similar to those shown. Several attempts to design different procedures to test efflux were unsuccessful: although differences between ABA-treated and control efflux rates could be observed, these were found not to be statistically significant with respect to random errors. This would appear to be a function of the low levels of radioactivity involved.

SECTION 4.

EXPERIMENTS ON WHOLE LEAVES

INTRODUCTION

It is well substantiated that ABA affects transpiration rates through stomatal closure when supplied *via* the transpiration stream (Mittelheuser and Van Steveninck, 1969; Raschke, 1975a). The evidence described in Section III of the Introduction suggests that this is a direct effect on the stomatal apparatus rather than an indirect effect as found with kinetin (Meidner, 1967). The results of the previous two sections verified this hypothesis using the epidermal segment incubation system developed in Section 1. However, although stomata of incubated epidermis tissue appeared to react in the same manner as they do on the whole leaf (Willmer and Mansfield, 1969; Experiments 1.5, 1.6 and 1.9), caution must be observed when extrapolating such results. Accordingly, a whole leaf system using *Commelina* was devised in which some of the effects of Section 2 could be re-examined. The advantage of this method over whole-leaf treatments using other plants, lay in the possibility of examining epidermal ABA levels by supplying radioactive hormone and taking epidermal peels.

Since there was no evidence in the literature that ABA in the transpiration stream reaches the stomatal apparatus or even the epidermis, this was examined. By utilising the freeze-drying process to 'fix' stomatal apertures (Experiment 1.10) it was also possible to obtain an 'improved' estimate of stomatal sensitivity to ABA based on the assumption that only hormone present in the epidermis affected stomatal movements.

RESULTS AND DISCUSSION

Experiment 4.1 Development of a Whole-leaf Experimental System.

As previously noted, it is necessary first to obtain consistently open stomata before examining the effects of ABA on closure. Preliminary

experiments on whole leaves were carried out in which leaves were pretreated by floating on water through which CO_2-free air was bubbled. Treated in this way, stomata opened evenly to about 10 µm after 2 h. However, after removal from the water, drying, and exposure to air, the stomata of control leaves closed rapidly during the experiment (within 45 min). ABA treatment caused faster closure (complete within 15 min), but it was, nevertheless, thought that this system was unsatisfactory.

Besides low CO_2 levels, another easily-manipulated environmental parameter which can result in stomatal opening, is the relative humidity of the atmosphere (Sheriff, 1977). Consequently, a test was made on the stomatal apertures of *Commelina* leaves placed in Perspex 'cells'(Fig.6) in vials of distilled water, and fed with a stream of air of high humidity. Fig.38 illustrates the results obtained with up to 3 h incubation. The stomatal movements were similar to those which occurred in epidermal segments incubated on 50 mol m^{-3} KCl/PIPES (Fig.17). Since apertures were relatively constant between 2-3 h, it was decided to carry out a 2 h pre-incubation to open stomata of leaves used in further experiments.

Stomatal opening was also found to occur if CO_2-free air was supplied instead of normal air. However, the apertures attained after 2-3 h were generally greater at around 11-12 µm.

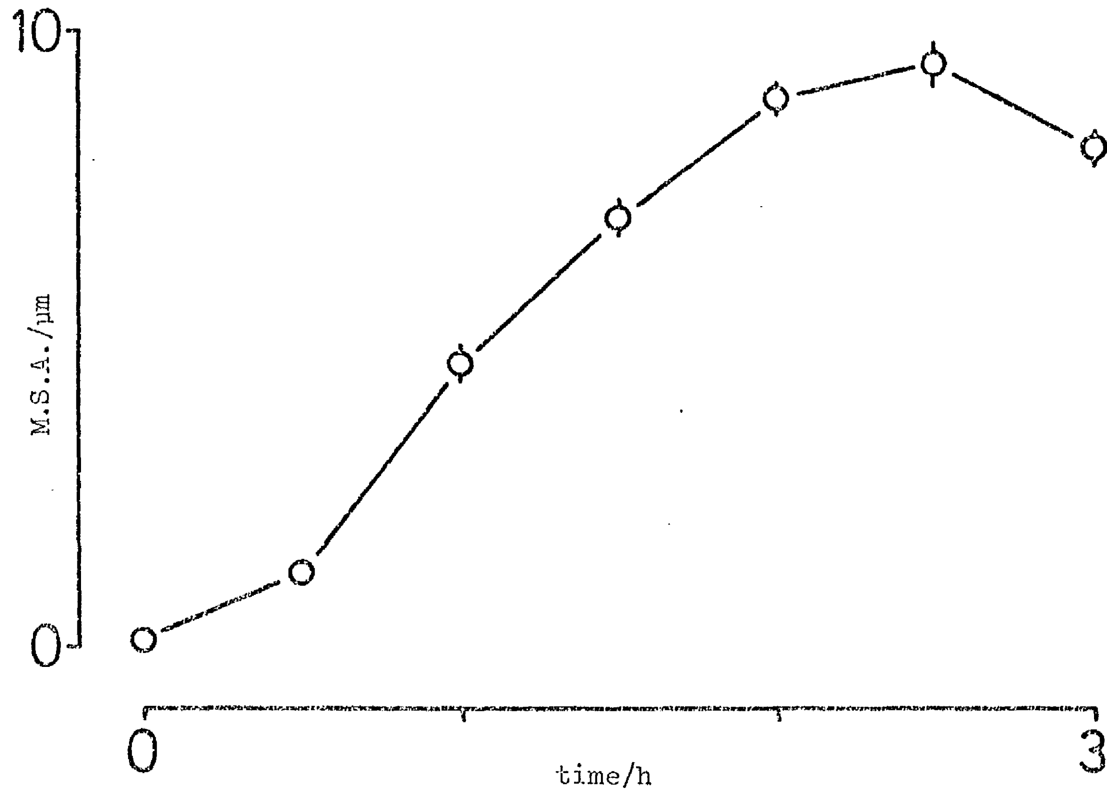
Experiment 4.2 Effect of ABA on Stomatal Aperture of Whole Leaves at Different CO_2 Concentrations.

In this experiment effects of ABA at 0.1 and 0.01 mol m^{-3} on stomatal aperture were examined with and without CO_2.

After 2 h preincubation using the relevant gas supply, leaves were rapidly transferred to another vial containing distilled water (controls) or ABA solution. Figs. 39a and b show the effects of this treatment over the following hour.

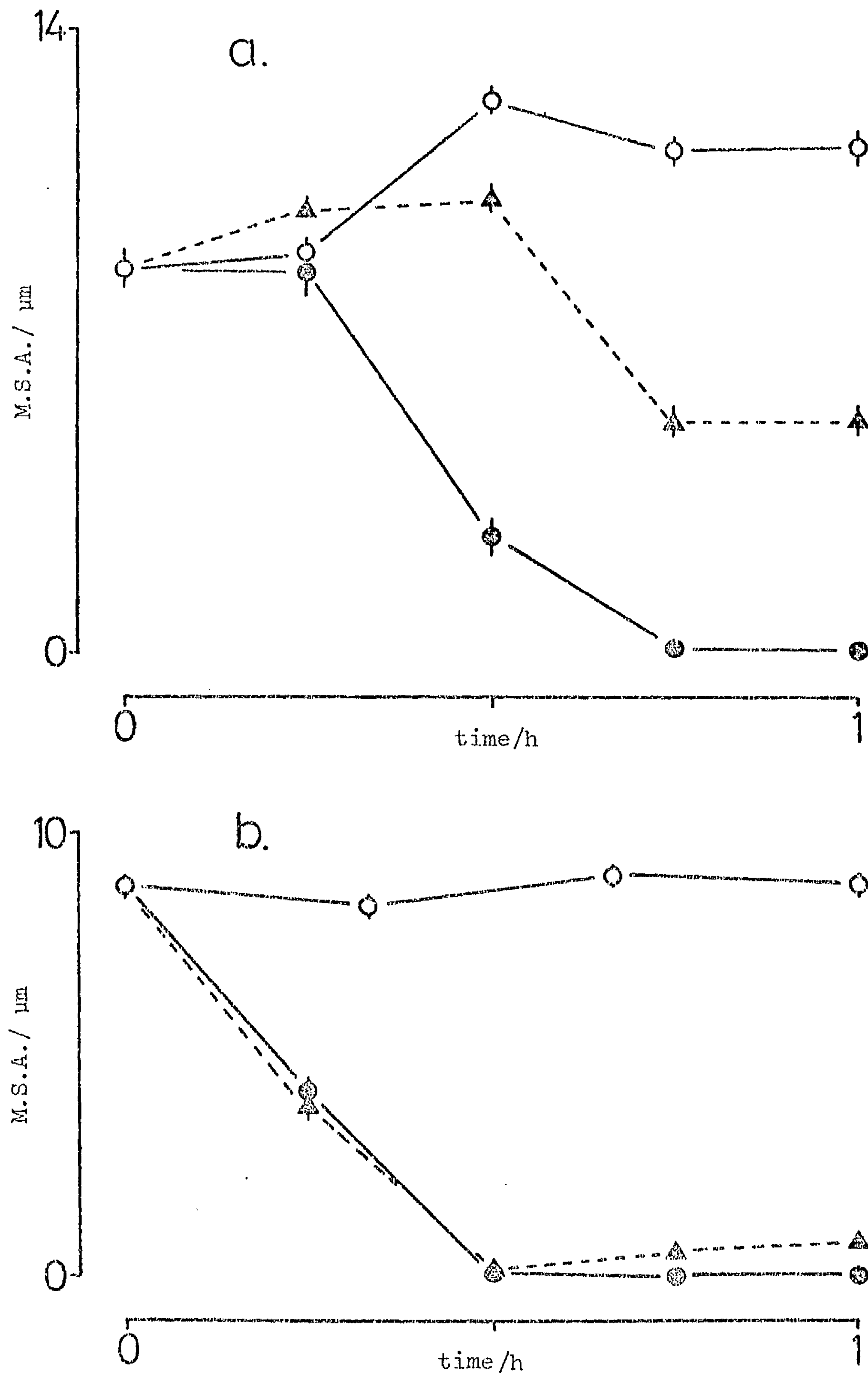
When normal air (*ca.* 300 vpm CO_2) was used (Fig. 39b), the two ABA concentrations had almost identical effects, although slight re-opening at the lower level was noted after 45 min. With CO_2-free air (< 1 vpm CO_2), however, only 0.1 mol m^{-3} ABA caused complete closure

Fig. 38. Stomatal Apertures Attained on Whole Leaves in Humid Air.



n = 50, s = 1.

Fig.39. Effect of ABA on Stomatal Aperture at Different CO_2 Concentrations.



a. CO_2-free air, n = 50, s = 1.
Open circles: distilled water control
Closed triangles: 0·01 mol m^{-3} ABA.
Closed circles: 0·1 mol m^{-3} ABA

b. Normal air, n = 50, s = 1.
Symbols as in a.

Results shown in a and b were obtained in separate experiments.

after 1 h. 0.01 mol m^{-3} ABA resulted in roughly 50% closure at this time. Furthermore, the effects of 0.1 mol m^{-3} ABA were delayed by approximately 15 min when CO_2-free air was used. Such a delay was also found by Mansfield (1976a) in *Xanthium*.

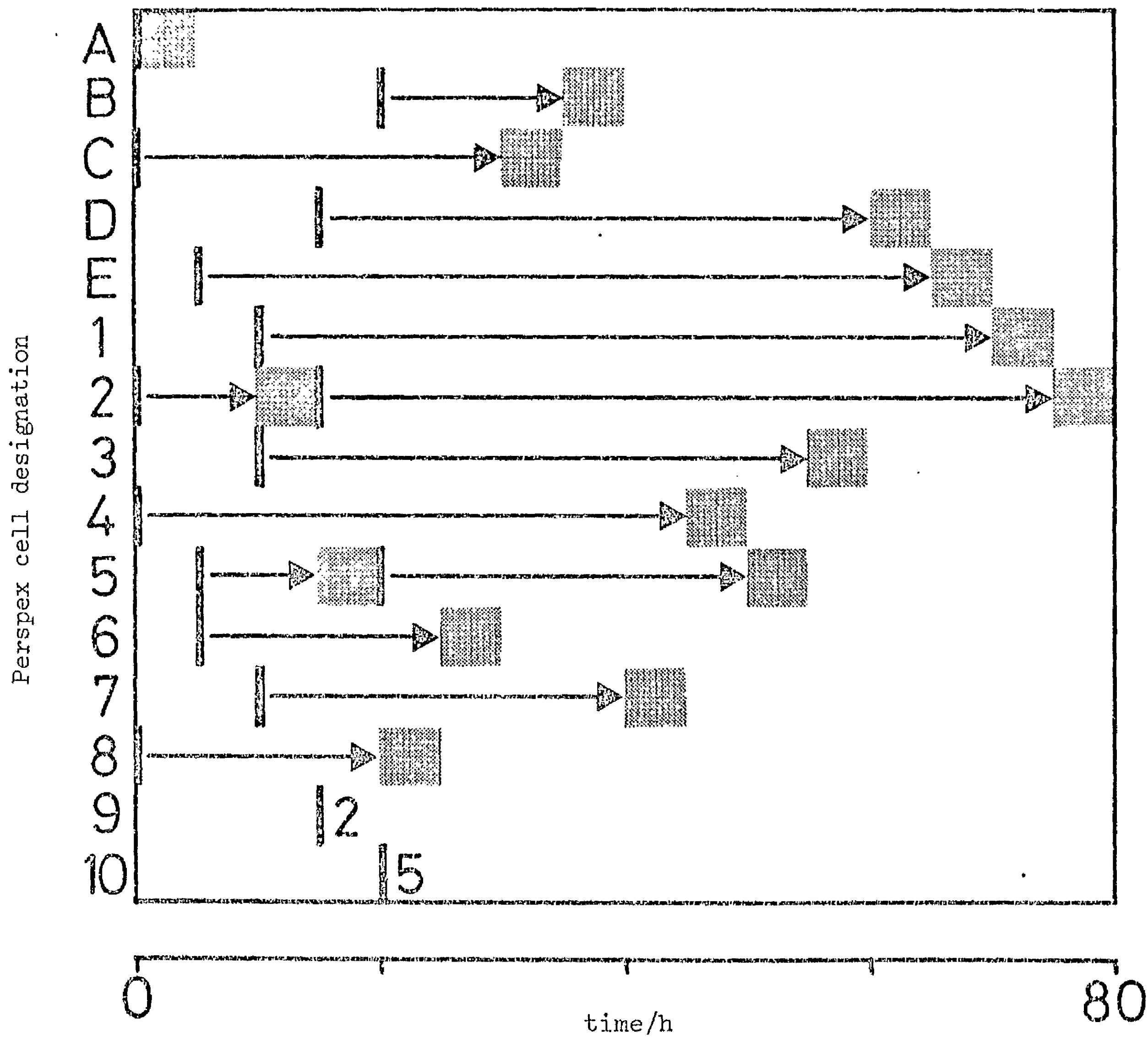
To investigate further ABA effects using 2-^{14}C-ABA, it was decided to use normal air, since results obtained with this presumably had more relevance to the normal environment of the plant. This treatment would also permit analysis of ABA effects of low concentrations.

Experiment 4.3 Effects of 2-^{14}C-ABA Applied to Whole Leaves I. Use of Different Treatment Times.

This experiment was basically similar to that shown in Fig.39b, with two major modifications. Firstly, 2-^{14}C-ABA at 0.1 mol m^{-3} was employed instead of unlabelled compound, and secondly, a leaf harvesting procedure was adopted so that both stomatal aperture could be measured and epidermal and mesophyll samples could be assayed for radioactivity. In these results, 'epidermis' refers to the abaxial epidermis alone and 'mesophyll' to the mesophyll layers *plus* the adaxial epidermis.

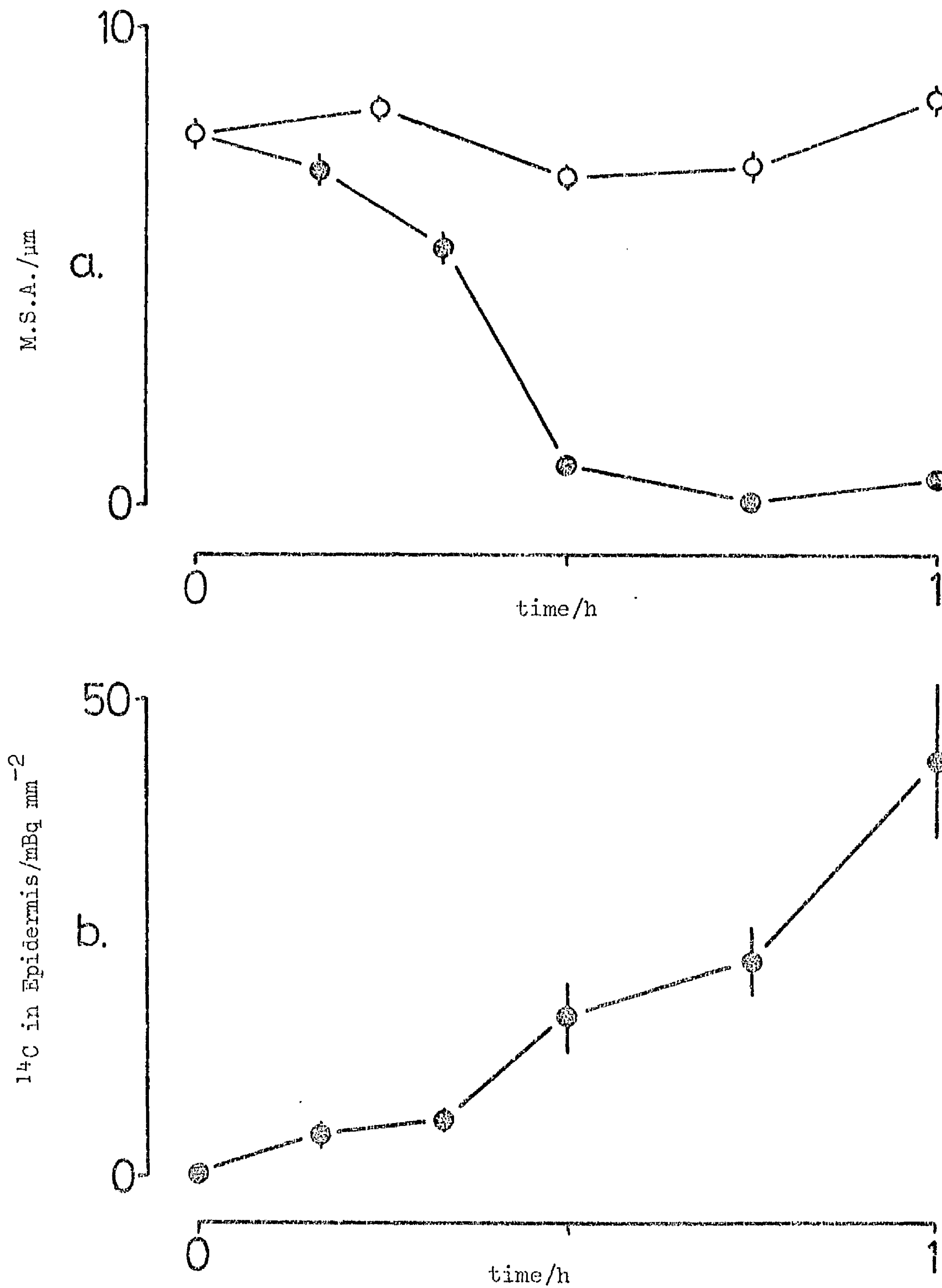
The treatment protocol used is illustrated in Fig.40. A-E and 1-10 represent the perspex cells, in each of which a leaf could be pre-incubated and treated. Pre-incubations (2 h) of individual leaves were staggered to correspond to the treatment starting times represented by heavy vertical lines. Treatments lasted for the time indicated by the length of the arrow. Peeling manipulations and freeze-drying of epidermal samples were carried out during 5 min (shaded squares). Cells A-E contained control leaves (1 leaf each of 0, 15, 30, 45 and 60 min), and 1-8 were used for treatment with radioactive solution (2 leaves each of 10, 20, 30, 45, 60 min). This procedure was carried out twice on consecutive days (t_o = 1200 h) and the results combined (Fig.41, Table 18).

Fig. 40. Treatment Protocol for Experiment 4·3.



For Explanation, see text.

Fig.41. Effects of 2-^{14}C-ABA on Stomatal Aperture; ^{14}C Accumulation in Epidermis (I).



a. Open circles: control (distilled water)
Closed circles: 0·1 mol m^{-3} 2-^{14}C-ABA
n = 100 (controls), 150-200 (ABA)
s = 2 (controls), 3-4 (ABA)

b. For details, see Table 18.

Table 18: ^{14}C *Partitioning in Commelina Leaves following 2-^{14}C-ABA Application.*

Treatment time/ min	Total area sampled/ mm^2	No. of Samples	Mean ^{14}C activity (S.E.)/ $mBq\ mm^{-2}$			^{14}C in epidermis % total
			Epidermis	Mesophyll	Total	
10	950	14	4.4 (1.2)	75.1(17.5)	79.5	5.5
20	900	14	6.0 (1.2)	104.8(20.2)	110.8	5.4
30	1075	15	15.6 (3.8)	201.5(32.0)	217.2	7.2
45	950	16	22.2 (3.8)	247.5(35.7)	269.7	8.2
60	1175	16	43.7 (8.0)	299.1(25.5)	342.8	12.8

Fig. 41a again demonstrated the effects of ABA in normal air, full closure occurring after 30 min as in Fig.39b. Fig. 41b shows that radioactivity was found in the lower epidermis before significant closure had occurred, indicating that ABA did reach the epidermal cells before reaction.

Since the specific activity of the ABA was known (1.328 TBq mol^{-1}) it was possible to calculate an estimate of stomatal sensitivity based on these results. Because closure was complete (i.e. below 1 µm) after 30 min, values of ^{14}C levels at this time were used. The stomatal density was assumed to be 50 per mm^2 . Using the *whole leaf* ^{14}C content, the estimated stomatal sensitivity was 163 fmol per mm^2 lamina. This is a value compatible with other estimates of stomatal sensitivity. Raschke (1975b) calculated that 90-180 fmol per mm^2 leaf area was required to give 5% stomatal closure in *Commelina*, while Kriedemann *et al.*, (1972) estimated that 89-349 fmol of ABA per mm^2 leaf area was required to initiate closure in French beans, rose, and *Zea* leaves.

If, however, the *epidermal* ^{14}C content was used, the estimate was lower, since only 7.2% of the whole leaf radioactivity was present in the epidermis at 30 min (Table 18). Thus, in this experiment, the stomata had closed after receiving *no more than* 236 amol per stomatal complex. Multiplying by Avogadro's number, this is equivalent to

ca. 142 million molecules per complex. It should be emphasised that such an estimate of stomatal sensitivity to ABA is an *upper limit estimate*. Clearly, not all the label present in the epidermis would be expected to be at active sites: Some may have been present in the free space; some may have been metabolised to non-active forms. However, in the absence of accurate quantification of these factors adjustment of the estimate cannot be justified.

The proportion of the total leaf radioactivity present in epidermis rose during the course of the experiment from 5.5% to 12.8% (Table 18). It was not clear whether this was a function of the method of hormone presentation or whether it represented accumulation by the epidermis.

TLC of methanol extracts of leaves fed $2\text{-}^{14}C$-ABA for 1 h (Methods and Solvent Systems as Fig.27) gave a single peak of radioactivity (always greater than 90% of the total) which co-chromatographed with standard ABA (Results not shown).

Experiment 4.4 Effects of $2\text{-}^{14}C$-ABA Applied to Whole Leaves II. Use of Different Concentrations of ABA.

In this experiment, it was intended to further investigate stomatal sensitivity to ABA. Since 0.01 mol m^{-3} ABA had caused full closure (Fig. 39b), in theory, it should have been possible to obtain stomatal sensitivities an order of magnitude lower than that obtained in Experiment 4.3 using 0.1 mol m^{-3} ABA. The time of treatment was therefore kept constant but the *concentration* of $2\text{-}^{14}C$-ABA supplied altered.

The time of treatment was maintained at 40 min to ensure maximal closure at low ABA concentrations. Leaves were treated with from 5×10^{-4} mol m^{-3} to 10^{-1} (0.1) mol m^{-3} $2\text{-}^{14}C$-ABA. Fifteen leaves were detached at 5 min intervals and preincubated for 2 h. The order of treatments was as follows (C = control leaf; concentrations in mol m^{-3}): C1, 5×10^{-4}, 10^{-3}, 5×10^{-3}, 10^{-2}, 5×10^{-2}, 10^{-1}, C2 5×10^{-4}, 10^{-3}, 5×10^{-3}, 10^{-2}, 5×10^{-2}, 10^{-1}, C3. At the time of peeling samples of epidermis were freeze-dried to fix stomatal apertures.

Epidermal samples were kept as large as possible (i.e. 150 mm^2) at the lower hormone concentrations in order to maximise the amount of radioactivity present per sample. The procedure was carried out twice on consecutive days and the results pooled.

Fig.42 shows the ^{14}C-activity present in the epidermis and stomatal aperture as a function of concentration.

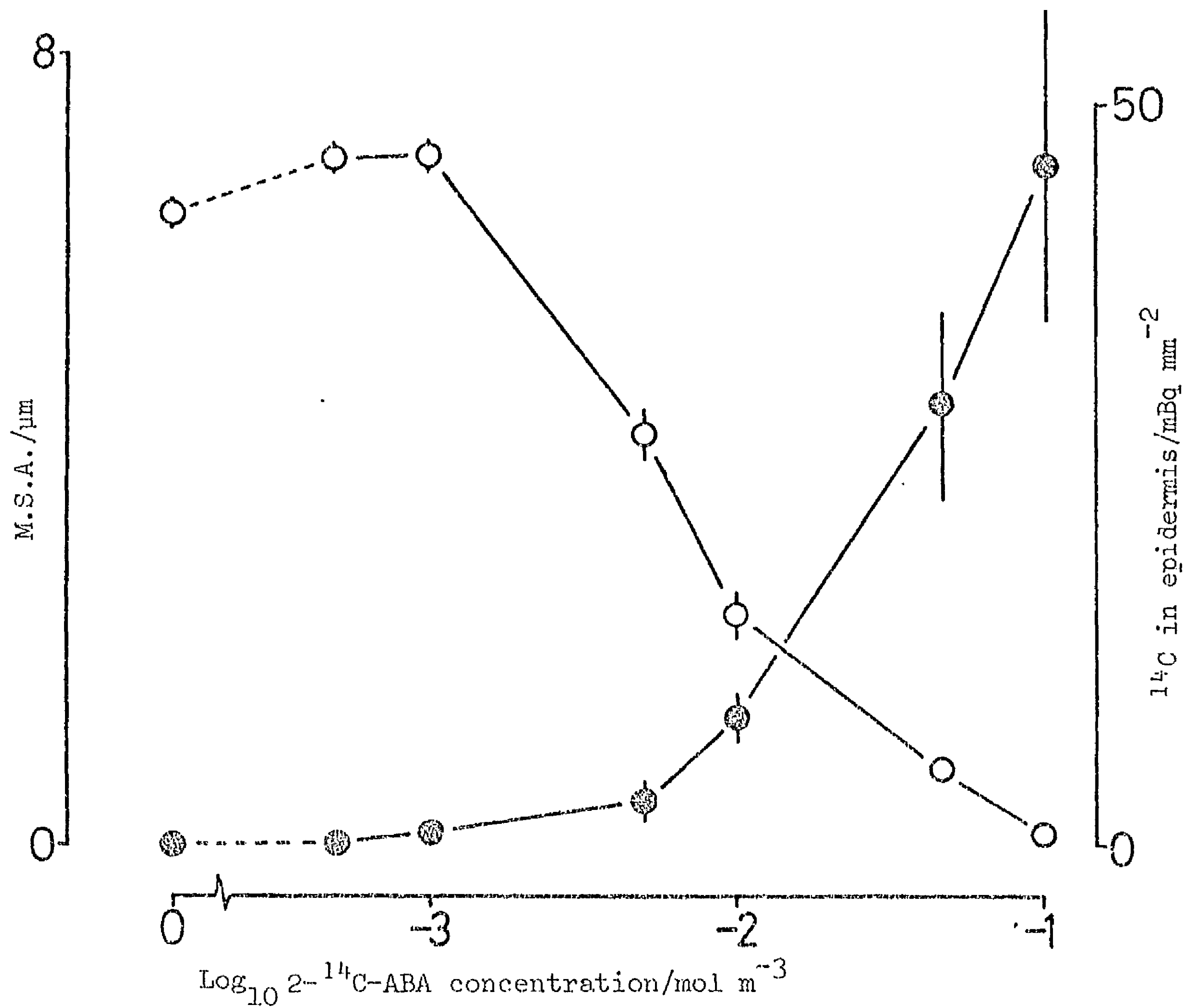
At the ABA concentrations employed, stomatal closure below 1 μm was only obtained at 0.1 mol m^{-3} and 5×10^{-2} mol m^{-3}. The upper limit estimates of stomatal sensitivity, calculated by methods similar to that used in Experiment 4.3 were 401 and 695 amol ABA per complex respectively (Table 19). These values are higher than that obtained in the previous experiment. The differences may have been due to the increase in treatment time considered (ie. 40 min *vs*.30) or to variability of plant material and experimental conditions. The latter possibilities were supported by the mean stomatal aperture obtained with 10^{-2} mol m^{-3} ABA (0.01 mol m^{-3}) which was higher than those found in Experiments 4.2 and 4.3 at similar treatment times.

The data of this experiment can be used to estimate the minimum amount of ABA required to cause a statistically significant alteration in stomatal aperture, a calculation not justified in the previous experiment due to the non-equivalence of treatment times. Since 5×10^{-4} and 10^{-3} mol m^{-3} ABA obviously had no effect on aperture, values after 40 min being, in fact, slightly higher than that of the control leaves, the figures obtained at 5×10^{-3} mol m^{-3} were tested. At this concentration, the difference between the mean stomatal aperture obtained and the controls was highly significant ($t = 8.10$, $P < 0.001$) and the amount of ^{14}C-activity present in the epidermis was equivalent to 45.4 amol per complex (Table 19). It thus appeared that the minimum amount of ABA required to cause significant reduction in stomatal aperture, in this system and given this treatment, lay between 12.6 and 45.4 amol ABA (7.6 - 23.3 million molecules) per stomatal complex.

The proportion of total leaf radioactivity present in the epidermis was, on average, 12.7%, a figure similar to that found after 60 min in Experiment 4.3.

A novel observation was made on freeze-dried tissue exposed to ABA concentrations which caused intermediate stomatal closure after 40 min

Fig.42. Effects of 2-^{14}C-ABA on Stomatal Apertures; ^{14}C Accumulation in Epidermis (II).



Open circles: stomatal apertures (n=200, s=4 except control where n = 300, s = 6)

Closed circles: ^{14}C in epidermis (see Table 19 for details)

Table 19 ^{14}*C-partitioning in Commelina Leaves after 2-*^{14}*C-ABA Treatment*

$2-^{14}C$-ABA concentration /mol m^{-3}	No. of samples taken	Total area sampled /mm^2	Mean ^{14}C activity (S.E.) mBq mm^{-}		ABA present in epidermis /amol $complex^{-1}$*	^{14}C in epidermis /% total
			Epidermis	Mesophyll		
5×10^{-4}	11	1225	0.2(0.2)	1.7(0.5)	2.5	9.1
10^{-3}	10	1300	0.8(0.3)	5.0(1.2)	12.6	14.3
5×10^{-3}	7	1000	3.0(1.3)	21.3(7.2)	45.4	12.3
10^{-2}	9	950	8.7(1.7)	58.2(10.8)	131.0	13.0
5×10^{-2}	12	1150	26.5(6.7)	173.7(39.0)	401	13.2
10^{-1}	11	900	46.0(10.3)	273.3(54.8)	695	14.4

*Assuming ^{14}C is 100% ABA and based on 50 stomata mm^{-2}.

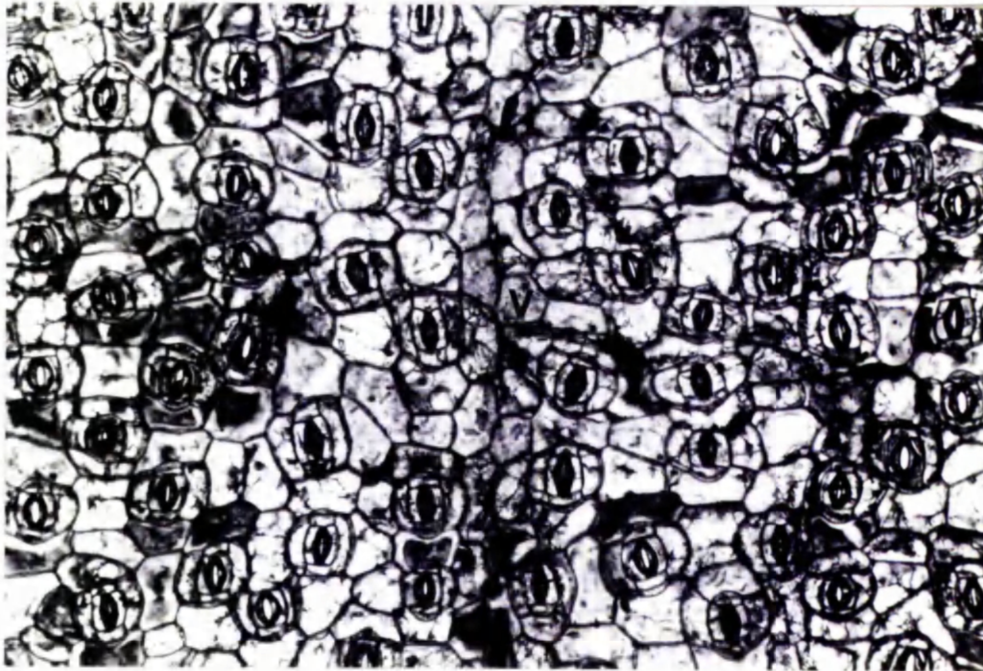
(10^{-2} and 5×10^{-2} mol m^{-3}). Fig.43 illustrates that this partial closure was not uniform throughout the epidermis. Rather, there was a gradient of stomatal opening depending on distance from the vascular tissue. This could have reflected the existence of a concentration gradient of ABA within the epidermis, assuming that stomatal closure reflected the presence of the hormone at the stomatal complex. Such a gradient would presumably reflect the transport pathway of compounds in the transpiration stream within the epidermis. In tissue treated with 0.1 mol m^{-3} ABA for the same time, closure was complete and even, an observation which reduces the alternative possibility of a distinct solute 'front' within the epidermis. Of course, the ABA treatment itself may alter the path of solutes within leaf tissues because of localised reductions in transpiration rate.

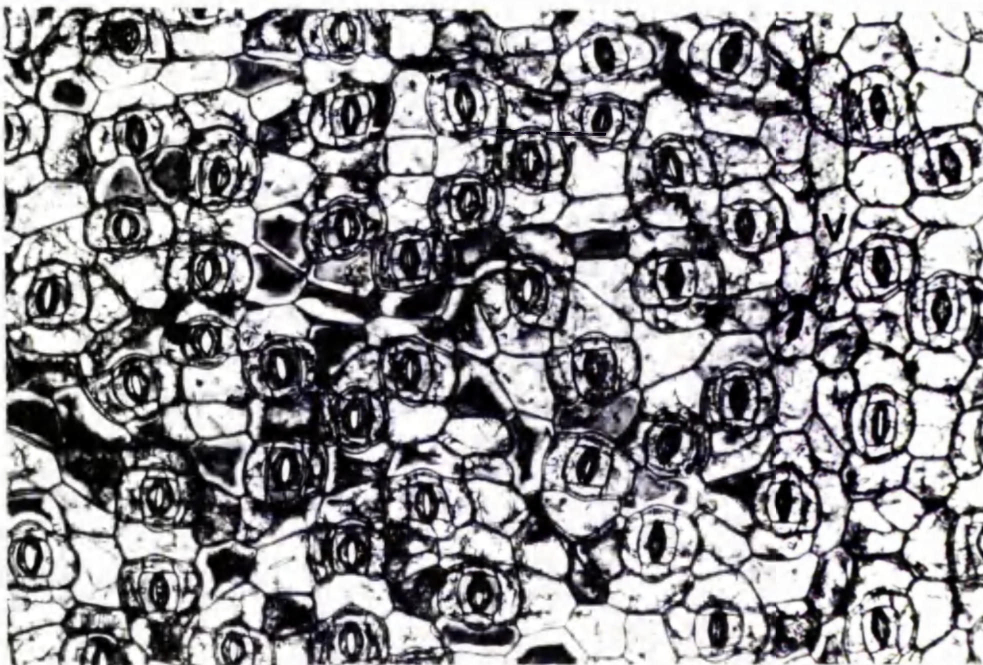
Experiment 4.5 Effects of 2-^{14}C-ABA Applied to Whole Leaves III. Microautoradiography of Epidermal Tissue.

Notwithstanding the low amounts of radioactivity present in the epidermis after the treatments of Experiments 4.3 and 4.4, the tissue freeze-dried for stomatal aperture fixation was subjected to microautoradiography using the methods of Experiment 2.4. Epidermal material from the 0.1 mol m^{-3} treatment of Experiment 4.4, having mean ^{14}C-activity of 46 mBq mm^{-2}, was used. The calculated exposure time (see Materials and Methods) was therefore approximately 100 d. In practice, this was increased to 125 d to allow for variability in ^{14}C contents of tissues and the results of latent image fading.

Fig.44 demonstrates the distribution of radioactivity within the epidermis after 2-^{14}C-ABA treatment: a degree of accumulation at the stomatal complex is apparent, similar to that found in Experiment 2.4. This result therefore shows that ^{14}C from ABA can be accumulated within the stomatal complex of *Commelina* in both epidermal segment and whole leaf application systems.

Fig. 43. Stomatal Apertures on Tissue Treated with 0·01 mol m^{-3} ABA.



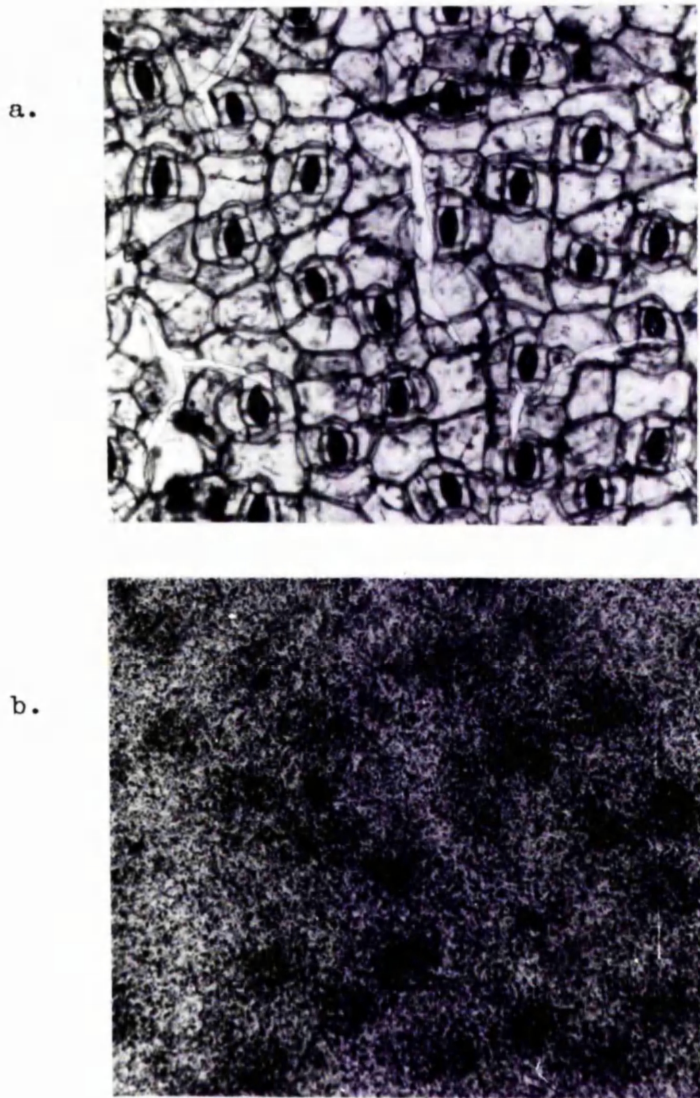


Two regions from freeze-dried epidermis tissue taken from leaves supplied with 0·01 mol m^{-3} ABA *via* the transpiration stream. Note closed stomata near to areas above vascular tissue (V), and increase in aperture according to distance away from this area; (112 x)

Fig. 44. Microautoradiographs of Epidermis from Leaves Supplied with 2-^{14}C-ABA.

a.

b.



a. Appearance of freeze-dried tissue under light miscroscopy.
b. Distribution of silver grains in the photographic emulsion. The leaf was fed 0·1 mol m^{-3} 2-^{14}C-ABA for 40 min; (112 x)

SECTION 5

ENDOGENOUS ABA IN COMMELINA

INTRODUCTION

Methods of quantification of ABA were discussed in the General Introduction, and it was clear from the evidence that the use of GLC with electron capture detection conferred several advantages. Methods of hormone purification were also generally reviewed. For this study, it was decided that to make efficient use of the sensitivity and selectivity of ECD, development of purification methods was required. The ideal sequence of purification steps had to be rapid, and had to adequately remove potentially interfering compounds in the original extract. It would also allow accurate measurement of purification losses and ABA breakdown during the procedure.

The first few experiments in this Section therefore describe results obtained in the development of the techniques eventually used to quantify ABA levels in *Commelina* tissue (Fig.8). In the introduction to this Section, the historical and theoretical background to the methods used will be discussed.

One of the first methodological problems encountered in the accurate quantification of any compound in plant material occurs in harvesting, where it is desirable to halt effectively all enzymatic processes. The extraction solvent itself is frequently used for this purpose (Harborne, 1973), but may take some time to penetrate the plant tissues. In this investigation, freeze-drying was used to obviate this problem by first immobilising and then removing the water present in the tissue. Extraction was then carried out in the normal manner, using methanol.

Subsequent purification of extracts has generally involved partition techniques and the use of adsorption and ion-exchange chromatography (Saunders, 1978), with the object of removing as much dry weight as possible whilst optimising ABA retention. Being a weak acid, the hormone is less dissociated at low pH values and therefore generally favours the organic phase in partition systems. Ciha *et al.*(1977) reported several partition coefficients for ABA, but they did not consider

the concurrent partition of dry weight. Thus, a particular set of solvent conditions may result in high transfer of ABA between solvents, but also of interfering compounds, resulting in no real benefit from the procedure. An experiment was carried out to investigate this aspect of the purification method.

Polyvinylpyrollidone (PVP) columns have been used to remove pigments and phenolic compounds from plant hormone extracts. Glen *et al.*, (1972) described the elution patterns of IAA, ABA and some gibberellins using this material, and reported high recovery of standards whilst reducing dry weight by up to 60-fold. Diethylaminoethyl cellulose (DE) also removes a high proportion of dry weight, particularly pigmented material, by ion exchange chromatography. Again, high plant hormone recoveries have been reported (McDougall and Hillman, 1978). Tests of column efficiency and elution characteristics were carried out on both these columns.

A critical aspect of quantification of phytochemicals is the assay of the losses of compound which inevitably occur during purification. Ideally, any purification standard used should be as chemically identical to the compound of interest as possible (Reeve and Crozier, in press). Although *t*-ABA has been used for this purpose (Lenton *et al.*, 1971) great care must be taken that the two geometric isomers are not separated during purification, and the absence of *t*-ABA from samples without internal standard must be established (Saunders, 1978). Milborrow (1968) described an elegant 'racemate dilution' method which utilised the optical differences between synthetic and natural ABA. This, however, required somewhat specialised equipment. The use of ^{14}C-ABA as internal standard (Alvim *et al.*, 1976) has many advantages (Saunders, 1978) and was adopted for this thesis. In the absence of convenient radio-GLC facilities, TLC was used to assay radiochemical purity at the end of the procedure.

In order to test the developed protocol a relatively simple experiment was designed to investigate the effects of wilting on ABA levels in *Commelina* tissue. For comparison with earlier results, the same short-term stress as used in Experiment 1.9 was employed. Since there was no previously published description of wilt-induced ABA production in *Commelina*, and in view of the report that a related plant

(*Tradescantia cv.*) did not produce ABA during wilting (Dörffling *et al.*, 1977) it was thought that the results would be valuable.

RESULTS AND DISCUSSION

Experiment 5.1 Characteristics of G- ^3H-ABA Partition between Aqueous and Organic Phases at Different pH Values.

This experiment was carried out for the reasons mentioned in the Section Introduction. G- ^3H-ABA was incorporated into extracts of *Ricinus* leaf material which had undergone filtration and PVP column stages or into an 'artificial' phloem sap. The results of partition of leaf extracts at pH 2, 4 and 8 are gathered in Tables 20, 21 and 22 respectively, and of partition of sucrose solution at pH 2 in Table 23.

Table 20 *^3H-ABA and Dry Weight Partition Characteristics at pH2.*

Organic Phase	^3H. ABA		Dry Weight		Separation Factor β
	D_{ABA}	E_{ABA}	D_{DW}	E_{DW}	
n -hexane	0.009	0.009	0.055	0.052	*
diethyl ether	5.056	0.835	0.019	0.019	263
chloroform	2.600	0.722	0.056	0.047	48
ethy acetate	22.89	0.952	0.029	0.028	795
n -butanol	39.16	0.975	0.090	0.083	484

* less than 1: $^1/_β = 6.3$

Table 21 ^{3}*H-ABA and Dry Weight Partition Characteristics at pH4.*

Organic Phase	^{3}H-ABA		Dry Weight		Separation Factor
	D_{ABA}	E_{ABA}	D_{DW}	E_{DW}	β
n-hexane	0.006	0.006	0.129	0.115	*
diethyl ether	5.334	0.842	0.017	0.017	313
chloroform	2.624	0.724	0.085	0.078	31
ethyl acetate	16.64	0.943	0.018	0.018	898
n-butanol	31.06	0.969	0.045	0.044	683

* less than 1: $^{1}/_{\beta} = 22$

Table 22 ^{3}*H-ABA and Dry Weight Partition Characteristics at pH 8.*

Organic Phase	^{3}H-ABA		Dry Weight		Separation Factor
	D_{ABA}	E_{ABA}	D_{DW}	E_{DW}	β
n-hexane	0.001	0.001	0.30	0.231	*
diethyl ether	0.017	0.017	0.06	0.052	**
chloroform	0.019	0.019	0.27	0.212	***
ethyl acetate	0.075	0.070	0.05	0.048	1.5
n-butanol	1.68	0.628	0.06	0.053	30

*,**,***, less than 1; $^{1}/_{\beta} = 266$; 3.2; 13.9 respectively.

Table 23 *^{3}H-ABA and Sucrose Partition Characteristics at pH2*

Organic Phase	^{3}H-ABA		Sucrose (D.W.)		Separation factor
	D_{ABA}	E_{ABA}	D_{DW}	E_{DW}	β
n-hexane	0.004	0.004	0.004	0.004	1.1
diethyl ether	4.72	0.825	0.003	0.003	1384
chloroform	1.78	0.640	0.004	0.004	440
ethyl acetate	19.1	0.950	0.002	0.002	8464
n-butanol	41.9	0.977	0.013	0.013	3115

For ABA, the results obtained are consistent with those found by Ciha *et al.*, (1977), although the same pH values were not employed. For all but *n*-hexane, the curve of E_{ABA} *vs.* pH for each solvent followed a sigmoidal pattern when these data and those of Ciha *et al.*, were combined. The E_{ABA} values obtained at pH 2 and 4 were all similar, but ABA transfer to the organic solvent was uniformly less efficient at pH 8, confirming theoretical prediction from the molecular structure of the hormone.

Dry weight transfer between the aqueous and organic phases was never above 25% efficient. However, consideration of this effect through the separation factor led to differences in the partition system which would be chosen on the basis of ABA distribution ratios alone. For exmaple, *n*-butanol at pH 4 had a higher E_{ABA} than ethyl acetate (0.969 *vs.* 0.943, Table 21), but the latter solvent had the higher dry weight: ABA separation factor (898 *vs.* 683).

In the 'simulated' phloem sap at pH 2, ethyl acetate was extraordinarily efficient at removing ABA from sucrose (β = 8464). The differences in separation factors found with this system and the leaf extract at pH 2 (Tables 20 and 23) emphasised the inadequacy of 'dry weight' as an estimator of interfering compounds in the extract; better estimates would be obtained if interfering compounds themselves

could be identified and measured for use in parallel tests. The possibility exists that an interfering compound may be removed by a solvent system having a low separation factor. Some advantage may therefore be conferred if several systems are used in series.

A number of points concerning solvent systems for purification of plant extracts for ABA emerge from these data, but practical problems may influence the eventual choice. Thus, although ethyl acetate gave consistently high β-values, it is known to degrade (to ethanol and acetic acid) upon storage (Merck Index, 8th Ed.) and must always be freshly redistilled; After partition and separation of phases, a retained organic solvent must be removed, and this is usually accomplished by rotary film evaporation; Certain advantages in terms of speed are hence conferred on solvents with low boiling points (e.g. diethyl ether); the chemical composition of the organic solvent may also influence choice: in ECD work traces of halogenated compounds are undesirable. This rules out the use of chloroform or methyene chloride for this reason; Finally, certain solvents may be hazardous or noxious. Risks encountered range from explosion (diethyl ether, petroleum ether) to narcosis (Diethyl ether, chloroform) and carcinoma (benzene).

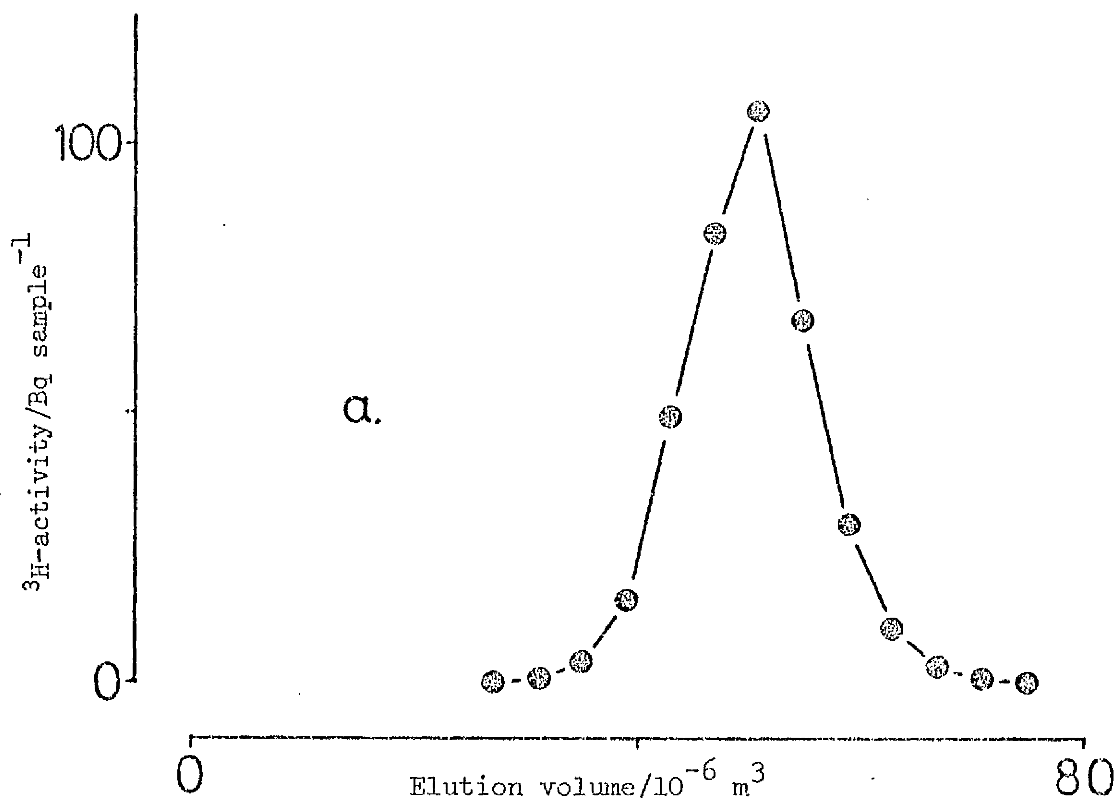
Experiment 5.2 ABA Elution Characteristics for PVP and DE Column Chromatography.

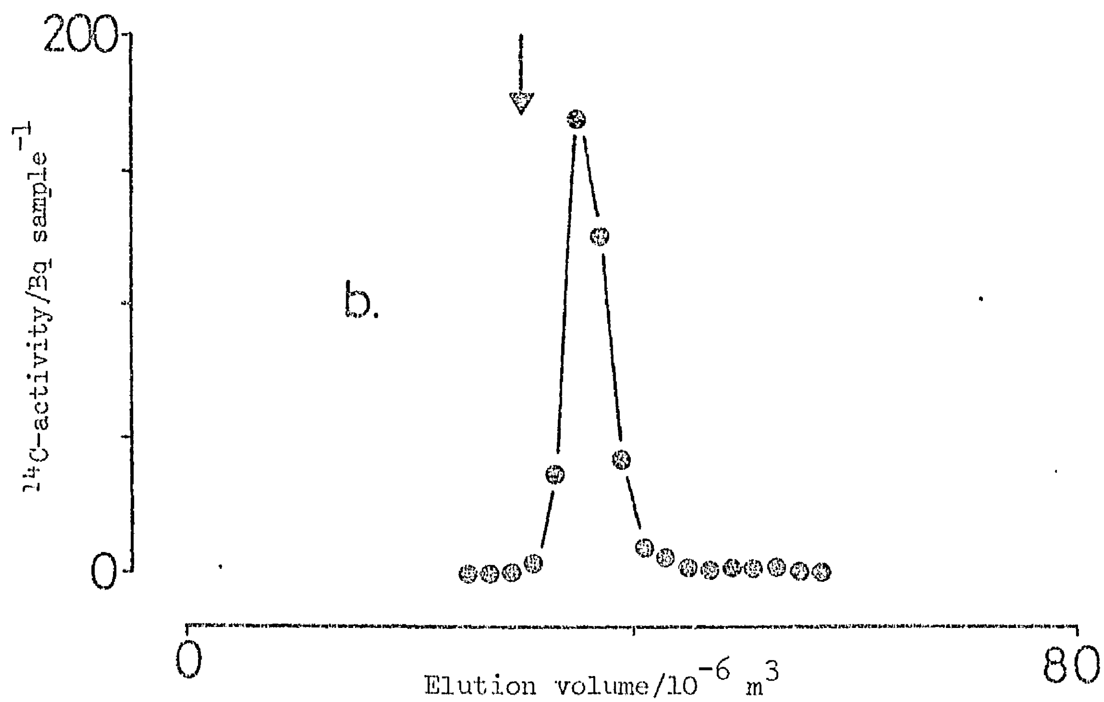
The elution of G-^{3}H-ABA through a standard PVP column (without the presence of plant material) is shown in Fig.45a. The efficiency of this column was estimated to be 0.89. A similar peak was found when plant extract was present but some peak tailing occurred.

Fig. 45b shows the elution profile of 2-^{14}C-ABA from a standard DE column. The efficiency of this column was estimated to be 0.93.

These results were used in the determination of the elution procedure normally used (see Materials and Methods).

Fig.45. ABA Elution Characteristics of PVP and DE Columns.





a. Standard PVP column

b. Standard DE column. Arrow indicates start of elution with 500 mol m^{-3} Na_2 SO_4.

Experiment 5.3 Linearity of Attenuation and Detection of the Electron Capture Detector.

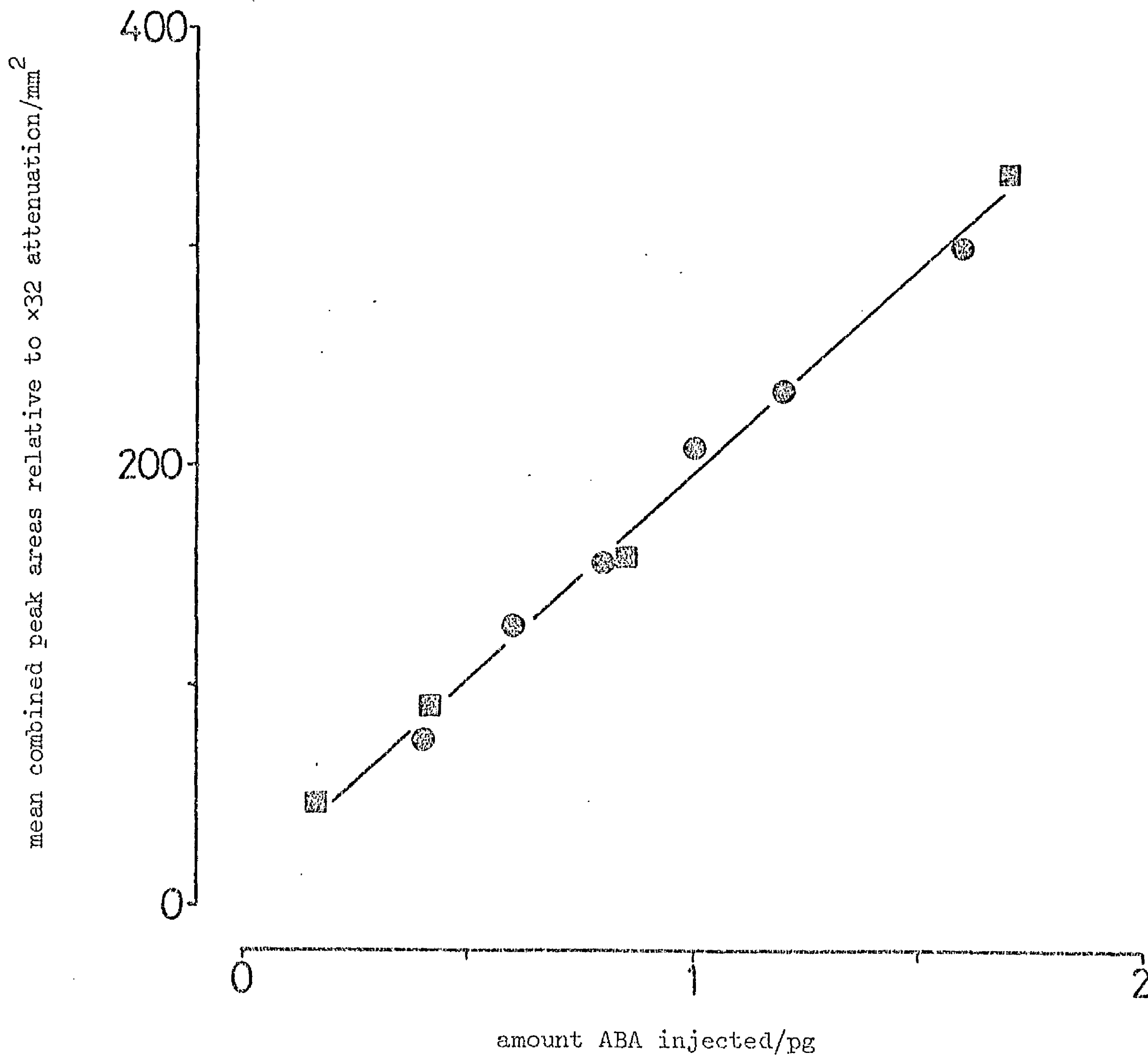
An essential element of quantification in any system where responses due to samples are compared with those of standards is the establishment of standard curves. If such curves are shown to be consistently linear over the portion of interest, then further quantification can be made by simple proportional comparison between responses of standards of known size and samples. In this Experiment the linearity of ECD response to both Me ABA and Me $2\text{-}^{14}C$-ABA was investigated. Before this, however, the linearity of attenuation was tested. Because amounts of injected ABA varied considerably, the electronic attenuation facility of the GLC-ECD was frequently utilised to adjust peak sizes on the chart recorder. To test the linearity of this procedure, 500 pg Me ABA was repeatedly injected and the attenuation altered from x8 to x128. Peak areas were measured and expressed as relative to x32 attenuation, and were found to have a low standard error (S.E. = 1.8% of total area; $n = 10$, 2 at each of 5 attenuation steps).

Response linearity is illustrated in Fig.46. A typical standard peak is shown in Fig.47. It is clear from Fig.46 that Me $2\text{-}^{14}C$-ABA gave similar responses to unlabelled ABA. The amounts of Me^{14}C- ABA injected were calculated from the specific activity supplied by the manufacturer. After about 1.75 ng the response curve was not lienar, but in the portion 160-1700 pg, the least squares linear fit had a high coefficient of correlation ($r = 0.996$, $p < 0.001$). This result was repeatable, and hence estimates of sample ABA contents were made by proportional comparison with standards. The amount injected was restricted to 0 - 1.5 ng.

Experiment 5.4 Effect of 0-5 h Wilting Treatment on Endogenous ABA Content of Commelina Leaves.

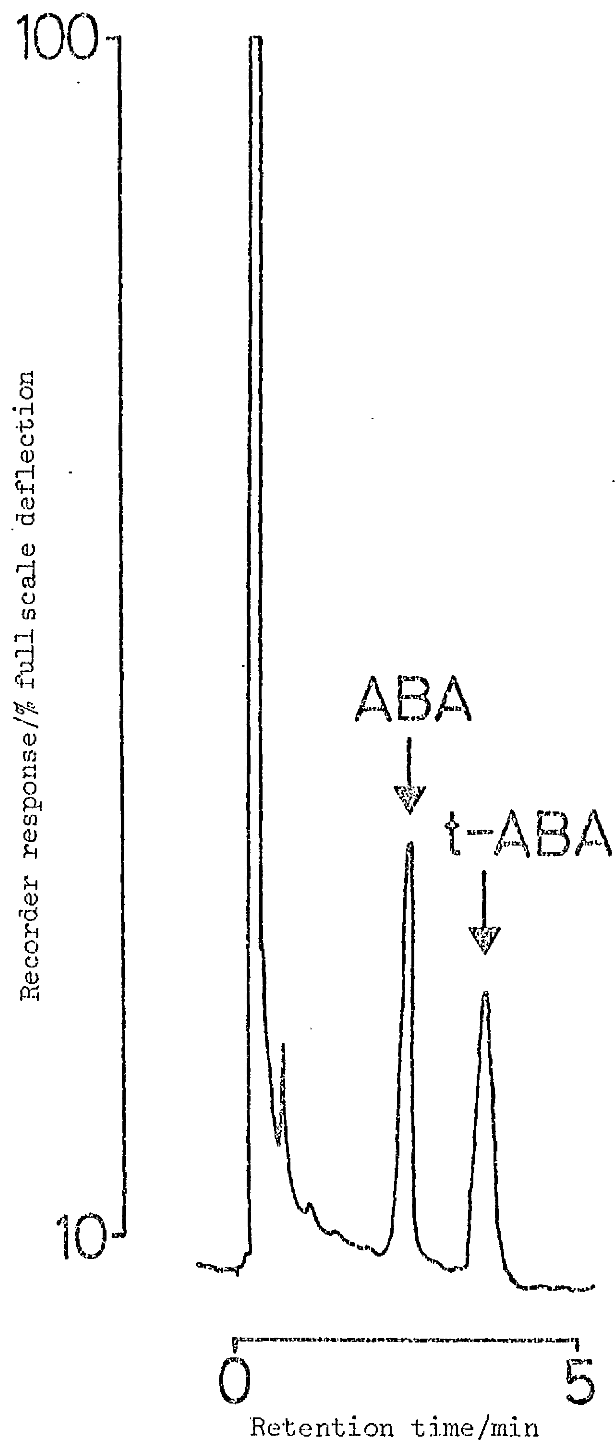
The treatment used in this experiment was the same as that employed in Experiment 1.9 to examine the effects of short-term stress on stomatal aperture attainment.

Fig.46. Linearity of ECD Response to MeABA and 2-^{14}C-MeABA.



Circles: MeABA
Squares: 2-^{14}C-MeABA
Least squares fit shown is $y = 187x + 9\cdot8$, $r = 0\cdot996$ ($p < 0\cdot001$)

Fig.47. Typical ECD Response to MeABA Standard.



Oven temperature 240 C

Commelina leaves were removed from healthy plants (*ca.* 10 g per treatment) and treated as described in the Materials and Methods. The fresh, wilted, and dry weights, and other details of treatment are given in Table 24.

Table 24 *Details of Commelina Leaf Wilting Treatments*

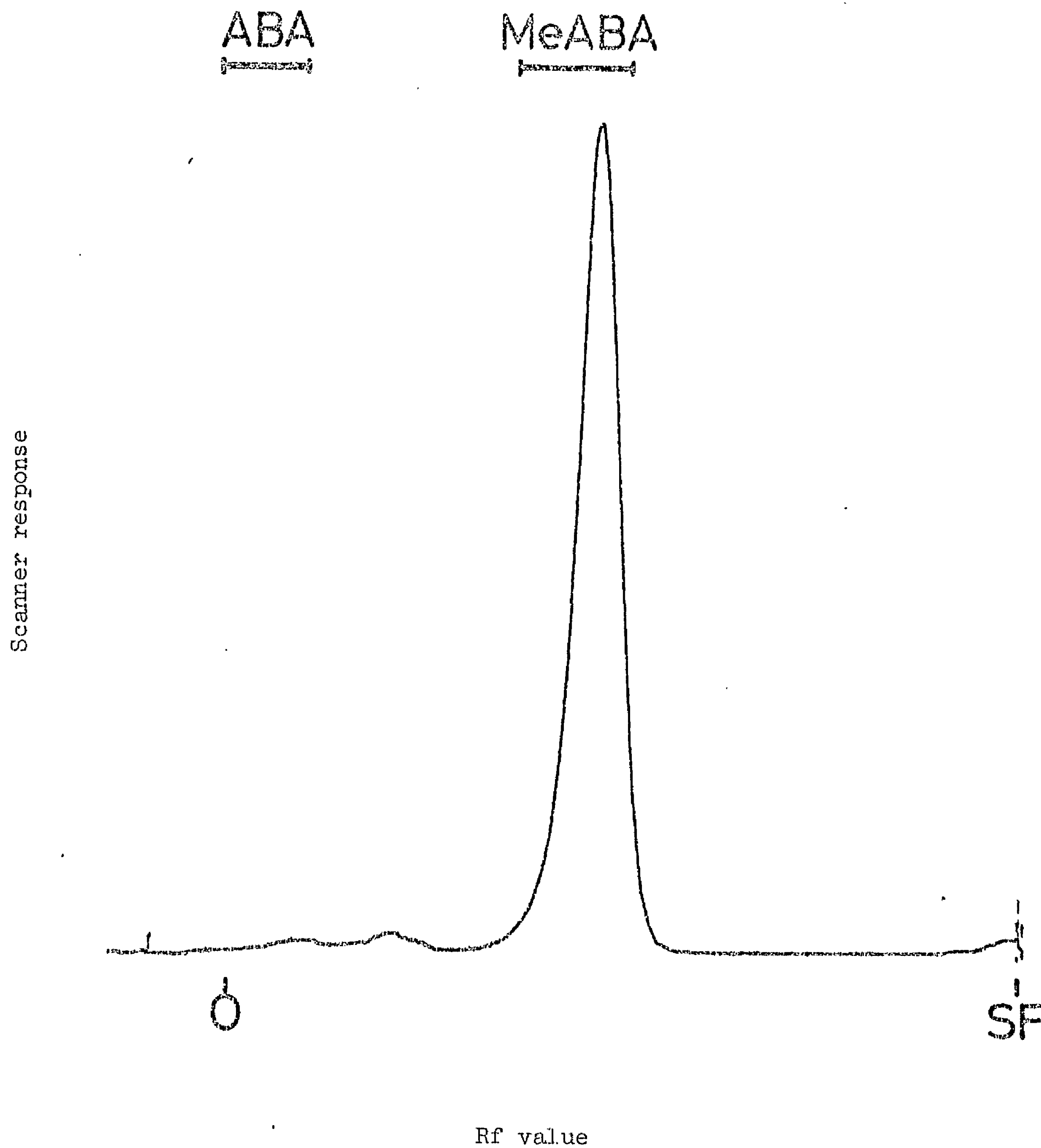
Extract Designation	Incubation time/ h	Weight of leaves/g.f.w.	10% loss weight/g.f.w.	Time taken for loss/min	Weight of leaves/ g.d.w.
C0	0	10.28	control	-	1.16
C1	1	10.94	control	-	1.83*
T1	1	10.03	9.03	10	1.05
C3	3	10.28	control	-	1.28
T3	3	10.08	9.07	45	1.08
C5	5	10.61	control	-	1.28
T5	5	10.03	9.03	14	1.59*

* observed to be not fully dry.

After methanol extraction, 2-^{14}C-ABA internal standard was added to each extract (439 Bq to controls, 2489 Bq to T1 and T5, and 2610 Bq to T3). Purification was then carried out using the standard procedure (Fig.8). After TLC of methylated samples in *n*-hexane: ethyl acetate 1:1, v/v, the TLC plates were examined using the Panax scanner. A typical result (that for T1) is shown in Fig.48. In all cases the single major peak of radioactivity corresponded to the position of Me 2-^{14}C-ABA standards. It was concluded that there was no evidence for ABA breakdown during purification. Purity of the internal standard was thus assumed to be 100% for the purpose of calculating purification efficiencies.

Aliquots of each sample (10%) were taken for radioassay and the efficiency of purification calculated. AFter GLC - ECD, the proportion of the response calculated to be due to the internal standard was subtracted from the total response. The remainder was assumed to

Fig.48. TLC of *Commelina* Leaf Extract.



O = origin (Rf=0)

SF = solvent front (Rf=1)

Bands corresponding to positions of standard 2-^{14}C-ABA and 2-^{14}C-MeABA are shown. Panax scanner operating conditions: time constant 100s, range 30, chart speed 120 mm h^{-1}, slit width 2mm.

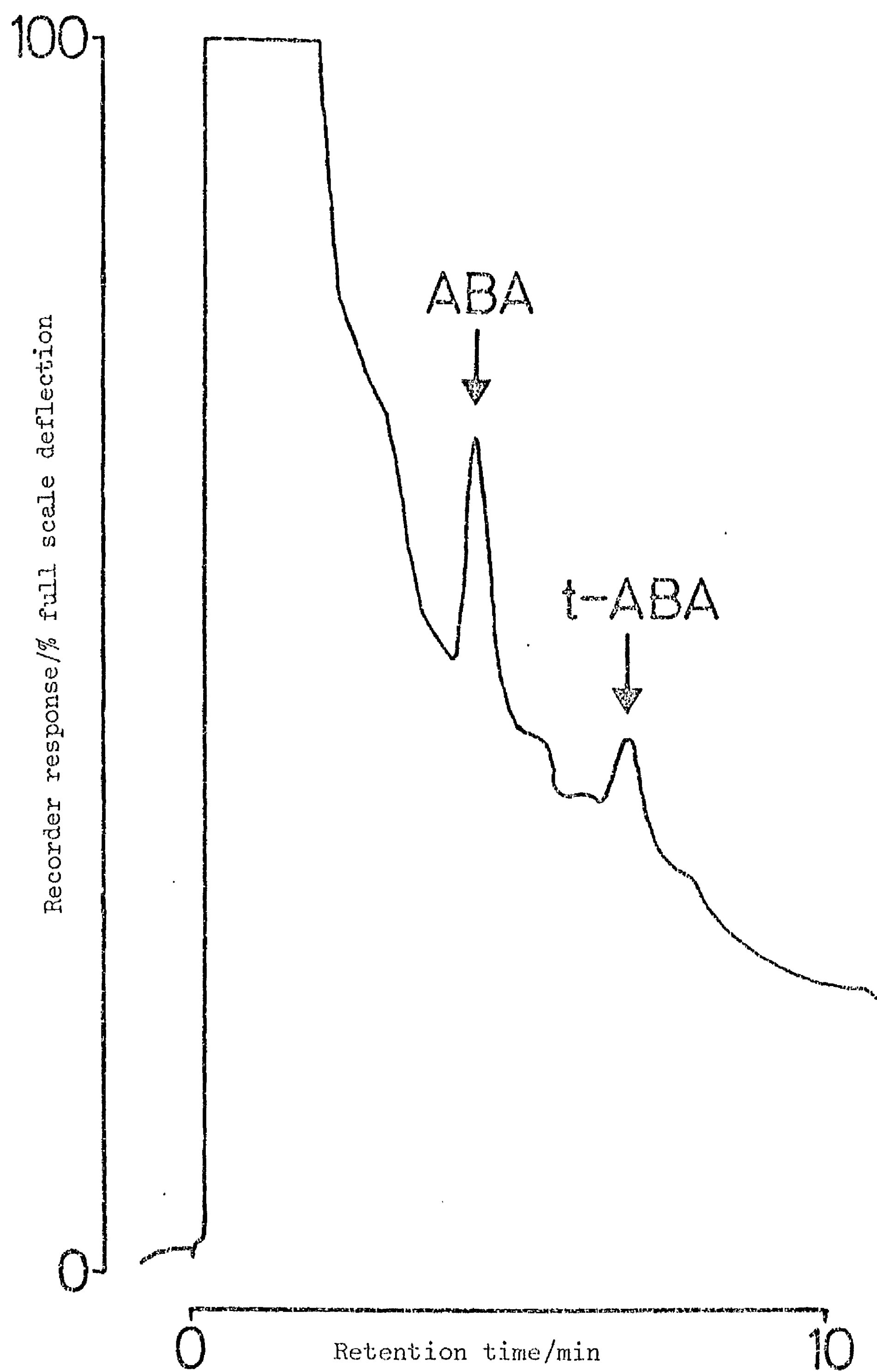
represent the endogenous ABA content. A typical GLC - ECD trace (that for T1) is shown in Fig.49 . The purification and quantitation parameters are given in Table 25 and the leaf ABA contents illustrated graphically in Fig.50.

The purification efficiency was found to vary between 0.072 and 0.768, thus demonstrating the value of an internal standard.

It was clear that the wilting treatment caused an increase in the endogenous ABA levels of the leaf tissue. For 1, 3 and 5 h, the wilting treatment gave ABA contents 9.2, 6.8 and 8.8 times those of the relevant controls. Since variability between samples was not assessed by replication, the significance of the apparent fall in ABA contents after 5 h is unclear. Levels appear to have risen during 1 h treatment and remained relatively constant thereafter. This may be a function of the relative rates of synthesis and metabolism of the hormone (Harrison and Walton, 1975).

The values obtained for the wilted treatment are similar to others reported for other species and tissues (see Table 2). For instance, Hoad (1975) found a 13.2-fold increase in *Ricinus* leaf ABA contents after a wilting treatment from 31 ng $g.f.w.^{-1}$ to 410 ng $g.f.w.^{-1}$; Simpson and Saunders (1971) noted that ABA levels in wilted pea epicotyls rose from 46 ng $g.f.w.^{-1}$ to 314 ng $g.f.w.^{-1}$ during an incubation period of eight hours (6.8 times increase). The values found in this experiment were slightly lower than these results, but obviously any differences may reflect both species- and treatment-dependent factors.

Fig.49. Typical ECD Response to *Commelina* Leaf Extract.



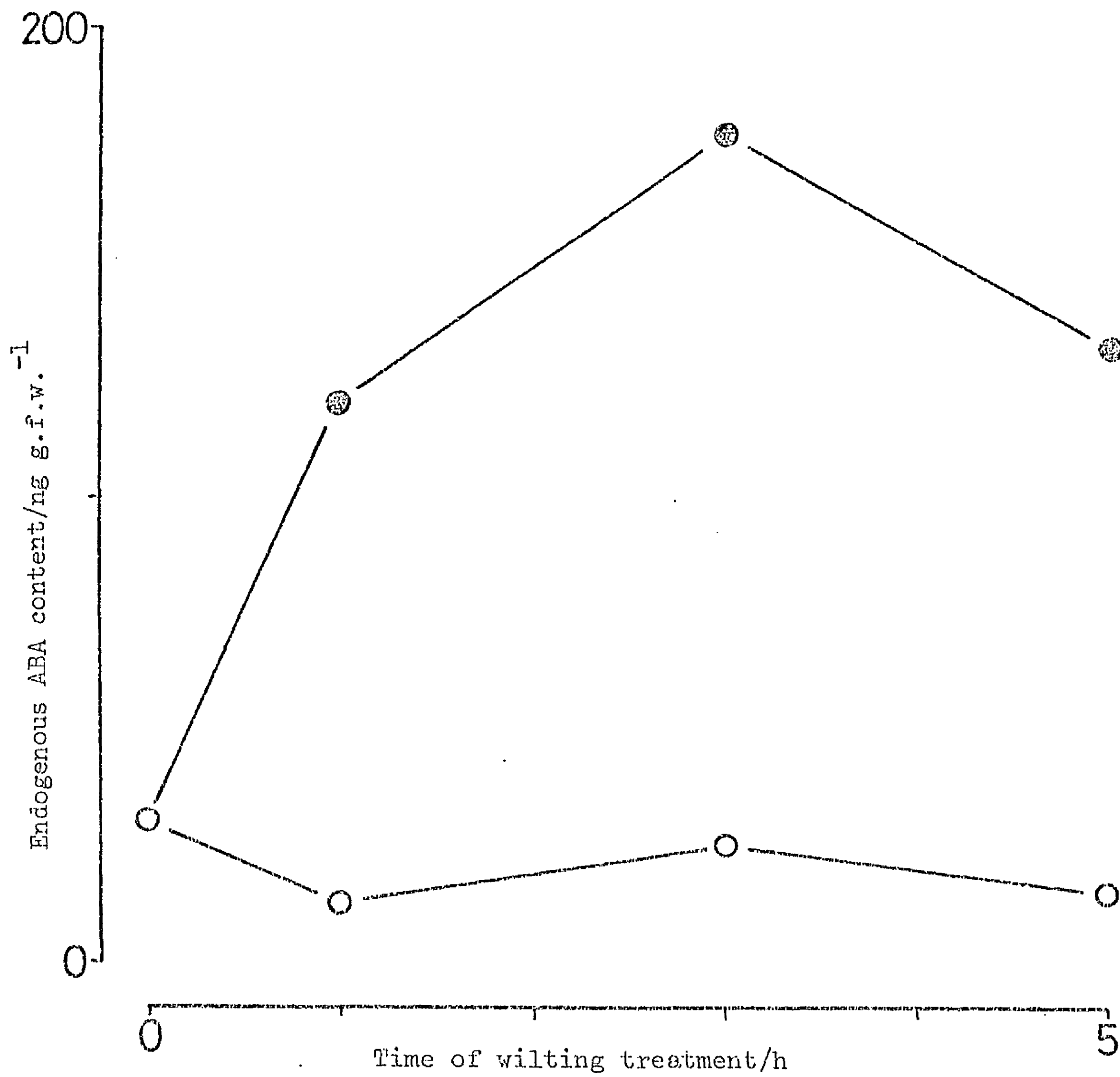
Oven temperature 220 C.

Table 25 *Details of Commelina Leaf Extract Purification and Quantification*

Extract Designation	Purification Efficiency	Peak areas $/mm^{-2}$			Endogenous ABA/ ng injection^{-1}	Injection factor	Total ABA in extract/ng	ABA in leaves/ ng.g.f.w.$^{-1}$*
		Expected due to internal standard	Actual found	Difference (Endogenous ABA)				
C0	0.244	99	171	72	0.78	100	319	31
C1	0.140	70	95	24	0.27	75	145	13
T1	0.072	157	236	79	0.87	100	1208	120
C3	0.133	68	112	44	0.48	75	270	26
T3	0.768	172	298	126	1.38	1000	1797	178
C5	0.485	136	181	45	0.50	150	155	15
T5	0.333	142	222	80	0.88	500	1321	132

* Expressed in terms of original fresh weight

Fig.50. Engodenous ABA Content of *Commelina* Leaves after Short-term Wilting Treatments.



Open circles: control treatments
Closed circles: wilted treatments

SECTION 6

ELECTRON MICROSCOPE STUDIES OF COMMELINA EPIDERMAL TISSUE

INTRODUCTION

The data reported in this short section are the results of an electron microscope investigation of the relationships between *Commelina* epidermis cells in terms of the presence or absence of plasmodesmatal connections.

Plasmodesmata are believed to act as channels for symplastic transport, although the evidence for such transport is largely circumstantial (Robards, 1976). In the absence of inter-cellular connections, any transport phenomena between cells must be assumed to involve an apoplastic component. This study was carried out to aid interpretation of the mode of solute accumulation by guard cells observed in this study (Sections 2, 3 and 4) and by many other authors (see later discussion). Plasmodesmatal connections between guard cells and subsidiary cells were examined as were those between other epidermis cells and those between epidermal and mesophyll cells. The latter were of interest firstly because of reports of intra-leaf transport of solutes (e.g. Dittrich and Raschke, 1977b; Loveys, 1977; Willmer *et al.*, 1973), and secondly because of damage which might be caused by the peeling of epidermis.

Carr (1976) reviewed the plasmodesmatal relations between guard cells and other epidermis cells as a function of development. He concluded from the evidence of many studies that such connections were present in immature tissues but did not exist when the epidermis was mature. Because the tissues used in the preceding experiments were always mature, the following experiment was confined to first fully-expanded leaves of *Commelina*.

RESULTS AND DISCUSSION

Experiment 6.1 Electron Microscope Studies of Commelina Epidermis

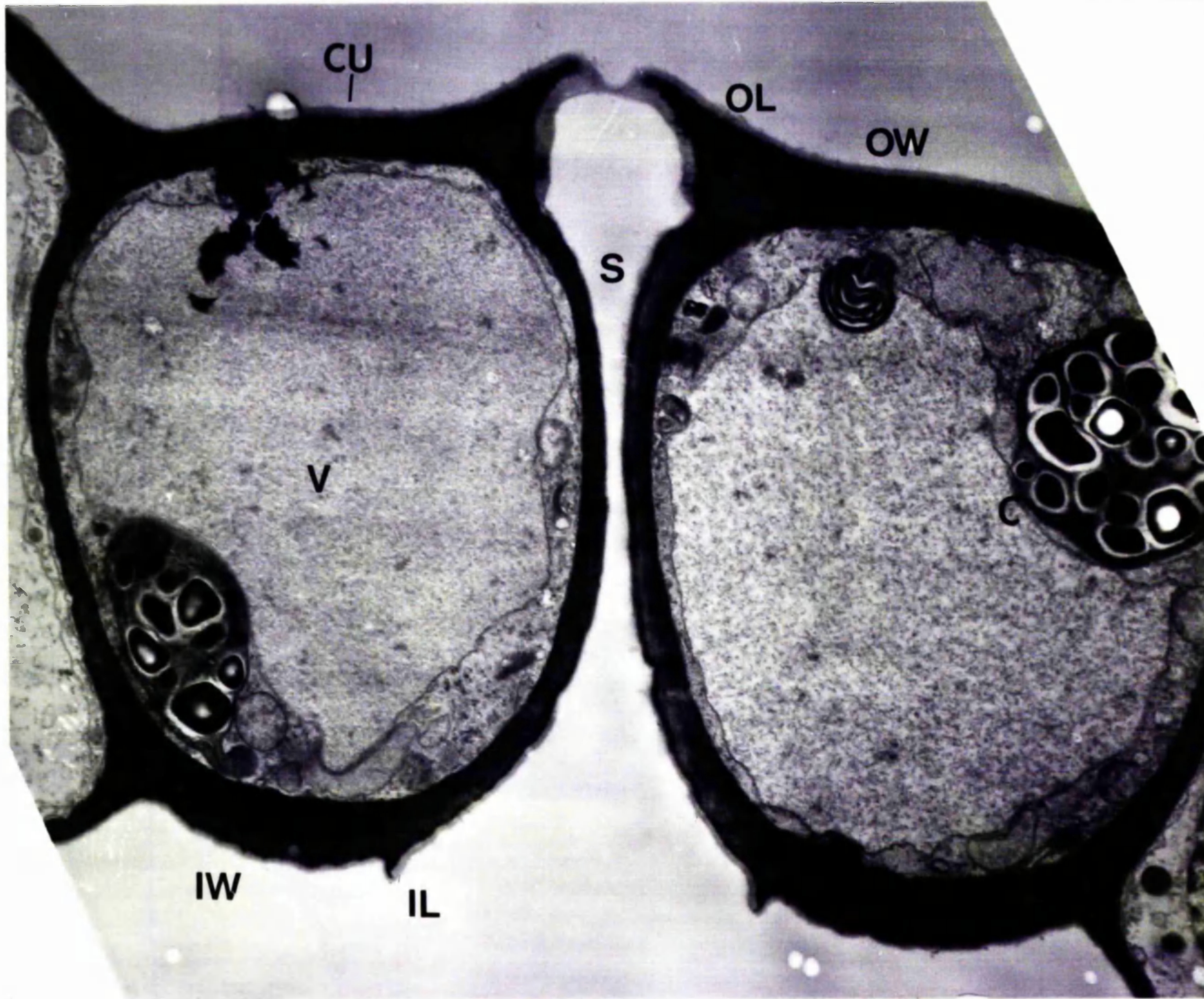
Leaf tissue was taken from *Commelina* plants on three separate occasions and subjected to the fixing, sectioning, and examination procedure described in the Materials and Methods Section.

Fig. 51 shows a typical guard cell pair (see also frontispiece). The classic hour-glass shape of the stoma in transverse section is evident. The outer and inner tangential guard cell walls are thickened, having large outer ledges and smaller inner ledges. These are thought to be adaptions to facilitate stomatal function (Meidner and Mansfield, 1968). In Fig. 52, the wall between a guard cell and an inner lateral subsidiary cell is magnified. No plasmodesmata are visible. In this study, over 30 guard cell/inner lateral subsidiary cell walls were photographed and many more observed; in no case was a structure resembling a plasmodesma discerned. Heavy cuticular deposits were found on the guard cells (Fig. 51). These extended to the sub-stomatal cavity surfaces of the subsidiary cells (Fig. 52).

In contrast, when cell walls between other epidermis cells were examined, many plasmodesmatal structures were visible (Figs. 53-57). Fig. 53 illustrates connections between the inner and outer lateral subsidiary cells (refer to Fig.9). Some distortion appears to have occurred during fixation or sectioning, but note the strands of material(ST) connected to the plasmodesmata in the upper portion of the micrograph. This may be endoplasmic reticulum material. Fig.54 shows a pair of plasmodesmata in the cell wall between the outer lateral subsidiary cell and an epidermal cell. An irregularity (I) in the plasmodesmatal cavity at the position of the middle lamella is visible. Four connections between two epidermal cells are presented in Fig.55, one of which is branched (BPD).

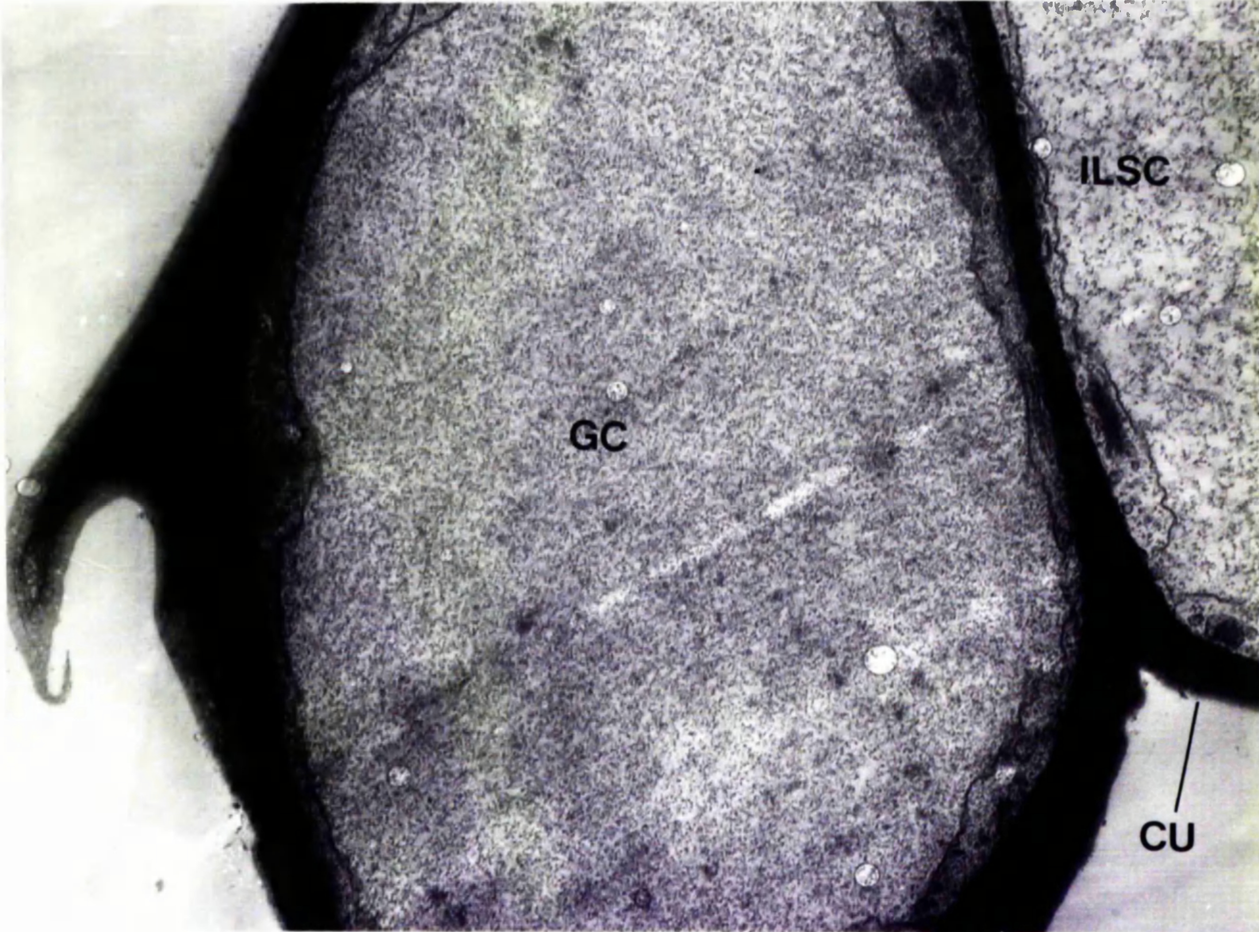
A particularly high density of plasmodesmata between mesophyll and epidermis cells was generally observed. Examples are shown in Figs. 56 and 57. In the former, the desmotubules (DT) are clearly seen within the plasmodesmata, whilst the latter illustrates the relationship between cell wall structure and the pore.

Fig. 51. Typical Guard Cell Pair of *Commelina*



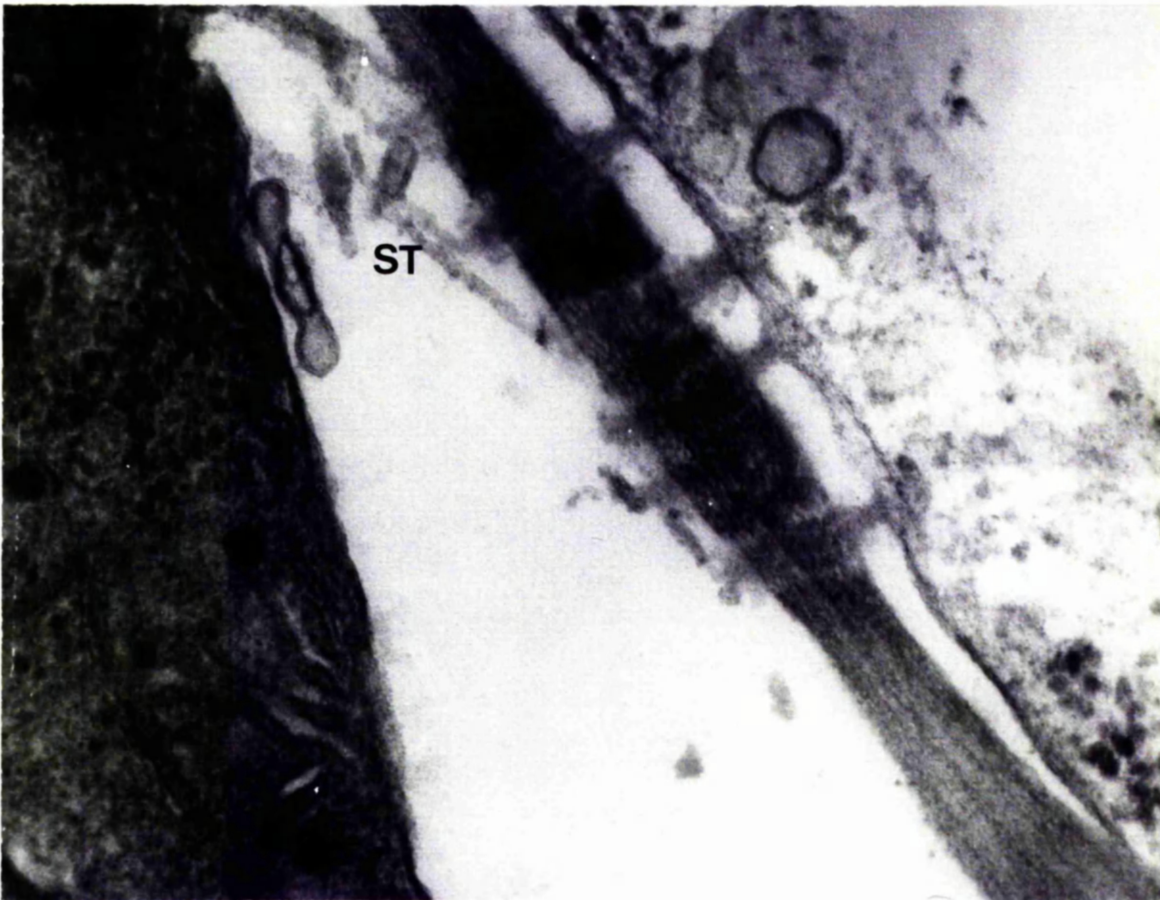
TS (7060 x) C = Chloroplast IL = Inner wall ledge
IW = Inner guard cell wall OL = Outer wall ledge
OW = Outer guard cell wall V = Vacuole
CU = Cuticle S = Stoma

Fig.52. Wall between Guard Cell and Inner Lateral Subsidiary Cell



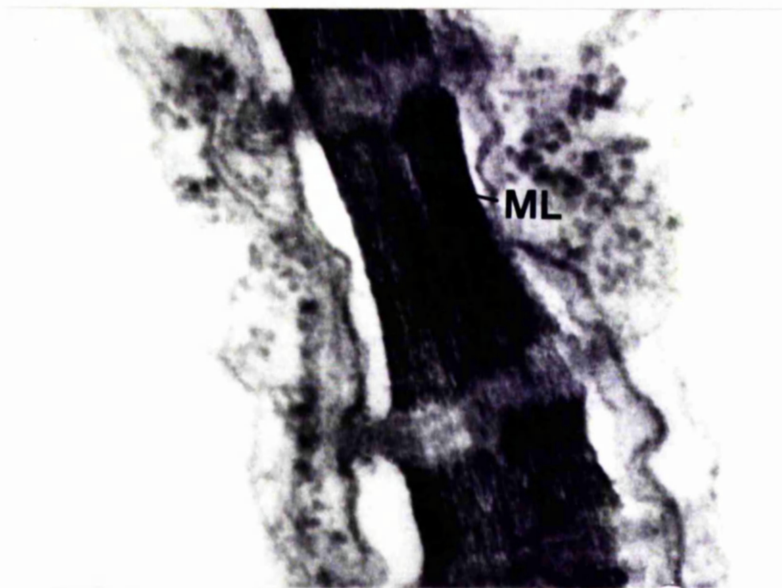
TS (11,290 x) CU = Cuticle GC = Guard Cell
ILSC = Inner Lateral Subsidiary Cell

Fig.53. Plasmodesmata in Cell Wall between Inner and Outer Lateral Subsidiary Cells.



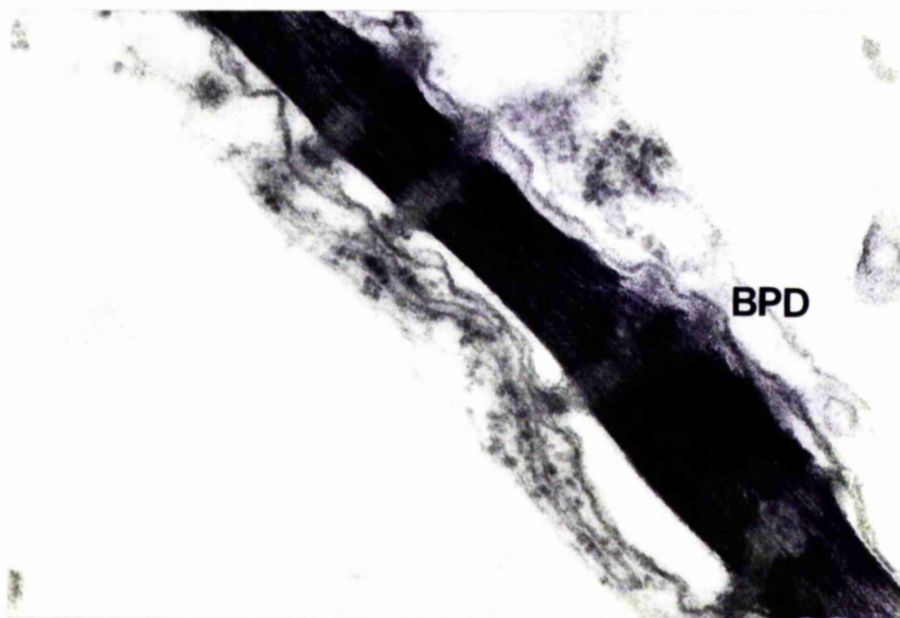
TS (89,880 x) PD = Plasmodesmata ST = Strands

Fig. 54. Plasmodesmata in Cell Wall between an Outer Lateral Subsidiary Cell and an Epidermal Cell



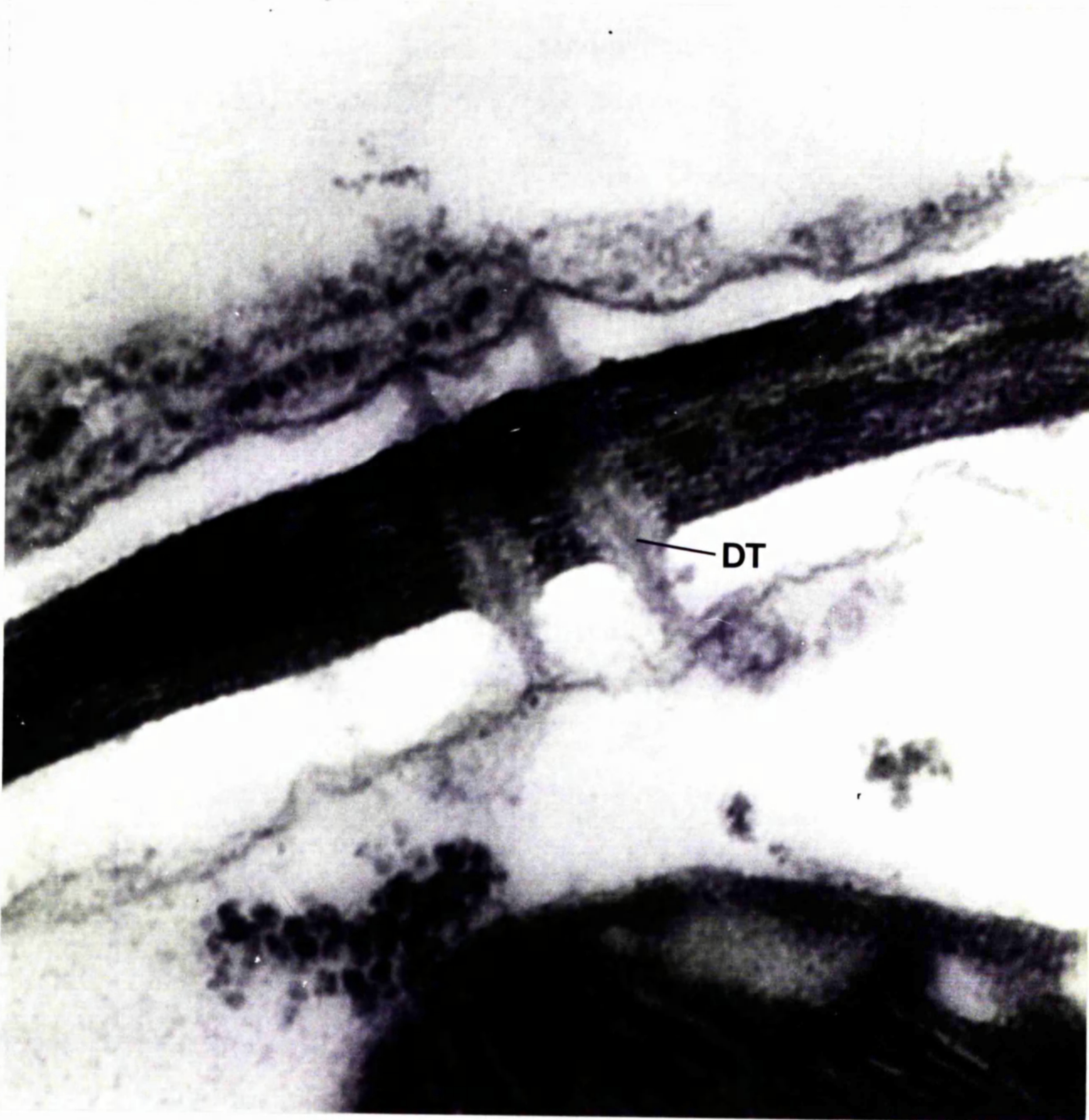
TS (86820 x) ML = Middle lamella I = Irregularity

Fig.55. Plasmodesmatal Connections between Two Epidermal Cells



TS (57880 x) BPD = Branched plasmodesma

Fig.56. Plasmodesmata in Cell Wall between Epidermal and Mesophyll Cells



TS (119060 x) DT = Desmotubule

Fig.57 Plasmodesmatal Connections between Epidermal and Mesophyll Cells.

TS (89650 x)

It was concluded that plasmodesmatal connections existed between all epidermis cells in *Commelina* except inner lateral subsidiary cells and guard cells, although connections between terminal subsidiary cells and other cells were not examined. It thus appears that the symplastic pathway could carry a solute from the mesophyll tissue as far as the stomatal complex *but not to the guard cells:* Guard cell solute uptake would appear to have been apoplastic.

SECTION 7

TRANSPORT OF ABSCISIC ACID IN RICINUS

INTRODUCTION

The transport of the plant hormones is of considerable interest with respect to the integration of many aspects of higher plant growth and metabolism. In the case of ABA, many studies have been made with explant material (see Milborrow, 1974), but relatively few involving whole plants.

King (1976) emphasised the possible importance of the phloem and xylem pathways for hormone transport. ABA has been quantified in both phloem and xylem saps (Table 2), and an increase in levels present in these pathways has beén correlated with the effects of water stress (Hoad, 1973; Hoad, 1975; Zeevaart, 1977; Hoad, 1978).

Hocking *et al.*, (1972) performed the first reported study of ^{14}C-ABA transport in whole (*Phaseolus*) plants. By the use of steam girdles, they were able to show that movement of ^{14}C (from ABA) from the leaves to the roots occurred *via* the phloem. Accumulation of label in root nodules and the shoot apex was observed. Friedlander *et al.* (1976) applied ^{14}C-ABA in detergent solution to cucumber leaves, but found little movement or metabolism of the tracer. That which did move, went largely to the shoot apex. Using similar methods, Goldbach *et al.* (1977) and Goldbach and Goldbach (1977) examined ^{14}C-ABA translocation in wheat and barley. Like Hocking *et al.* and Friedlander *et al.*, they found most movement occurred into the important sinks for photosynthates. The transport of ABA in relation to the distribution of assimilates from photosynthesising leaves was therefore of interest. Also, in view of hypotheses concerning the conjugation of hormones and translocation (Wareing, 1977), a system whereby molecular changes in translocated substances could be studied was required.

The *Ricinus* phloem exudation phenomenon has been used to study mechanisms of assimilate transport (Milburn, 1975). In addition, endogenous ABA has been found in *Ricinus* phloem exudate (Hoad, 1973). In this section a system for the study of hormone loading and transport which utilises this phenomenon is described with particular reference to ABA.

RESULTS AND DISCUSSION

Experiment 7.1 Movement of ^3H from ABA in the Phloem Sap of Ricinus

Hocking (1973) studied several methods of application of ABA to plant leaves and concluded that leaf abrasion was the most efficient method for hormone uptake by the leaf. This technique was therefore adopted for investigation of ABA transport in *Ricinus*.

During abrasion of *Ricinus* leaves, it was noticed that the colouration of the abraded area altered. This was thought to be due to loss of anthocyanin-like pigments present in the gland cells of the leaf epidermis. Since this inferred rupture of these cells, and because structural damage to the vascular tissue might also occur, a study was made of the effects of abrasion on cell structure. Leaf segments from abraded and nearly normal leaf surfaces were fixed, sectioned and stained with toluidine blue. Observation of the sections (Fig. 58) revealed two significant differences between abraded and normal leaves:

(1) The outer peridermal wall of the upper epidermis cells was often broken although the inner wall was usually intact. Where the epidermal cells *were* still intact, the cuticle appeared much thinner than the controls.

(2) A few palisade mesophyll cells were ruptured with cell contents missing. The majority were still intact, but cellular disruption appeared to have occurred. All other cells, including those of the vascular tissue, appeared normal.

It was concluded that application of tracer after abrasion would result in speedy appearance of the compound in the inner-leaf free space.

G-^{3}H-ABA was applied to the abraded leaf surface of a *Ricinus* plant in an agar donor block and exudate collected from the stem and assayed for radioactivity. The exudation rate (Fig. 59a) rose to a peak at 40 min and subsequently declined. ^{3}H-activity was found in the phloem sap, indicating that translocation of the labelled ABA had occurred (Figs. 59b and c). When expressed per unit volume (Fig. 59b) or per unit time (Fig. 59c), the radioactivity present in exudate showed similar characteristics, rising to a peak at about 80 min.

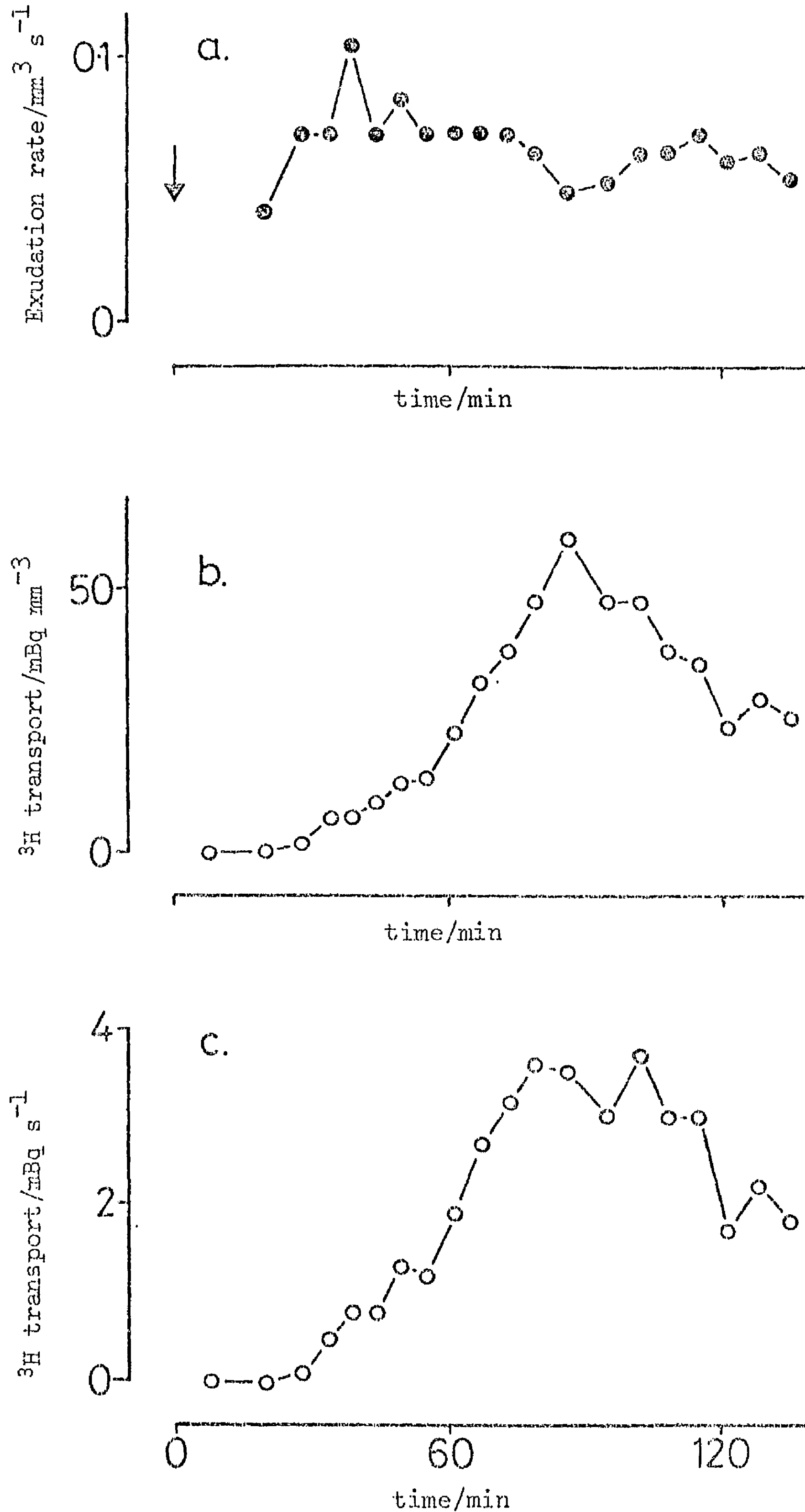
Fig.58. Effect of Abrasion on Leaf Cell Structure

a.

b.

a. Appearance of normal epidermis;
b. " " abraded " ; (315 x).

Fig.59. Appearance of ^{3}H from ABA in the Phloem Sap



1667 Bq G-^{3}H-ABA was applied in a 157 mm^{3} agar block to the first alternate leaf of a *Ricinus* plant having one expanded alternate leaf. Sap was collected from an incision opposite the treated leaf in the bark between the first alternate leaf and the primary leaves. Samples were collected in 25 mm^{3} microcapillary tubes. The arrow indicates the time of incision.

In order to examine further the significance of these data, the molecular state of the ^3H label transported in this system was investigated (Experiment 7.2) and the relationship between sucrose and ABA transport studied using double-label techniques (Experiment 7.4).

Experiment 7.2 State of ^3H Label Translocated in the Phloem Sap.

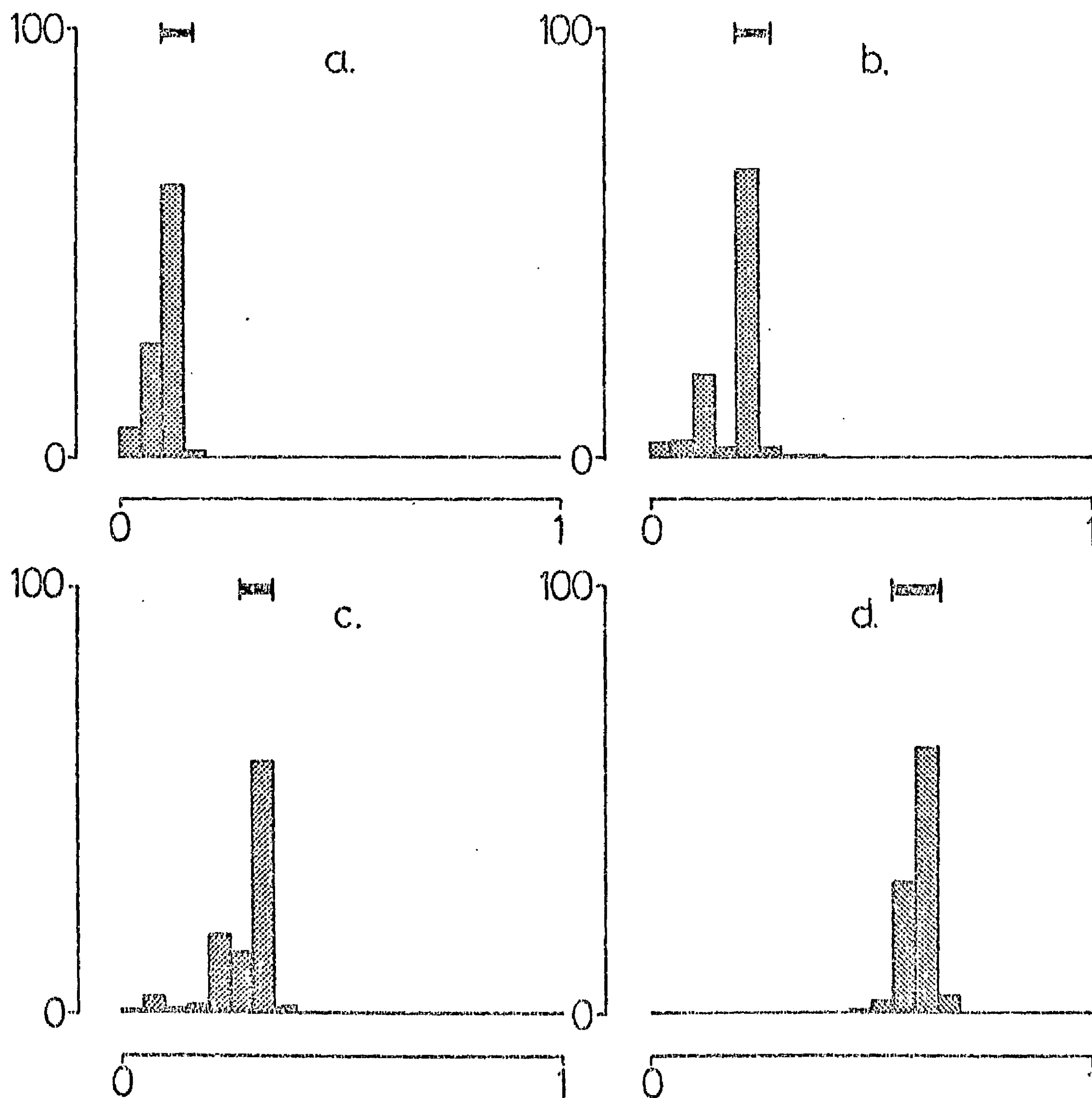
^3H-ABA (43,000 Bq) as 25 mm^3 aqueous solution was applied to abraded leaves of each of four *Ricinus* plants and sap collected from a series of ascending cuts made over 4 h. The mean percentage recovery of applied label was 9.62. This represented a much higher rate of uptake and recovery than that found in other studies (Friedlander *et al.* 1976). In a similar experiment with three plants, the sap collected was subjected to TLC analysis of the molecular state of the ^3H label (Fig. 60). Sap was collected for 4 h and after this period the shoots were removed and xylem sap collected for a further 12 h. This was found to contain a significant amount of radioactivity (on average 0.28% of that applied), which indicated that some recycling of ABA (Hoad, 1975) had occurred. However, no analysis of the state of xylem exudate label was carried out.

Fig. 60 shows that at least 65% of the ^3H label recovered from phloem sap was chromatographically identical to standard ABA in 4 solvent systems. However, in contrast to Fig. 27, more than one peak was visible in two of the systems (b and c). It was not clear whether this was due to the presence of a genuine metabolite. Cummins (1973) studied the metabolism of ABA in barley leaves. Using solvent system C in the proportion 50:5:2, he found that leaves extracted after 35 min exposure to ^{14}C-ABA exhibited little metabolism, but after 2 h a second peak appeared at a lower Rf value which he concluded was phaseic acid (Fig. 2e). His results were therefore similar to those obtained in this study.

Experiment 7.3 Endogenous ABA in Ricinus Phloem. Sap.

In order to make a positive identification of free ABA in the phloem sap, a sample (10 ml) was partititoned at pH2 with ethyl acetate

Fig.60. TLC of Phloem Sap extract.



X-axes: Rf value; y-axes: ^{3}H activity in Rf zone/% total recovered.
Bars represent position of 2-^{14}C-ABA standard.

Solvent systems, all v/v (with total recovered ^{3}H):

a. *n*-hexane:ethylacetate; 1:1 (878 Bq)
b. Ethyl acetate: toluene; acetic acid, 1:10:2 (783 Bq)
c. Benzene: acetone: acetic acid; 70:30:1 (848 Bq)
d. Chloroform: methanol; 1:1 (771 Bq).

and the components of the organic phase subjected to GLC-MS analysis. The following observations were made which supported the hypothesis that ABA was present in the extract.

(1) Two peaks found in the sample co-chromatographed on GLC with authentic ABA and *t*-ABA.

(2) When sample and standard were co-injected only two peaks having the same retention times as in (1) were found.

(3) Single-ion detection of the base peak at m/e 190 gave responses having identical retention times to those of standards. When tuned to m/e 194 the responses of the hexadeuterated internal standard were found to have the same retention times.

(4) When a full-scan mass spectrum was carried out, the characteristic fragmentation pattern of standard ABA (peaks at M-56 (208), M-74 (190), M-102 (162), M-130 (134)) was found, although the relative abundance of the peaks were not identical to those of standards, probably as a result of the presence of contaminating compounds.

The internal standard was used to quantify the endogenous ABA present in the extract which was estimated at 500 mg m^3. This is relatively high for a non-stressed *Ricinus* plant compared with other findings (Table 2) but is lower than values found for stressed plants. It was concluded that free ABA was present in the phloem extract.

Experiment 7.4 Movement of ^3H from ABA and ^{14}C from Sucrose in Ricinus Phloem Sap.

Although results such as those of Experiments 7.1 and 7.2 can show that ABA is readily transported in the phloem, kinetic analysis of such translocation is relatively meaningless unless compared with that of other substances. In particular, the relationships between ABA movement and that of the normal photosynthates are of interest. Since sucrose is the major translocated carbohydrate in *Ricinus* (Milburn, 1975),

double-label techniques were used to analyse the characteristics of ABA and sucrose transport simultaneously.

Fig. 61 illustrates the appearance of radioactivity from ^{3}H-ABA and ^{14}C-sucrose in phloem exudate after application together to an abraded area of leaf. As found in Fig.59a, the exudation rate (Fig. 61a) declined from a maximum which occurred soon after the incision as previously described (Hall *et al.*, 1971). Correlations between secondary peaks in exudation rate of 35 and 65 min with small fluctuations in growth-room temperature (± 2 C) were noted: higher rates of exudation corresponded to high ambient temperatures.

Radioactivity from both substances appeared rapidly in the exudate (Fig. 61b). From knowledge of the distance from the point of application to the incision, it was possible to calculate that the fastest component of the translocation stream had a velocity of about 1.1 m h^{-1}. This value is compatible with other estimates of phloem transport rates which range from 0.2 - 1.0 m h^{-1} (Nobel, 1975). The concentration of radioactivity from each substance increased to an asymptote during 90 min. However, whilst the concentration of ^{14}C (sucrose) continued to rise slowly even after 60 min, that of ^{3}H (ABA) reached a plateau after 30 min. This meant that the relative concentrations of the labels altered. Thus, the initial ratio of labels

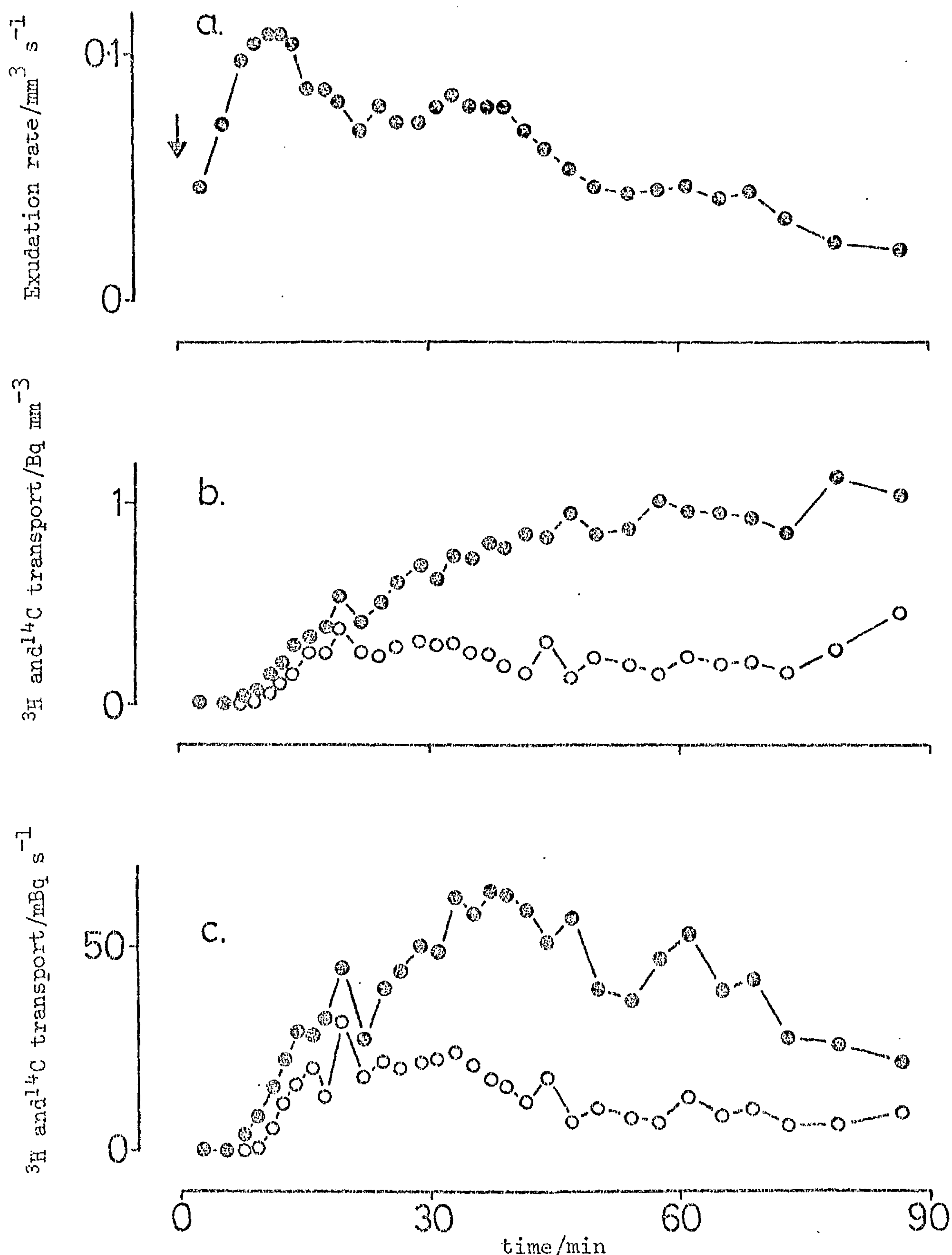
$$\left(\frac{^{14}\text{C (sucrose)}}{^{3}\text{H (ABA)}} \right)$$

was 0.74 in the applied buffer solution, but on the appearance of radioactivity in the exudate was roughly 1.0 and rose to over 4.0 after 60 min. This indicated either preferential loading of sucrose into the phloem sap, or removal of ABA to leaf compartments distant from the site of loading, or both.

In a unit time basis (Fig. 61c), the flux of label from both substances declined after 35 min, as the exudation rate fell. These values are thought to reflect the actual phloem loading rates more accurately than those expressed on a per volume basis, since they take into account fluctuations in exudation rate (Smith, personal communication).

When more than one incision was made during the course of the collection of sap, an increase in exudation rate was noted. This was

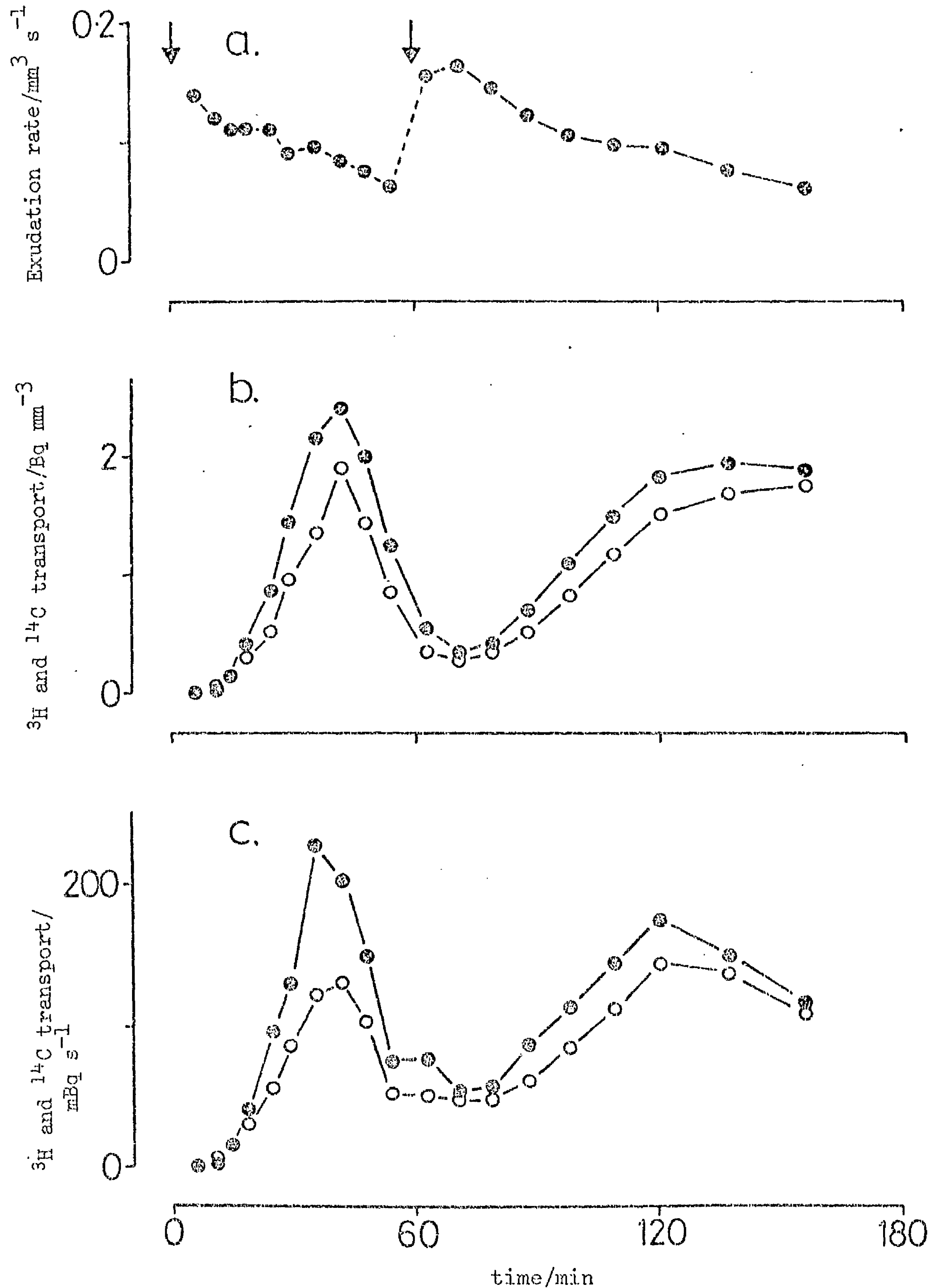
Fig.61. Appearance of ^{3}H from ABA and ^{14}C from Sucrose in the Phloem Sap (One Incision).



10689 Bq ^{3}H-ABA and 7936 Bq ^{14}C-sucrose were applied in 50 mm^3 MES buffer (10 mol m^{-3}, pH 5·5) to the second alternate leaf of a *Ricinus* plant having two expanded alternate leaves. Sap was collected from an incision under the treated leaf in the bark between the cotyledons and the primary leaves. Samples were collected in 25 mm^3 microcapillary tubes. The arrow indicates the time of incision.

followed after a lag period by an increase in the transport of both labelled compounds (Fig. 62). It has been implied from such results that the phloem loading process responds to the decrease in turgor within the phloem caused by excision and other factors (Smith, 1978). These results indicate rather more close linking of rates of sucrose and ABA transport, although the ratio of labels varied between about 1.0 to 2.0 during the course of exudation. It is clear that further investigations of such transport phenomena are required before precise conclusions can be made about the mode of hormone loading. Indeed, the results obtained in these experiments are consistent with passive movement of ABA as well as with active loading.

Fig.62. Appearance of ^{3}H from ABA and ^{14}C from Sucrose in the Phloem Sap (Two incisions).



10689 Bq ^{3}H-ABA and 7936 Bq ^{14}C-sucrose were applied in 50 mm^{3} MES buffer (10 mol m^{-3}, pH 5·5) to the second alternate leaf of a *Ricinus* plant having two expanded alternate leaves. Sap was collected from an incision under the treated leaf in the bark between the cotyledons and the primary leaves. Samples were collected in 25 mm^{3} microcapillary tubes. The arrows indicate the times of incision.

GENERAL DISCUSSION

This General Discussion brings together the results of the 7 experimental Sections, summarising the main findings, and relating them to the published literature wherever the points are not dealt with in the independent Section discussions (Section A).

In Section B, the results are briefly discussed within the specific remit of the hormonal nature of wilt-induced ABA action. Section C outlines a selection of potential research themes on ABA action on stomata.

SECTION A

SYNOPSIS AND INTEGRATION OF RESULTS SECTIONS.

The first Results Section was concerned with the development and characterisation of systems whereby the effects of ABA on stomata of isolated epidermal segments could be examined. An important initial conclusion from the results of Experiments 1.1 and 1.2 was that the existence of morphological subsidiary cells around the stomata of *Commelina communis* conferred an advantage in studies of stomatal movements using this species: the high viability of cells in the stomatal complex after peeling resulted in uniform and repeatable opening and closing upon incubation.

Since stomatal movements are the result of changes in turgor relations between guard cells and their neighbouring epidermis cells (Meidner and Willmer, 1975; Raschke, 1975), death of functional or morphological epidermal cells (causing zero turgor) would be predicted to lead to stomatal opening if the guard cells remained alive having positive turgor. This was demonstrated by Squire and Mansfield (1972) using *Commelina,* and is confirmed in this study in Figs. 13 and 14. In normal *Commelina* epidermis (as used in all other experiments), however, high proportions ($> 95\%$) of living subsidiary cells were found (Fig. 12b). These correlated with the low initial apertures found after peeling, at the start of the incubation period (e.g. Fig.17), presumably reflecting the on-plant aperture before separation (Fig. 24a), and implying that the cells of the complex were at least capable under

certain conditions of contributing to complete stomatal closure. This is not the case with epidermis tissue from certain other plants, notably *Vicia*, which do not possess morphological subsidiary cells. Thus, in peeled *Vicia* epidermis, the proportion of living epidermal cells is usually low (Fischer, 1972). Consequently, initial stomatal apertures are normally relatively high and variable (e.g. between 4.2 and 6.5 μm, Horton, 1970), which may explain the variability of results with this species reported by Willmer and Mansfield (1969). Horton (1970) described effects of ABA on *Vicia* epidermis. Although apertures were reduced compared with controls, they did not narrow beyond the high initial value. Comparable results were obtained by Itai and Meidner (1978a; 1978b) in *Commelina* epidermis treated to kill subsidiary cells: in their tissue ABA had no effects on aperture. This is not to say that ABA could not have had effects on the guard cells in terms of ion fluxes and cell metabolism; rather, that if it did, the effects were prevented from being manifest in stomatal movement by predominating turgor effects of cell death. Nevertheless, in this study, it was thought desirable to develop an epidermal segment system in which ABA effects similar in all respects, including movements, to those found on whole leaves could be examined. The untreated *Commelina* tissue seemed ideal for this purpose.

It is of interest to speculate about the factors leading to death of epidermal cells of *Vicia* and *Commelina*. The cause obviously does not affect *Commelina* subsidiary cells, which could rule out the stresses of tissue bending and pulling - these are perhaps relatively evenly distributed. A more likely possibility concerns the effects of breaking intercellular plasmodesmatal connections between the epidermis and mesophyll tissues, since these only occur between epidermal cells and mesophyll cells (personal observations), and do not involve cells of the stomatal complex.

The failure of stomata to close in response to ABA when subsidiary cells were dead emphasises the importance of live *and turgid* subsidiary cells in stomatal movements. The lack of ABA effect at high KCl concentrations (Fig. 16) may be explained in a similar fashion. Since plasmolysis of subsidiary cells was observed at high salt concentrations, turgor would have been reduced and stomatal closure

inhibited. Squire and Mansfield (1972) speculated that *Commelina* stomatal opening at high salt concentrations might be the result of subsidiary cell plasmolysis rather than guard cell ion uptake. This would explain the relative unspecificity of *Commelina* opening for different ionic species compared with, say, *Vicia* (Thomas, 1975), which might therefore be due to osmotic effects rather than guard cell ion uptake. It is not clear why ABA should have different effects if supplied continuously or after opening in $NaNO_3$ buffer (Table 7), but this may relate to secondary induced ion movements. Willmer and Beattie (1978) discussed plasmolysis techniques for examining epidermis cell osmotic potentials. They cautioned that cells of the epidermis were capable of osmotic adjustment during treatment. Certainly, subsidiary cell plasmolysis would not appear to account for all of the opening found at high KCl concentrations, in view of the guard cell $^{86}Rb^+$ uptake observed in Fig.33. Further observations involving plasmolysis studies and autoradiographic assay of sodium ($^{22}Na^+$) uptake and movement are required. Notwithstanding, it is obvious that results of assays of sensitivity to ABA performed at high salt concentrations (e.g. Lancaster *et al.*, 1977) will be dubious.

Responses of stomata to incubation over 2 h in 50 mol m^{-3} KCl/PIPES buffer were found to be remarkably consistent, leading to similar aperture values for controls between experiments (see Figs. 17, 18, 19, 20, 22, 23, 30, 31 and Tables 8, 9), possibly related to the care taken to pre-equilibrate and water correctly the experimental plant material. The importance of such treatments were emphasised in Experiment 1.9, where prior water stress was found to affect greatly apertures attained by epidermis material. In general, results obtained with Incubation System 2 were slightly less predictable than those found with System 1, which may have been due to the control of the carbon dioxide concentration in the latter system.

ABA was able to close pre-opened stomata both completely and rapidly at 50 mol m^{-3} KCl (Fig.17). Indeed, only Willmer *et al.* (1978) appear to have obtained such rapid movements in an epidermal segment system. Some criticism may be made of the relatively high ABA concentrations used throughout this thesis. Such high levels were required for autoradiographic studies owing to the low specific

activity of the available labelled ABA. It was thought that the sensitivity of stomata of *Commelina* to low ABA concentrations was sufficiently established (Ogunkanmi *et al.*, 1973) to allow the use of relatively high hormone concentrations. Some plant hormones exhibit so-called 'optimum curves' for response (e.g. IAA in extension growth). This is not precisely the case with ABA effects on stomata which generally follow a sigmoidal concentration-response pattern (Experiment 1.8; Ogunkanmi *et al.*, 1973). Stimulatory effects of ABA on stomatal aperture have never been reported.

No doubt, had ABA dilution series been employed in the PIPES buffer system used for this thesis, similar results to those of Ogunkanmi *et al.* would have been obtained.

The effects of divalent cations on stomatal aperture (Experiment 1.7) have been found in many other studies (Thomas, 1975) but no adequate explanation of their inhibitory action has been forthcoming. In normal tissue, calcium may be inactivated in the form of calcium oxalate raphides, observed to be abundant in *Commelina* epidermis. For certain, the use of Ca^{++} 'to maintain membrane integrity', at the concentrations advised by some authors, would be totally impractical in *Commelina* segment systems. In view of Fischer's (1972) report that Ca^{++} effects were diminished at high KCl concentrations (100 mol m^{-3}), the constraints earlier discussed with respect to ABA-induced closure must also be borne in mind.

In Experiments 1.8 and 1.9, the physiological state of the leaf used for peeling was noted to have a pronounced effect on the responses of stomata of incubated segments, and from the previous discussion, it is plain that any environmental factor which might affect epidermal ion distributions and concentrations, could also possibly have an effect. In particular, the long-term osmotic potential adjustment of leaves in response to prolonged water stress (Hsiao, 1973) may be important.

The effects of the short-term stress treatment, both in terms of stomatal re-opening after incubation (Fig. 23), and on whole-leaf ABA levels (Fig. 50), exhibited parallelism. It is thus possible to infer that the effects of Fig.23 were a result of the presence of

elevated ABA levels in the epidermis. Another similarity to be noted was that found between the characteristics of stomatal re-opening after ABA pretreatment (Fig. 29) and those found after stress (Figs. 22 and 23), indicating that efflux of ABA from pre-stressed tissue may explain the latter phenomenon. Re-opening after stress or ABA treatment was never found to be complete, however, indicating that the effects of the hormone may not be entirely reversible, at least within 3 h.

The quantitative relationships found between on-leaf aperture and those of peeled epidermis tissue (Experiment 1.10) confirmed the reliability of the *Commelina* epidermis system. The use of freeze-drying to 'fix' apertures whilst other operations were being carried out was convenient for experimental design.

The question arises of the relevance of stomatal aperture measurements to transpiration fluxes and stomatal movement on the whole plant. On whole leaves of a related species (*Zebrina)*, the relationship between pore diameter and transpiration rate is near-linear in moving air (Bange, 1953). In still air, a state rarely found in nature (Meidner and Mansfield, 1968), stomatal control is exerted mainly at low apertures. Stomatal aperture measurements seem therefore to be relevant to normal plant water-loss. A more pertinent point concerns whether the ionic and metabolic changes observed on epidermal segments are relevant to the whole-plant situation.

In the isolation of part of an organism for experimentation , it is inevitable that the normal functions of the separated component(s) are affected; the chief benefits to be obtained being ease of observation and greater control over experimental conditions. Isolated epidermis segments suffer cell death as a consequence of peeling, and the cells of segments floating on dilute solutions are likely to have a higher turgor pressure than those in the epidermis of the intact leaf. This does not appear to affect the ability of *Commelina* stomata to respond to stimuli in much the same way as they do on the leaf (Willmer and Mansfield, 1969), but care is necessary in the choice of medium used for incubation.

Advantages from the use of epidermis material include ease of measurement and the ability to maintain precise environmental conditions and carry out precise changes in them. It is also possible to control and alter experimental variables not readily modified in whole leaves, and to avoid effects caused by other tissues in the leaf. Epidermal segments are thus a convenient tool for the study of stomatal action. However, caution must be observed when extrapolating results to the whole plant, and, where possible, experiments should also be carried out using whole leaves to substantiate any conclusions.

In summary, then, Section 1 described aspects of the stomatal behaviour of Commelina epidermis segments. The importance of the ionic content of the incubation medium, the physiological state of the source leaf, and methods of aperture measurement were described. A system was evolved in which short-term ABA effects could be reliably assessed.

The experiments of Section 2 were designed to investigate the uptake and movement of ABA within epidermal tissue. Radioactivity from ABA was found to be taken up by the tissue (Fig. 25), and this process was dependent upon the presence of living cells (Table 13). No metabolism of the hormone was detected by TLC after 1 h incubation (Fig. 27). Making allowances for the physical processes involved in microautoradiography, the autoradiographs of Experiment 2.4 showed clearly that radioactivity from ABA was taken up by the guard cells. This phenomenon was noticeable after 20 min and became more exaggerated after 40 and 60 min.

In accordance with the above discussion, the experiments were also carried out with whole leaf tissue (Experiments 4.3, 4.4, and 4.5) and essentially similar results were obtained (Fig. 44) - accumulation of ABA in the stomatal complex was found after 40 min when the compound was fed *via* the transpiration stream. The following discussion concentrates on the relevance of these results to endogenous ABA movement during stress.

Part of the answer to this problem lies in the predicted pathway of endogenous ABA to the epidermis, as Lovey's(1977) experiments demonstrated that intra-leaf ABA transport is required for wilt-induced

ABA effects on stomata. There is, however, little evidence to indicate whether transport of endogenous ABA from the mesophyll during stress follows an apoplastic or a symplastic route to the site of action.

If transported *via* the apoplast solution, the hormone must first be released by the synthesising mesophyll cells. Limited support for this occurrence was provided by the ABA efflux demonstrated in Experiment 2.1, but better evidence has been obtained from studies of water-stress effects of ABA levels in sterile liquid-culture of grape pericarp tissue (Loveys *et al.*, 1975) where a proportion of stress-induced ABA was found in the culture medium. Similarly, ABA was released into the incubation medium when *Vicia* leaf mesophyll tissue was stressed *in vitro* (Loveys, 1977).

Once present in the apoplast, the hormone could move to the epidermis by diffusion or in the transpiration stream. The former process would appear to be rapid enough to account for apparently hormonal responses of stomata to stress. Estimating the maximum pathway for diffusion in a typical mesophyte leaf to be 1 mm, over one third of ABA molecules initially present at a point source could have travelled this distance by diffusion within 4.2 min (at 20 C, see General Introduction). The rate of transpiration flow would also appear to be adequate.

Mass flow of water through non-vascular leaf tissue was discussed by Meidner and Sheriff (1976), and probably depends on the extent of leaf vascularisation. In plants tending towards xerophytic habit, flow through mesophyll and epidermal tissues seems inevitable. Some evidence for such flow in *Commelina* was seen in the results shown in Fig. 43, although this was obtained with leaf tissue of high water potential. Meidner (1975) and Sheriff (1977) have suggested that an important portion of the water supply from major veins in leaves travels within the epidermis tissue to sites of evaporation close to the stomatal pores. It is thus perhaps not surprising that a solute such as ABA should accumulate in this region when supplied *via* the transpiration stream. On the other hand, uptake of the hormone in the epidermal segment incubation system suggested that uptake could proceed without evaporative deposition.

The alternative route for ABA transport is symplastic, and for this reason an electron microscope study of plasmodesmatal distribution was made (Section 6). It was found that symplastic transport from mesophyll cells to inner lateral subsidiary cells was feasible, but that transfer between the inner lateral subsidiary cells and guard cells (at least in *Commelina*) had to be apoplastic, because there were no plasmodesmatal connections between these two types of cell.

Within epidermal tissue, study of the movement of the vital stain neutral red (Willmer and Mansfield, 1969) has revealed that a solute transport system exists in *Commelina* which is apparently symplastic. When epidermal segments were placed in neutral red solution, the dye was first taken up by epidermal cells. If the epidermis was then transferred to distilled water, the stain was seen to accumulate in the guard cells, *without first accumulating in the subsidiary cells*. Later, the dye moved out of the epidermal cells and accumulation was noted in subsidiary cells. This seems to implicate the subsidiary cells in a symplastic transfer process. Itai and Meidner (1978a; 1978b) have shown that ABA can affect this uptake of neutral red. ABA-closed stomata did not show such pronounced guard cell uptake of the dye as controls. In the experiments of Section 2, ABA itself is the solute studied, but it seems unfeasible to assess the effects of the hormone on its own distribution.

It appears, therefore, that both pathways are plausible. It may be that both contribute to ABA movement in leaves. What does seem certain, is that to be taken up by guard cells, ABA must cross the guard cell plasmalemma.

The specificity of the ABA uptake phenomenon is doubtful, since guard cells are known to accumulate a number of other substances such as metal ions, sugars, organic acids, and dyes (Willmer and Mansfield, 1969; Fischer, 1972; Dittrich and Raschke,1977a; Willmer and Rutter, 1977; Dittrich and Mayer, 1978). ABA may follow the same route as the carbohydrates from the mesophyll which are apparently necessary for stomatal function (Dittrich and Raschke, 1977b). It should be emphasised that a lack of specificity of the process need not preclude physiological significance. Indeed, it is highly probable that the epidermis is normally supplied with dilute

ABA solution in the form of the transpiration stream, since the hormone has been shown to occur in xylem sap (Davison and Young, 1972). These levels can rise dramatically during stress (Hoad, 1975). Implicit in this statement is the assumption that uptake is related to stomatal movements, and this will now be discussed.

It may be argued that ABA accumulation occurs in an intracellular compartment distant from its site of action. Notwithstanding this possibility, it is almost inconceivable that the hormone could be taken up or transported across a particular cell without coming into contact with these sites, which are presumably located at the plasmalemma.

As seen from the relatively rapid efflux of the hormone from the storage compartments, this contact would be repeated were uptake reversed. The physiological meaning of the efflux pattern of Fig.25 is unclear. In some respects, removal of the active moiety in this way would be advantageous after a stress had ceased. Certainly, efflux was correlated with stomatal reopening in the epidermal incubation system (Fig.29). It is also difficult to assess the efflux phases of Fig.25. Apart from cytoplasm and vacuole, these could correspond to a number of other potential sequestration sites and even to the active sites themselves. Precise conclusions cannot be made until the intracellular site(s) of accumulation and of action are identified.

In brief, Section 2 established the possibility of epidermal ABA accumulation, and characterised this in terms of rate of uptake, site of uptake, and ease of efflux. The hormone appeared to accumulate in the guard cells.

The third Results Section was related to the mode of action of ABA in stomatal closure, using $^{86}RbCl$ to study the effects of the hormone on ion movements. $^{86}Rb^{+}$ was found to accumulate in the guard cells when presented in KCl/PIPES solutions (Fig. 33). The patterns of stomatal response to KCl/PIPES and RbCl/PIPES media were very similar (Fig. 30), supporting the hypothesis that the two alkali metal ions were not discriminated by the tissue. In theory, it would have been possible to carry out the experiments of this section using $^{42}K^{+}$. However, the short half-life of this isotope would have made radioassay much more complicated.

The relationship between stomatal aperture and $^{86}Rb^+$ uptake was near-linear in two experiments (Figs. 31b and 32b), as found by Fischer (1972) in *Vicia* with both $^{42}K^+$ and $^{86}Rb^+$. In *Vicia* , at least, this result is expected on theoretical grounds if it is assumed that the tracer equilibrates with the major guard cell ion pool (Raschke, 1976). In Fischer's study, epidermal segments having few living epidermal cells were selected and it was *assumed* that segment tracer uptake was therefore attributable to the guard cells. In the present study, it has been possible to confirm that epidermis uptake was largely into guard cells, using autoradiography. It is believed that this is the first report of guard cell ion uptake using this technique.

There were, however, some unsatisfactory aspects to the phenomenon of *Commelina* epidermis $^{86}Rb^+$ uptake, the chief of which was the 'hot spot' syndrome found on several segments subjected to autoradiography (Fig.34). It was not evident whether these areas were artifacts of either the microautoradiographic process or the segment rinsing procedure, or whether they represented genuine tissue ion uptake. A large part of the variability found between segments might possibly by due to 'hot spot' effects (e.g. in Figs. 31, 32, and 35).

The effect of ABA on $^{86}Rb^+$ uptake (Experiment 3.4) was interesting as it paralleled the earlier non-quantitative results of Mansfield and Jones (1970). These authors showed, using staining techniques, that the location of endogenous K^+ in *Commelina* was altered by ABA treatment, in favour of the epidermal and subsidiary cells. Such dye location is certainly difficult to quantify (Fischer, 1972) and even the use of quantitative autoradiography (Table 16) can be problematical (Rogers, 1973). More reliable estimates of endogenous cell ion contents can be obtained with ion-specific microelectrodes and ion microprobe analyses (Humble and Raschke, 1971; Penny and Bowling, 1974), although these techniques also have inaccuracies.

The effect of ABA on $^{86}Rb^+$ efflux (Experiment 3.6) is a novel observation which requires corroboration. Similar results have been obtained by MacRobbie *et al.*,(1978; Personal communication). It is not known whether enhanced efflux represented what would normally (in

a whole leaf) be rapid expulsion of ions from the guard cells to cell wall sites or the apoplast fluids, or whether it reflected increased transfer of ions between the guard and subsidiary cells which had been 'short-circuited' by the presence of the incubation medium. Certainly, exchange of ions between the epidermis apoplast and the medium seems likely, although there is evidence that at least part of the stomatal complex is cuticularised on the inner wall which would be in contact with the solution (Fig. 52).

Sites for ions outwith the plasmalemma have been postulated in the fern *Polypodium* (Stevens and Martin, 1977). Results of Saftner and Raschke (1978) indicate that the ion-exchange capacity of the guard cell wall is large. However, the presence of ions in such sites may be temporary before they are taken up by other cells.

It is difficult to postulate in molecular terms how ABA might affect membrane permeability to ions, especially since it may affect different cells in different ways. Thus, the same compound may stimulate guard cell ion release but also stimulate subsidiary epidermal ion uptake. A preliminary study (Collins, 1978) on synthetic membranes has shown that ABA may act like an 'ionophore' to increase membrane conductance. A second possibility is that the hormone affects the ion uptake ATPases (Hsiao, 1976). Until more is known of the mechanism of trans-membrane ion movement it seems that little progress will be made in this area.

To summarise, Section 3 was a description of the ionic relations of the Commelina epidermis in terms of $^{86}Rb^{+}$ *uptake and distribution. The effects of ABA on these processes were examined and the hormone was found to alter both the pattern of uptake and the rate of efflux of the tracer.*

The fourth Results Section involved the development of a whole-leaf system which was then used for re-examining the effects and movement of ABA found in Sections 1 and 2.

Leaves supplied with free water presumably have a high leaf water potential approaching zero. In such conditions combined with high humidity, the stomata opened consistently (Figs. 38, 39, 42 and 43). The stomatal response to high relative humidity has been characterised for a number of species and treatments (e.g.

Lange *et al.*, 1971; Sheriff, 1977; Edwards and Meidner, 1978; Lösch and Schenk, 1978); it appears to result from decreased transpiration from the inner epidermal surfaces, allowing hydropassive movement. A slight disadvantage of the experimental system was therefore that the rate of transpiration was probably low. On the other hand, under certain conditions, transpiration can actually be higher for a given (low) water potential in moist air than in dry air (see Raschke, 1975). An alternative method to induce stomatal opening would have been the use of CO_2-reduced air. However, this would have introduced a further variable to the experiment. Besides this point, it was also not possible to obtain complete stomatal closure with ABA at lower concentrations in CO_2-free air (e.g. Fig.39b).

In normal air, ABA had effects on stomata of whole leaves similar to those obtained with epidermal segments (Figs. 39 and 41, *cf*. Fig.18). An important difference was that the time taken for complete stomatal closure when the hormone was applied *via* the transpiration stream was longer (30-45 min *vs*. 15 min). This was probably related to both the time taken for the hormone to travel to the epidermis in the vascular tissue and to the eventual hormone concentration at the site of action; in the epidermal segment system the 'latent period' for ABA application was almost zero and the concentration of hormone at the guard-cell plasmalemma must have rapidly reached that of the outside medium. These effects may explain why the apparent stomatal sensitivities to ABA estimated in both systems were different by an order of magnitude: in the segment system an excess of ABA was probably present. From Fig. 42 it appears that the number of molecules per stoma required for complete closure estimated from Fig. 41 was a true minimum and not a function of the kinetics of closure: use of lower hormone concentrations did not give a lower value. These very low estimates of stomatal sensitivity coupled with the ABA uptake phenomenon indicate that hormone-induced stomatal movements may take place when even minor adjustments of ABA compartmentation take place (Mansfield *et al* ., 1978).

A problem which has not yet been discussed concerns the possible differential activity of the (+) and (-) optical isomers present in synthetic ABA on both stomatal assay systems. In many other bioassays,

both isomers have near equivalent effects (Milborrow, 1974) but there seems to be some controversy over the situation with stomatal assays (Milborrow, 1974; Raschke, 1975). Cummins and Sondheimer (1973) found (-) - ABA was less active but did have residual activity on excised barley leaves. Kriedemann *et al.* (1972) compared the activity of ABA from a plant extract (presumably only (+) - ABA) with that of the synthetic racemic mixture and obtained a two-fold difference which they explained by inactivity of the (-)-isomer It therefore seems that it may be necessary to halve the sensitivity estimates obtained in Sections 2 and 4 with the synthetic compound. On the other hand, this may not be justified until the effects of (-)-ABA on *Commelina* stomatal movements have been evaluated. Allowance for the small amounts of *t*-ABA present in the synthetic ABA used was not made.

As earlier mentioned, the evidence for guard-cell ABA uptake was strengthened by the fact that it occurred in both stomatal assay systems used. This evidence was not only obtained from microautoradiography; the partitioning of leaf ^{14}C-ABA content varied through the course of application (Tables 18 and 19) in a manner consistent with sequestration within the epidermis. The question of the 85-95% of applied activity which remained in the mesophyll tissue is of interest. It is possible that this was largely present in the vascular tissue but the fact that the hormone accumulated in the adhering mesophyll cells when epidermis was incubated in ABA (Fig. 28) indicated that a certain amount of cellular uptake was possible. It was not possible to estimate what proportion of the ABA present in the epidermis tissue arrived in the transpiration stream, compared with that which might have entered from a pûtative symplastic pathway *via* primary uptake by mesophyll cells.

In outline, Section 4 produced corroborative evidence, using a whole leaf system, to support the conclusions of Section 2. Additionally, a lower 'upper limit estimate' of stomatal sensitivity was obtained.

The fifth Results Section dealt with measurement of endogenous ABA levels in *Commelina*. Several aspects of the purification stage of analysis were examined (Experiments 5.1, 5.2 and 5.3), and a short,

effective procedure adopted (Fig.8). The short-term wilting procedure used in Experiment 1.9 was applied to *Commelina* leaf material and the ABA content estimated using this purification method. Endogenous Me ABA was identified by comparison with the retention times of Me ABA and *t*-Me ABA standards after isothermal GLC with EC detection. It must be emphasised, though, that GLC-ECD is essentially a chromatographic separatory process, and although highly sensitive and selective, it cannot be entirely specific. GLC provides little information content on the configuration and structure of the molecule being analysed. Thus, although it was regarded as highly probable that the peaks measured were a result of a response to Me ABA, ideally, further identification procedures were required. It was considered, however, that the ECD purification technique used was inadequate for further analysis by, for instance, GLC-MS (see discussion on Section 7). On the same grounds, the TLC procedure for analysis of the internal standard (Fig. 48) was also not entirely satisfactory.

Assuming, then, that the measured peaks *were* due to the presence of Me ABA, the calculated ABA contents (Table 25) revealed that ABA levels of *Commelina* whole leaf tissue reopened to stress in the same manner as has been described in many other species (see discussion of Experiment 5.4). For a strict comparison of these results (Fig. 50) with the effects of the same stress on epidermal segment aperture attainment (Fig. 23), or of ABA-pretreatment on epidermal segment aperture attainment (Fig. 29), *epidermis* ABA levels should have been quantified. This would probably have been relatively easier with *Commelina* rather than *Vicia* (i.e. Loveys, 1977), since the former is both easier to peel and carries less contamination on the epidermal tissue.

Section 5 culminated in the estimation of endogenous ABA contents of Commelina whole leaf tissue using GLC-ECD. Levels were found to rise following a wilting treatment.

An attempt was made in Section 6 to examine Commelina epidermis ultrastructure with the specific aim of investigating the plasmodesmatal junctions between cells. Most cells were joined by these connections, but none were observed between guard cells and inner lateral subsidiary cells. The relevance of these results was discussed with those of Section 2.

The seventh and final Section involved a different experimental plant, *Ricinus communis*, which was used to look at the long distance transport of ABA. In some respects, these results may appear to be out of context, but nonetheless, in a study of the hormonal control of a particular phenomenon, the side effects of the response mechanism may be relevant. In the case of wilt-induced ABA, the possibilities of effect of the increased levels of the hormone on other processes besides stomatal movements should be considered. Several of the normal plant responses to stress can be effected by ABA application (e.g. Pitman *et al.*,1974; Quarrie and Jones, 1977). Since the major organ of ABA synthesis during stress is the leaf (Table 2), it seems logical to assume that some degree of transport of the hormone around the plant may occur. This seemed to be the case in a study by Hoad (1975) on sunflower plants. In parallel, very little is known of hormone loading and translocation.

This Section involved the description of a whole plant system in which these phenomena could be observed. ABA appeared to be readily transported and the co-transport of the hormone with sucrose was investigated.

Mass spectrometric identification of ABA in the phloem corroborated earlier results. The fact that full-scan analysis of apparently clean and symmetrical GLC peaks did not result in an $^m/e$ relative abundance spectrum the same as standards, underlined the inadequacy of the GLC technique for assessment of compound identity.

These investigations of ABA transport seemed to result in the formulation of more questions rather than yielding answers, perhaps as a function of their brief and preliminary nature. Some of these were as follows.

(a) are the kinetics of hormone loading determined by the rate of sucrose loading?

(b) can the hormone(s) influence the loading and transport of assimilate?

(c) why was ABA translocated in the free form and at relatively high concentrations?

(d) what are the correlative functions of the hormones in the phloem?

Obviously, further study is required for a full understanding of these points.

Section Seven, in summary, demonstrated that radioactivity from ABA could be transported in the phloem sap of Ricinus following foliar application. ABA was identified in Ricinus phloem sap and the co-transport of radioactivity from both ABA and sucrose was analysed using double-label techniques; A system for the analysis of hormone transport phenomena has thus been described.

In essence, these experiments on ABA in *Commelina* and *Ricinus* showed that the compound was taken up, transported, and exerted demonstrable physiological effects on plant tissues. Ancillary experiments and observations on ion movement and plant structure aided the interpretation of these results on the physiology of ABA. That the closure response of stomata could be initiated by as little as 45.5 amol ABA per stomatal complex (23 million molecules) emphasised the potentially crucial rôle of the substance in the functioning of the intact plant. Whether or not ABA can be regarded as a hormone in the specific instance of stomatal control in water stress forms the point of the following Section.

SECTION B.

THE RESULTS IN RELATION TO THE CONCEPT OF THE HORMONAL NATURE OF WILT-INDUCED ABA ACTION.

This Section of the Discussion presents the work of this thesis in relation to the concepts of hormone action considered in the General Introduction. The evidence that ABA controls stomatal aperture during water stress in a hormonal manner was summarised in Table 3. The results of this thesis will be treated in a similar order.

The data supporting the concept of *parallelism* were re-inforced by the correlation between the increase in endogenous ABA found in Experiment 5.4 and the responses of stomata from similarly stressed leaves in Experiment 1.9. No true additions were made to the evidence upholding *excision*. Corroborative evidence was obtained from the observation that stomata opened when stress was removed

(Experiment 4.1) but closed when water was not supplied (Experiment 1.9).

ABA application clearly resulted in *substitution* for the stimulus of water stress by causing stomatal closure in a number of systems (Experiments 1.5, 1.6, and 4.2). Removal of the hormone (Experiment 2.5 also allowed stomatal re-opening.

In a sense, all the experiments involving epidermal segments and ABA were examples of *isolation*. $^{86}Rb^{+}$ was used to identify the sites and mode(s) of action of the hormone (Experiments 3.4 and 3.6).

The results of Section 5 added another species to the list of those known to respond to stress by ABA production, enforcing the rule of *generality*. Even though *Commelina* is such a common experimental plant in studies of ABA action, the data on ABA effects can also be seen as substantiating these well-proven observations.

The rationale of the *specificity* of ABA uptake has been discussed and it was concluded that lack of specificity in this case need not preclude physiological relevance. No experiments were carried out to examine this aspect of hormone action.

No evidence was obtained to support the criterion of *spatial separation* apart from that which confirmed the effects of ABA on epidermis material.

Transport between tissues was investigated directly with *Ricinus* and indirectly with *Commelina* . It was shown that ABA was readily translocated in the phloem of the former plant (Section 7). The uptake of ABA by the stomatal complex was envisaged as a manifestation of the normal transport route for the hormone between the mesophyll and epidermis or between the xylem contents and the epidermis (Experiments 2.2, 2.4, 4.3 and 4.5).

The observed speed of ABA formation (Milborrow, 1974) could be correlated with the speed of stomatal closure in both experimental systems (Experiments 1.5 and 4.3) to give further corroboration of the nature of the *temporal separation* between the stress stimulus and the stomatal response.

Very little was known about *receptors* in epidermal cells at the time of this investigation. One of the events which presumably

must precede 'reception' is the uptake of a hormone into the target cell. This is a prerequisite unless the receptors are known to exist on the outer surface of the plasmalemma or in the cell wall structure. The guard cell ABA uptake phenomenon observed in this thesis may therefore be analysed as an adaption to allow small amounts of the hormone to reach the active sites. The sensitivity estimates (Experiments 1.5, 2.2, 4.3 and 4.5) can be seen as giving some estimate of the number of receptors/active sites present in the *Commelina* stomatal complex.

In conclusion, the results of this thesis have been not only in general agreement with other studies, but have also validated the hypothesis that abscisic acid acts in a hormonal manner during water deficit stresses.

SECTION C.

FURTHER STUDY OF ABA ACTION ON STOMATA.

Few, if any, of known plant physiological phenomena have been shown conclusively to involve plant hormone action. The evidence that ABA controls stomatal aperture during water stress is relatively good (Table 3), but a number of areas merit further investigation. Some of these are summarised below.

The first topic to be considered is that of endogenous ABA measurement. The relevance of whole-leaf ABA estimations appears to be limited, as discussed in Section III of the General Introduction. Quantification of epidermis ABA levels before and after stress on a number of species is thus required to confirm the observations of Loveys (1977) on *Vicia*. In particular, this type of study needs to be extended to provide a kinetic analysis of stomatal closure during stress combined with assay of the appearance, in the epidermis tissues, of wilt-induced ABA synthesised in the mesophyll. Furthermore, subsequent post-stress stomatal closure and later re-opening should be correlated with epidermis ABA levels. A potential pitfall in this work might be the discrimination between hydropassive and hydroactive

stomatal closure caused by the stress treatment. Presumably, the former would be almost immediately reversed by re-supply of water, whilst the latter would not. Possible side effects of air humidity and CO_2 levels during treatment should be avoided, since these would add additional variables capable of altering stomatal responses independently.

Values of epidermis ABA levels obtained as above could be used to obtain estimates of the sensitivity of stomatal movements to *endogenous* hormone levels. These might then be compared with values obtained with synthetic ABA (as in Section 4). Agreement between the two estimates would support the hypothesis of hormonal involvement in stomatal reaction to stress.

Relatively few measurements of endogenous ABA have been made with adequate identification of the compound. The difficulties this entails for molecules present in plants in such low concentrations as the plant hormones have been discussed, and it seems impossible with present apparatus to reconcile rigorous criteria of identification with measurements on individual plants, organs or tissues. Some compromise involving pooled extracts may be possible. Alternatively, statistical treatment of estimates obtained at different stages in the purification procedure (successive approximation) may suffice.

Outlaw and Lowry (1977) described a method based on enzyme amplification in which it was possible to measure cell substrates (e.g. malate and citrate) in individual guard cell pairs cut from freeze-dried tissue. However, even if enzymes specific for transformations of plant hormones were available for such assays, an additional three orders of magnitude of sensitivity would be required to allow assay of e.g. ABA in guard cells. This is because of the differences in normal concentration between these different classes of compounds. It seems that for some time GLC-ECD will be the most sensitive tool available to plant physiologists for assay of ABA.

Several unresolved areas exist concerning the biosynthesis of ABA. Compared with, say, the gibberellins, the synthesis and breakdown of the ABA molecule is little understood. Knowledge of the intra-cellular compartmentation of ABA is also low. Investigations on chloroplast and cell-free preparations appear promising (Milborrow,1973;

Railton *et al* ., 1974; Loveys, 1977). The 'trigger' for stress-induced ABA synthesis may be leaf water potential or leaf turgor (Beardsell and Cohen, 1975) but the two have not yet been resolved.

Many of the problems of analysis of reactions of even an isolated tissue such as *Commelina* epidermis stem from the fact that several cell types exist in the tissue. A development which may facilitate investigation of responses of individual cells is the production of guard-cell protoplasts. Zeiger and Hepler (1976; 1977) and Schnabl *et al* . (1978) have isolated guard-cell protoplasts of *Vicia* and *Allium* and shown that they can react to stimuli in a manner consistent with that expected of the cells in whole epidermis. If the purity of such preparations can be ensured, it may prove possible to examine the mechanism of ion movement at the membrane level by isolating guard cell plasmalemmae. Compartment analysis of ion and hormone uptake and efflux phenomena would also be more reliable. Effects of these compounds on different cell types from the epidermis could also be investigated and the isolation of receptor proteins might be facilitated.

Control of crop-plant drought resistance by manipulation of stomatal movements has attracted much interest. This might be achieved by application of chemical antitranspirants (Mansfield, 1976b)or by breeding for appropriate characteristics (Jones, 1978b). In both cases a possible use for abscisic acid has been postulated. In the former instance, ABA or an analogue might be employed in a foliar spray applied at strategic points of growth or when certain environmental conditions were predicted. This has prompted the screening of chemicals for properties of stomatal closure (Orton and Mansfield, 1974) but no compound related to ABA has been found to have higher activity than the hormone itself (Mansfield, 1976b). On theoretical bases, it has been concluded that a given degree of stomatal closure would exert a larger proportional effect on transpiration than on CO_2 fixation (Meidner and Mansfield, 1968). This has been confirmed by experimental investigations using ABA spray treatments (e.g. Mizrahi *et al.*, 1974). Such treatments are also found to alter development and morphology in the long-term (e.g. Quarrie and Jones, 1977).

Whether changes such as reduced stomatal numbers and smaller leaf size area a direct consequence of ABA action or indirectly due to ABA-caused stomatal closure is not known.

Selection of plants for plant hormonal attributes is only one of the possible methods available to the plant breeder breeding for drought resistance (Jones, 1978b). ABA measurements have been made on stress tolerant *Zea* varieties, and a positive correlation between the formation of ABA and the tolerance was found (Laarque-Saavedra and Wain, 1974; 1976). Despite this apparent success, several other considerations must be taken into account. Examples are the time taken for recovery from short-term stresses and the eventual economic yield in cultivars which are relatively tolerant. Thus, the reduction in photosynthesis and harvestable yield which accompanies stomatal closure may outweigh any saving in water (Jones, 1978a).

The main themes of future long-term and short-term research would appear to lie in (a) the use of ABA as a tool for elucidation of the mechanism of stomatal functioning, (b) the applied facet of the use of ABA and related compounds for manipulation of crop growth and yield, (c) the investigation of the stress/ABA/stomatal closure system as a model physiological system to clarify aspects of phytohormone action, and (d) peripheral studies of the biochemistry and biophysics of ABA and its action.

REFERENCES

Papers and books not actually consulted but cited through another authority are marked with an asterisk after the date.

Abeles, F.B., 1974: Ethylene in Plant Biology. Academic Press, New York.

Addicott, F.T., Lyon, J.L., 1969: Physiology of Abscisic Acid and Related Substances. Ann. Rev. Pl. Physiol. 20, 139-164.

Addicott, F.T., Wiatr, S.M., 1977: in: Plant Growth Regulation. Ed. P.E. Pilet, Springer-Verlag, Berlin.

Aharoni, N., 1978: Relationship between Leaf Water Status and Endogenous Ethylene in Detached Leaves. Plant Physiol. 61, 658-662.

Allaway, W.G., 1973: Accumulation of Malate in Guard Cells of *Vicia faba* during Stomatal Opening. Planta 110, 63-70.

Allaway, W.G., Hsiao, T.C., 1973: Preparation of Rolled Epidermis of *Vicia faba* L. so that Stomata are the Only Viable Cells: Analysis of Guard Cell Potassium by Flame Photometry. Aust. J. Biol. Sci. 26, 309-318.

Allaway, W.G., Mansfield, T.A., 1970: Experiments and Observations on the After-effects of Wilting on Stomata of *Rumex sanguineus*. Can. J. Bot. 48, 513-521.

Alvim, R., Hewett, E.W., Saunders, P.F., 1976: Seasonal Variation in the Hormone Content of Willow 1. Changes in Abscisic Acid Content and Cytokinin Activity in the Xylem Sap. Plant Physiol. 57, 474-476.

Audus, L.J., 1972: in: Plant Growth Substances Vol.I: Chemistry and Physiology 3rd Edn. St. Pauls Press Ltd., Malta.

Bailey, L.H., 1925: The Standard Cyclopedia of Horticulture Vol.1. MacMillan Co., New York.

Baker, D.A., Hall, J.L., 1975: Ion Transport in Plant Cells and Tissues. North-Holland Pub. Co., Amsterdam.

Bandurski, R.S., Schulze, A., 1974*: Concentrations of Indole-3-acetic Acid and its Esters in *Avena* and *Zea*. Plant Physiol. 54, 257-262.

Bange, G.G.J., 1953: On the Quantative Explanation of Stomatal Transpiration. Acta. Bot. Neerlandia 2, 255-296.

Barrington, E.J.W., 1964: Hormones and Evolution. English Universities' Press, Ltd., London.

Bayliss, W.M., Starling, E.H., 1902: On the Causation of the So-called Periferal Reflex Secretion of the Pancreas. (Preliminary Communication). Proc. R. Soc., 69, 352-353.

Bayliss, W.M., Starling, E.H., 1904: The Chemical Regulation of the Secretory Process. Proc. R. Soc., 73, 310-322.

Beardsell, M.F., Cohen, D., 1975 : Relationship between Leaf Water Status, Abscisic Acid Levels and Stomatal Resistance in Maize and Sorghum. Plant Physiol., 56, 207-212.

Bennet-Clark, T.A., Kefford, N.P., 1953: Chromatography of the Growth Substances in Plant Extracts. Nature 171, 645-647.

Berrie, A.M.M., Don, R., Buller, D., Alam, M., Parker, W., 1975: The Occurrence and Function of Short Chain Length Fatty Acids in Plants. Plant Sci. Lett. 6, 163-173.

Birch, M., 1974: Pheremones. Frontiers of Biology, 32. Ed. Neuberger, A., Tatum, E.L., North-Holland Pub. Co., Amsterdam.

Bittner, S., Even-Chen, Z., 1975: A GLC Procedure for Determining Sub-nanogram Levels of Indol-3yl Acetic Acid. Phytochem. 14, 2455-2457.

Boussiba, S., Rikin, A., Richmond, A.E., 1975: The Role of Abscisic Acid in Cross-Adaption of Tobacco Plants. Plant Physiol., 56, 337-339.

Bowling, D.J.F., 1976: Malate-switch Hypothesis to Explain the Action of Stomata. Nature 262, 393-394.

Boysen-Jensen, 1913*: Über die Leitung des Phototropischen Reizes in der *Avena* -koleoptile. Ber. Deut. Bot. Ges, 31, 559-566.

Brown, K.W., Jordan, W.R., Thomas, J.C., 1976: Water Stress Induced Alterations of the Stomatal Response to Decreases in Leaf Water Potential. Physiol. Plant, 37, 1-5.

Brown, S.A., Wetter, L.R., 1972: in: Progress in Phytochemistry (Vol.3). Eds. Reinhold, L., Liwschitz, Y. Interscience Publishers, London.

Browning, G., Saunders, P.F., 1977: Membrane Localised Gibberellins A_9 and A_4 in Wheat Chloroplasts. Nature 265, 375-377.

Cahn, R.S., Ingold, C.K., Prelog, V., 1966: Specification of Molecular Chirality. Agnew. Chem. Int. Ed. Engl. 5, 385.

Carr, D.J., 1976: in: Intercellular Communication in Plants: Studies on Plasmodesmata. Eds. Gunning, B.E.S., Robards, A.W., Springer-Verlag, Berlin.

Carr, D.J., Reid, D.M., Skene, K.G.M., 1964: The Supply of Gibberellins from the Root to the Shoot. Planta 63, 382-392.

Ciha, A.J., Brenner, M.L., Brun, W.A., 1977: Rapid Separation and Quantification of Abscisic Acid from Plant Tissues Using High Performance Liquid Chromatography. Plant Physiol. 59, 821-826.

Collins, J.C. , 1978: Abscisic Acid Action in Ion and Water Transport at the Membrane Level. Fed. Eur. Soc. Pl. Physiol. Inaug. Meeting Edinburgh Abs. 138-139.

Colquhoun, A.J., 1974: Ph.D. Thesis, University of Glasgow.

Colquhoun, A.J., Hillman, J.R., 1972: The Effects of ABA on Senescence in Leaf Discs of Radish *Raphanus sativus L.* Planta 105, 213-224.

Coombe, B.G., Cohen, D., Paleg, L.G., 1967: Barley Endosperm Bioassay for Gibberellins II. Application of the Method. Plant Physiol. 42, 113-119.

Cornforth, J.W., 1969: Exploration of Enzyme Mechanisms by Asymmetric Labelling. Quart. Rev. Chem. Soc. 23, 125-140.

Cornforth, J.W., Milborrow, B.V., Ryback, G., 1965b: Synthesis of (±) - Abscisin II. Nature 206, 715.

Cornforth, J.W., Milborrow, B.V., Ryback, G., 1966: Identification and Estimation of (+) - Abscisin II ('Dormin') in Plant Extracts by Spectropolarimetry. Nature 210, 627-628.

Cornforth, J.W., Milborrow, B.V., Ryback, G., Wareing, P.F., 1965a: Chemistry and Physiology of 'Dormins' in Sycamore. Nature 205, 1269-1270.

Cram, W.J., 1976: in: Encyclopedia of Plant Physiology (New Edn. Vol.2A). Gen. Eds. Pirson, A., Zimmermann, M.H. Springer-Verlag, Berlin.

Cummins, W.R., 1973: The Metabolism of Abscisic Acid in Relation to its Reversible Action on Stomata in Leaves of *Hordeum Vulgare L.* Planta 114, 159-167.

Cummins, W.R., Kende, H., Raschke, K., 1971: Specificity and Reversibility of the Rapid Stomatal Response to Abscisic Acid. Planta 99, 347-351.

Darwin, C., Darwin, G., 1880*: The Power of Movement in Plants. Appleton-Century Inc., New York.

Davies, P.J., Probesting, W.M., Gianfagna, T.J., 1977: in: Plant Growth Regulation. Ed. P.E. Pilet, Springer-Verlag, Berlin.

Davis, L.A., Addicott, F.T., 1972: Abscisic Acid: Correlations with Abscission and with Development in the Cotton Fruit. Plant Physiol. 49, 644-648.

Davis, L.A., Heinz, D.E., Addicott, F.T., 1968: Gas-Liquid Chromatography of Trimethylsilyl Derivatives of Abscisic Acid and other Plant Hormones. Plant Physiol., 43, 1389-1394.

Davison, R.M., Young, H., 1972: Abscisic Acid content of Xylem Sap. Planta 109, 95-98.

Dennis, F.G., 1977: Growth Hormones: Pool Size, Diffusion, or Metabolism? Hort. Sci. 12, 217-219.

Dittrich, P., Mayer, M., 1978: Inhibition of Stomatal Opening during Uptake of Carbohydrates by Guard Cells in Isolated Epidermal Tissues. Planta 139, 167-170.

Dittrich, P., Raschke, K., 1977a: Malate Metabolism in Isolated Epidermis of *Commelina communis L.* in relation to Stomatal Functioning. Planta 134, 77-81.

Dittrich, P., Raschke, K., 1977b: Uptake and Metabolism of Carbohydrates by Epidermal Tissue. Planta 134, 83-90.

Dodoo, G., 1977: Ph.D. Thesis, University of Glasgow.

Dörffling, K., 1978: The Possible Rôle of Xanthoxin in Plant Growth and Development. Phil. Trans. R. Soc. Lond. B. in Press.

Dörffling, K., Streich, J., Kruss, W., Muxfeldt, B., 1977: Abscisic Acid and the After-effect of Water Stress on Stomatal Opening Potential. Z. Pflanzenphysiol. 81, 43-56.

Drury, R.E., 1969: Interaction of Plant Hormones. Science 164, 564-565.

Eagles, C.F., Wareing, P.F., 1964: The Role of Growth Substances in the Regulation of Bud Dormancy. Physiol. Plant. 17, 697-708.

Edwards, M., Meidner, H., 1978: Stomatal Responses to Humidity and the Water Potentials of Epidermal and Mesophyll Tissue. J. Exp. Bot. 29, 771-780.

El-Beltagy, A.S., Hall, M.A., 1974: Effect of Water Stress upon Endogenas Ethylene Levels in *Vicia faba*. New Phytol. 73, 47-59.

Epstein, E., 1972: in: Mineral Nutrition of Plants: Principles and Perspectives. John Wiley & Sons, Inc., New York.

Erdei, L., Zsoldos, F., 1977: Potassium Absorbtion by Rice at Different Levels of Organisation 1. Effects of Temperature and Calcium on K^+ Fluxes and Content.

Esau, K., 1965: Plant Anatomy 2nd Ed. John Wiley & Sons Inc., New York.

Evanari, M., 1961: in: Encyclopaedia of Plant Physiology 16, 691-736. Springer, Berlin.

Evans, L.T., 1976: in: Transport and Transfer Processes in Plants. Eds. Wardlaw, I.F., Passioura, J.D. Academic Press, New York.

Fensom, D.S., 1975: in: Encyclopedia of Plant Physiology (New Edn. Vol. 1). Gen. Eds. Pirson, A., Zimmermann, M.H. Springer-Verlag, Berlin.

Fenton, R., Davies, W.J., Mansfield, T.A., 1977: The Role of Farnesol as a Regulator of Stomatal Opening in *Sorghum*. J. Exp. Bot. 28, 1043-1053.

Fenton, R., Mansfield, T.A., Wellburn, A.R., 1976: Effects of Isoprenoid Alcohols on Oxygen Exchange of Isolated Chloroplasts in Relation to their Possible Physiological Effects on Stomata. J. Exp. Bot. 27, 1206-1214.

Fifield, F.W., Kealey, D., 1975: Analytical Chemistry. International Textbook Co., Ltd., London, U.K.

Fischer, R.A., 1968: Stomatal Opening in Isolated Epidermal Strips of *Vicia faba*. I. Response to Light and CO_2-free Air. Plant Physiol. 43, 1947-1952.

Fischer, R.A., 1970: After-effect of Water Stress on Stomatal Opening Potential II. Possible Causes. J. Exp, Bot. 21, 386-404.

Fischer, R.A., 1972: Aspects of Potassium Accumulation by Stomata of *Vicia faba*. Aust. J. Biol. Sci. 25, 1107-1123.

Fitting, H., 1909*: Die Beeinflussing der Orchideenblüten durch die Bestäubung und durch andere Umstände. Z. Bot. 1, 1-86.

Fitting, H., 1910*: Weitere Entwicklungsphysiologische Untersuchungen an Orchideenblüten. Z. Bot. 2; 225-266.

Fitzgerald, P.J., Ord, M.G., Stocken, L.A., 1961: A 'Dry' Mounting Autoradiographic Technique for the Localization of Water-Soluble Compounds. Nature 189, 55-56.

Folkers, K., Litterru, G.P., Ho, L., Runge, T.M., Havanonda, S., Cooley, D., 1970*: (Title Unknown) Int. J. Vit. Nut. Res. 40, 380.

Foster, R.J., McRae, D.H., Bonner, T., 1952*: Auxin-induced Growth Inhibitions, a Natural Consequence of Two-point Attachment. Proc. Nat. Acad. Sci. 38, 1014-1022.

Friedlander, M., Atsmon, D., Galun, E., 1976: Uptake, Transport and Stability of [2-^{14}C] Abscisic Acid in Cucumber Following Foliar Application. Plant & Cell Physiol 17, 965-972.

Frye, B.E., 1967: Hormonal Control in Vertebrates. The Macmillan Co., New York.

Fuijino, M., 1967*: Role of Adenosine triphosphate and Adenosinetriphosphatase in Stomatal Movement. Sci. Bull. Fac. Educa. Nagasaki Univ. 18, 1-47.

Gaff., D.F., Ogong'O-Ogola, 1971: The Use of Non-permeating Pigments for Testing the Survival of Cells. J. Exp. Bot. 22, 756-758.

Gaskin, P., MacMillan, J., 1968: Identification and Estimation of Abscisic Acid in a Crude Plant Extract by Combined Gas Chromatography - Mass Spectrometry. Phytochem. 7, 1699-1701.

Gertman, E., Fuchs, Y., 1972: Effect of Abscisic Acid and its Interactions with Other Plant Hormones on Ethylene Production in Two Plant Systems. Plant Physiol. 50, 194-195.

Gillard, D.F., Walton, D.C., 1976: Abscisic Acid Metabolism by a Cell-Free Preparation from *Echinocystis lobata* Liquid Endosperm. Plant Physiol. 58, 790-795.

Glenn, J.L., Kuo, C.C., Durley, R.C., Pharis, R.P., 1971: Use of Insoluble polyvinyl-pyrrolidone for Purification of Plant Extracts and Chromatography of Plant Hormones. Phytochem. 11, 345-351.

Glinka, Z., 1973: Abscisic Acid Effect on Root Exudation Related to Increased Permeability to Water. Plant Physiol. 51, 217-219.

Glinka,Z., Reinhold, L., 1971: Abscisic Acid Raises the Permeability of Plant Cells to Water. Plant Physiol. 48, 103-105.

Glinka,Z., Reinhold, L., 1972: Induced Changes in Permeability of Plant Cell Membranes to Water. Plant Physiol. 49, 602-606.

Goad, L.J., Knapp, F.F., Lenton, J.R., Goodwin, T.W. 1972*: Observations on the Sterol Side-chain Alkylation Mechanism in a *Trebouxia* Species.

Goldbach, H., Goldbach, E., 1977: Abscisic Acid Tranlocation and Influence of Water Stress on Grain Abscisic Acid Content. J. Exp. Bot. 28, 1342-1350.

Goldbach, H., Goldbach, E., Michael, G., 1977: Transport of Abscisic Acid from Leaves to Grains in Wheat and Barley Plants. Naturwissenschaften 64, 488.

Goldsmith, M-H.M., 1969: in: The Physiology of Plant Growth and Development. Ed. M.B. Wilkins. McGraw-Hill, London.

Goldsmith, M-H.M., 1977: The Polar Transport of Auxin. Ann. Rev. Pl. Physiol. 28, 439-478.

Good, N.E., Winget, G.G., Winter, W., Connolly, T.N., Izawa, S., Singh, R.M.M., 1966: Hydrogen Ion Buffers for Biological Research. Biochem. 5, 467.

Goodwin, T.W., 1973: in: 'Phytochemistry'. Ed. L.P. Miller, Van Nostrand Reinhold Co., New York.

Gray, R.J., Mallaby, R., Ryback, G., Williams, V.P., 1974*: Mass Spectra of Methyl Abscisate and Isotopically-Labelled Analogues. J. Chem. Soc. 8, 919-924.

Gross, D., 1975: Growth Regulating Substances of Plant Origin. Phytochem. 14, 2105-2112.

Hall, M.H., Baker, D.A., Milburn, J.A., 1971: Phloem Transport of ^{14}C-labelled Assimilates in *Ricinus*. Planta 100, 200-207.

Hall, S.M., Milburn, J.M., 1973: Phloem Transport in *Ricinus*: Its Dependence on the Water Balance of the Tissues. Planta 109, 1-10.

Harborne, J.B., 1973: Phytochemical Methods. Chapman and Hall, London.

Harrison, M.A., Walton, D.C., 1975: Abscisic Acid Metabolism in Water-stressed Bean Leaves. Plant Physiol. 56, 250-254.

Hartung, W., 1976*: Der Basipetale [2-^{14}C] Abscisinsäuretransport in Wurzeln intakter Bohnenkeimlinge und Siene Bedeutung für den Wurzelgeotropismus. Planta 128, 59-62.

Heddon, P., MacMillan, J., Phinney, B.O., 1978: The Metabolism of Gibberellins. Ann. Rev. Pl. Physiol. 29, 149-192.

Hemberg, T., Tillberg, E., 1978: The Yield of Auxin with Different Extraction Solvents and Methods. Fed. Eur. Soc. Plant Physiol. Inaug. Meeting Edinburgh Abs. 255-256.

Hiatt, A.J., 1970: An Anomoly in Potassium Accumulation by Barley Roots 1. Effects of Anions, Sodium Concentrations, and Length of Absorbtion Period. Plant Physiol. 45, 408-419.

Hiron, R.W.P., Wright, S.T.C., 1973: The Role of Endogenous Abscisic Acid in the Response of Plants to Stress. J. Exp. Bot. 24, 769-781.

Hillman, J.R., 1978: Isolation of Plant Growth Substances. Cambridge University Press, London.

Hillman, J.R., Hocking, T.J., McWha, J.A., 1973: in: Plant Growth Substances 1973. Hirokawa Publishing Co., Inc. Tokyo.

Hillman, J.R., Young, I., Knights, B.A., 1974: Abscisic Acid in Leaves of *Hedera helex L.* Planta 119, 263-266.

Hipkins, M.F., 1978: The Emission Yields of Delayed and Prompt Fluorescence from Chloroplasts. Biochem. Biophys. Acta 502, 161-168.

Hoad, G.V., 1973: Effect of Moisture Stress on Abscisic Acid Levels in *Ricinus communis L.* Planta 113, 367-372.

Hoad, G.V., 1978: Effect of Water Stress on Abscisic Acid Levels in White Lupin (*Lupinus albus L.*) Fruit, Leaves and Phloem Exudate. Planta 142, 287-290.

Hocking, T.J., 1974: Ph.D. Thesis, University of Glasgow.

Hocking, T.J., Hillman, J.R., Wilkins, M.B., 1972: Movement of Abscisic Acid in Phaseolus vulgaris plants. Nature New Biology 235,124-125.

Horton, R.F., 1970: Stomatal Opening: the Role of Abscisic Acid. Can. J. Bot. 49, 583-585.

Horton, R.F., Moran, L., 1972: Abscisic Acid Inhibition of Potassium Influx into Stomatal Guard Cells. Z. Pflanzenphysiol. 66, 193-196.

Housley, S., Taylor, W.C., 1958: Studies on Growth Hormones VI. The Nature of Inhibitor -β in Potato. J. Exp. Bot. 9, 458-451.

Hsiao, T.C., 1973: Plant Responses to Water Stress. Ann. Rev. Plant. Physiol. 24, 519-570.

Hsiao, T.C., 1976: in: Encyclopedia of Plant Physiology (New Edn. Vol. 2B). Gen. Eds. Pirson, A., Zimmermann, M.H. Springer-Verlag, Berlin.

Humble, G.D., Hsiao, T.C., 1969: Specific Requirement of Potassium for Light-activated Opening of Stomata in Epidermal Strips. Plant Physiol. 44, 230-234.

Humble, G.D., Hsiao, T.C., 1970: Light-dependent Influx and Efflux of Potassium of Guard Cells during Opening and Closing. Plant Physiol. 46, 483-487.

Humble, G.D., Raschke, K., 1971: Stomatal Opening Quantitatively Related to Potassium Transport: Evidence from Electron Probe Analysis. Plant Physiol. 48, 447-453.

Huxley, J.S., 1935: Chemical Regulation and the Hormone Concept. Biol. Rev. 10, 427-441.

Iljin, W.S., 1957: Drought Resistance in Plants and Physiological Processes. Ann. Rev. Pl. Physiol. 8, 257-274.

Imber, D., Tal, M., 1970: Phenotypic Reversion of *flacca*, a Wilty Mutant of Tomato, by Abscisic Acid. Science 169, 592-593.

Itai, C., Benzioni, A., 1976: in: Water and Plant Life Vol.19. Eds. Lange, O.L., Kappen, L., Schulze, E-D. Springer-Verlag, Berlin.

Itai, C., Meidner, H., 1978a: Effect of Abscisic Acid on Solute Transport in Epidermal Tissue. Nature 271, 653-654.

Itai, C., Meidner, H., 1978b: Functional Epidermal Cells are Necessary for Abscisic Acid Effects on Guard Cells. J. Exp. Bot. 29, 765-770.

Itai, C., Weyers, J.D.B., Hillman, J.R., Meidner, H., Willmer, C., 1978: Abscisic Acid and Guard Cells of *Commelina communis*. Nature 271, 652-653.

Jackson, M.B., Osborne, D.J., 1970: Ethylene, The Natural Regulator of Leaf Abscission. Nature 225, 1019-1022.

Jacob, F., Monod, J., 1961: Genetic Regulatory Mechanisms in the Synthesis of Proteins. J. Mol. Biol. 3, 318-356.

Jacobs, W.P., 1959: What Substance Normally Controls a Given Biological Process?:1. Formulation of Some Rules. Dev. Biol. 1, 527-533.

Jeffries, 1972: Unpublished results cited in Milborrow, 1972.

Jones, H.G., 1978: in: Stress Physiology of Crop Plants. Eds. Mussell, H., Staples, R.C. Wiley, New York.

Jones, R.J., Mansfield, T.A., 1970: Suppression of Stomatal Opening im Leaves Treated with Abscisic Acid. J. Exp. Bot. 21, 714-719.

Jones, R.L., 1973: Gibberellins: Their Physiological Role. Ann. Rev. Pl. Physiol. 24, 571-598.

Jones, W.W., Coggins, C.W. Jr., Embleton, T.W., 1976: Endogenous Abscisic Acid in Relation to Bud Growth in Alternate Bearing 'Valencia' Orange. Plant Physiol. 58, 681-682.

Kahn, A.A., 1975: Primary, Preventive and Permissive Roles of Hormones in Plant Systems. Bot. Rev. 41, 391-420.

Kefeli, V., Kadyrov, C., 1971: Natural Growth Inhibitors, their Chemical and Physiological Properties. Ann. Rev. Pl. Physiol. 22, 185-196.

Kende, H., Gardner, G., 1976: Hormone Binding in Plants. Ann. Rev. Plant Physiol. 27, 267-290.

King, R.W., 1976: in: Transport and Transfer Processes in Plants. Eds. Wardlaw, I.F., Passioura, J.D., Academic Press, New York.

Kozlowski, T.T., 1968: Water Deficits and Plant Growth Vols.I-III. Academic Press, New York.

Kriedemann, P.E., Loveys, B.R., 1975: in: Environmental and Biological Control of Photosynthesis. Ed. R. Marcelle, Dr. W. Junk b.v. Publishers, The Hague, Holland. pp 227-236.

Kriedemann, P.E., Loveys, B.R., Fuller, G.L., Leopold A.C., 1972: Abscisic Acid and Stomatal Regulation. Plant Physiol. 49, 842-847.

Lancaster, J.E., Mann, J.D., Porter, N.G., 1977: Ineffectiveness of Abscisic Acid in Stomatal Closure of Yellow Lupin, *Lupinus Luteus* var. Weiko III. J. Exp. Bot. 28, 184-191.

Lange, O.L., Lösch, R., Schulze, E-D., Kappen, L.,1971: Responses of Stomata to Changes in Humidity. Planta 100, 76-86.

Larque-Saavedra, A., Wain, R.L., 1974: Abscisic Acid in Relation to Drought Tolerance in Varieties of *Zea Mays L.* Nature 251, 716-717.

Larque-Saavedra, A., Wain, R.L., 1976: Studies on Plant Growth-Regulating Substances: XLII. Abscisic Acid as a Genetic Character Related to Drought Tolerance. Ann. Appl. Biol. 83, 291-297.

Larsen, P., 1955: Nomenclature of Plant Growth Substances. Plant Physiol. 30, 190-191.

Lenton, J.R., Bowen, M.R., Saunders, P.F., 1968: Detection of Abscisic Acid in the Xylem Sap of Willow (*Salix viminalis L.*) by Gas-liquid Chromatography. Nature 220, 86-87.

Lenton, J.R., Perry, V.M., Saunders, P.F., 1971: The Identification and Quantitative Analysis of Abscisic Acid in Plant Extracts by Gas-Liquid Chromatography. Planta 96, 271-280.

Lenton, J.R., Perry, V.M., Saunders, P.F., 1972: Endogenous Abscisic Acid in Relation to Photoperiodically Induced Bud Dormancy. Planta 106, 13-22.

Little, C.H.A., Eidt, D.D., 1968: Effect of Abscisic Acid on Budbreak and Transpiration in Woody Species. Nature 220, 498-499.

Liu, W-C., Carns, H., 1961: Isolation of Abscisin, an Abscission Accelerating Substance. Science 134, 384-385.

Livne, A., Vaadia, Y., 1965: Stimulation of Transpiration Rate in Barley Leaves by Kinetin and Gibberellic Acid. Physiol. Plant. 18, 658-664.

Lösch, R., 1978: Responses of Stomata to Environmental Factors - Experiments with Isolated Epidermal Strips of *Polypodium vulgare* 1. Temperature and Humidity. Oecologia 29, 85-97.

Löve, A., Löve, D., 1945: Experiments on the Effects of Animal Sex Hormones on Dioecious Plants. Arkiv. För Botanik 32, 1-60.

Lovelock, J.E., 1958: A sensitive Detector for Gas Chromatography. J. Chromatog. 1, 35-46.

Lovelock, J.E., 1961: Ionization Methods for the Analysis of Gases and Vapours. Anal. Chem. 33, 162-178.

Lovelock, J.E., 1963: Electron-capture Detectors and Technique for use in Quantitative and Qualitative Analysis by Gas Chromatography. Anal. Chem. 35, 474-481.

Loveys, B.R., 1977: The Intracellular Location of Abscisic Acid in Stressed and Non-stressed Leaf Tissue. Physiol. Plant 40, 6-10.

Loveys, B.R., Brien, C.J., Kriedemann, P.E., 1975: Biosynthesis of Abscisic Acid under Osmotic Stress: Studies Based on a Dual Labelling Technique. Physiol. Plant 33, 166-170.

Loveys, B.R., Kriedemann, P.E., 1974: Internal Control of Stomatal Physiology and Photosynthesis. 1. Stomatal Regulation and Associated Changes in Endogenous Levels of Abscisic and Phaseic Acids. Aust. J. Plant Physiol. 1, 407-415.

Loveys, B.R., Kriedemann, P.E., Törökfalvy, E., 1973: Is Abscisic Acid Involved in Stomatal Response to Carbon Dioxide? Pl. Sci. Lett. 1, 335-338.

McDougall, J., Hillman, J.R., 1978: in: Isolation of Plant Growth Substances. Ed. Hillman, J.R. Cambridge University Press, London.

Machus, L., 1972: The Coming of Age of Sex Hormones in Plants. Mycologia 64, 235-247.

MacKinney, G., 1941: Absorbtion of Light by Chlorophyll Solutions. J. Biol. Chem. 140, 315-322.

MacRobbie, E.A.C., Lettau, J., Bray, M., 1978: Ionic Relations of Stomatal Guard Cells. Fed. Eur. Soc. Pl. Physiol. Inaug. Meeting Edinburgh Abs. 341-342.

McWha, J.A., 1973: Ph.D. Thesis, University of Glasgow.

McWha, J.A., Philipson, J.J., Hillman, J.R., Wilkins, M.B., 1972: Molecular Requirements for ABA Activity in Two Bioassay Systems. Planta 109, 327-336.

Mahan, B.H., 1970: University Chemistry 2nd ed. Addison-Wesley Publishing Co., Reading, U.S.A.

Mansfield, T.A., 1976a: Delay in the Response of Stomata to Abscisic Acid in CO_2-free Air. J. Exp. Bot. 27, 559-564.

Mansfield, T.A., 1976b: Stomatal Behaviour: Chemical Control of Stomatal Movements. Phil. Trans. R. Soc. Lond. B. 273, 541-550.

Mansfield, T.A., Jones, R.J., 1971: Effects of Abscisic Acid on Potassium Uptake and Starch Content of Stomatal Guard Cells. Planta 101, 147-158.

Mansfield, T.A., Wellburn, A.R., Morieira, T.J.S., 1978: The Role of Abscisic Acid and Farnesol in the Alleviation of Water Stress and their Mechanism of Action on Stomata. Phil. Trans. Roy. Soc. Lond. B. In press.

Marrè, E., 1977: in: Regulation of Cell Membrane Activities in Plants. Eds. Marre,E., Cifferi, I. Elsevier/North-Holland Biomedical Press, Amsterdam.

Meidner, H., 1965: Stomatal Control of Transpirational Water Loss. Symp. Soc. Exp. Biol. 19, 185-204.

Meidner, H., 1967: The Effect of Kinetin on Stomatal Opening and the Rate of Intake of CO_2 in Mature Primary Leaves of Barley. J. Exp. Bot. 18, 556-561.

Meidner, H., 1975: Water Supply, Evaporation, and Vapour Diffusion in Leaves. J. Exp. Bot. 26, 666-673.

Meidner, H., Mansfield, T.A., 1968: Physiology of Stomata. McGraw-Hill, New York.

Meidner, H., Sheriff, D.W., 1976: Water and Plants. Blackie, Glasgow.

Meidner, H., Willmer, C., 1975: Mechanics and Metabolism of Guard Cells. Current Advances in Plant Science 17, 1-15.

Mer, C.L., 1968: What is an Inhibitor? Z. Pflanzenphysiol. 59, 415-419.

Milborrow, B.V., 1967: The Identification of (+)-Abscisin II [(+)-Dormin] in Plants and Measurement of its Concentrations. Planta 76, 93-113.

Milborrow, B.V., 1970: The Metabolism of Abscisic Acid. J. Exp. Bot. 21, 17-29.

Milborrow, B.V., 1972: in: Plant Growth Substances 1970. Ed. D.J. Carr. Springer, Berlin. pp 281-290.

Milborrow, B.V., 1973: Biosynthesis of Abscisic Acid by a Cell-free System. Phytochemistry 13, 131-136.

Milborrow, B.V., 1974: Chemistry and Physiology of Abscisic Acid. Ann. Rev. Pl. Physiol. 25, 259-307.

Milborrow, B.V., Mallaby, R., 1975: Occurrence of Methyl (+)-Abscisate as an Artefact of Extraction. J. Exp. Bot. 26, 741-748.

Milborrow, B.V., Noddle, R.C,, 1970: Conversion of 5(1'2'-Epoxy-2'6'6'-trimethyl- 1-cyclohexyl) - 3 methyl-penta -cis-2-trans-4-dienoic acid in Abscisic Acid in Plants. Biochem. J. 119, 727-734.

Milborrow, B.V., Robinson, D.R., 1973: Factors Affecting the Biosynthesis of Abscisic Acid. J. Exp. Bot. 24, 537-548.

Milburn, J.A., 1975: in: Encyclopedia of Plant Physiology (new Edn. Vol.1) Gen. Eds. Pirson, A., Zimmermann, M.H. Springer-Verlag, Berlin.

Mittelheuser, C.J., Van Steveninck, R.F.M., 1969: Stomatal Closure and Inhibition of Transpiration Induced by (RS) - Abscisic Acid. Nature 221, 281-282.

Mittelehuser, C.J., Van Steveninck, R.F.M., 1971: Rapid Action of Abscisic Acid on Photosynthesis and Stomatal Resistance. Planta 97, 83-86.

Mizrahi,Y., Richmond, A.E., 1972a: Abscisic Acid in Relation to Mineral Deprivation. Plant Physiol. 50, 667-670.

Mizrahi,Y., Richmond, A.E., 1972b: Hormonal Modification of Plant Responses to Water Stress. Aust. J. Biol. Sci. 25, 437-442.

Mizrahi,Y., Scherings, S.G., Malisarad, S., Richmond, A.E., 1974: Aspects of the Effect of ABA on the Water Status of Barley and Wheat Seedlings. Physiol. Plant. 31, 44-50.

Monod, J., Changeux, J., Jacob, F., 1963: Allosteric Proteins and Cellular Control Systems. J. Mol. Biol. 6, 306-329.

Morton, R.A., 1974: The Vitamin Concept. Vitam. Horm. 32, 155-166.

Moody, W., Zeiger, E., 1978: Electrophysiological Properties of Onion Guard Cells. Planta 139, 159-165.

Moorby, J., 1977: Integration and Regulation of Translocation within the Whole Plant. Symp. Soc. Exp. Biol. 31, 425-454.

Morris, J.G., 1974: A Biologist's Physical Chemistry 2nd Ed. E. Arnold Ltd., London.

Mousseron-Canet, M., Mani, J-C., Dalle, J-P., Olivé, J.L. 1966: Photoxydation Sensibilisée de Quelque Composés Apparentes à la Déhydro-β-inone, Synthèse de l'ester Méthylique de la (±) Abscisine. Bull. Soc. Chim. Fr. 12, 3874-3878.

Nevo, Y., Tal, M., 1973: Metabolism of Abscisic Acid in *Flacca*, a Wilty Mutant of Tomato. Biochem. Genet. 10, 79-90.

Nishida, K., 1963: Studies on Stomatal Movement of Crassulacean Plants in Relation to the Acid Metabolism. Physiol. Plantarum 16, 281-298.

Nobel, P.S., 1974: Introduction to Biophysical Plant Physiology. W.H. Freeman and Co., San Fransisco.

Ogunkanmi, A.B., Tucker, D.J., Mansfield, T.A., 1973: An Improved Bioassay for Abscisic Acid and Other Antitranspirants. New Phytol. 72, 277-282.

Ohkuma, K., Addicott, F.T., Smith, O.E., Thiessen, W.E., 1965: The Structure of Abscisin II. Tetrahedron Lett. 29, 2529-2535.

Ohkuma, K., Lyon, J.L., Addicott, F.T., 1963: Abscisin II, An Abscission Accelerating Substance, from Young Cotton Fruit. Science 142, 1592-1593.

O'Malley, B.W., Means, A.R., 1974: Female Steroid Hormones and Target Cell Nuclei. Science 183, 610-620.

Orton, P.J., Mansfield, T.A., 1974: The Activity of Abscisic Acid Analogues as Inhibitors of Stomatal Opening. Planta 121, 263-272.

Osborne, D.J., 1977: Ethylene and Target Cells in the Growth of Plants. Sci. Progr. 64, 53-65.

Osborne, D.J., Jackson, M., Milborrow, B.V., 1972: Physiological Properties of Abscission Accelerator from Senescent Leaves. Nature 240, 98-101.

Osmond, C.B., 1976: in: Encyclopedia of Plant Physiology (New Edn. Vol.2A). Gen. Eds. Pirson, A., Zimmermann, M.H. Springer-Verlag, Berlin.

Outlaw, W., Kennedy, J., 1978: Anion Synthesis in Guard Cells. Plant Physiol. Supp. 61, 86.

Outlaw, W.H., Lowry, O.H., 1977: Organic Acid and Potassium Accumulation in Guard Cells during Stomatal Opening. Proc. Nat. Acad. Sci. 74, 4434-4438.

Paál, A., 1919*: Ueber Phototropische Reizleitung. Jb. Wiss. Bot. 58, 406-458.

Pallaghy, C.K., Raschke, K., 1972: No Stomatal Response to Ethylene. Plant Physiol. 49, 275-276.

Pate, J.S., 1976: in: Transport and Transfer Processes in Plants. Eds. Wardlaw, I.F., Passioura, J.D. Academic Press, New York.

Peel, A.J., 1975: in: Encyclopedia of Plant Physiology (New End. Vol.1) Gen. Eds. Pirson, A., Zimmermann, M.H. Springer-Verlag, Berlin.

Penny, M.G., Bowling, D.J.F., 1974: A Study of Potassium Gradients in the Epidermis of Intact Leaves of *Commelina communis* in Relation to Stomatal Opening. Planta, 119, 17-25.

Penny, M.G., Bowling, D.J.F., 1975: Direct Determination of pH in the Stomatal Complex of *Commelina*. Planta 122, 209-212.

Penny, M.G., Kelday, L.S., Bowling, D.J.F., 1976: Active Chloride Transport in the Leaf Epidermis of *Commelina communis* in Relation to Stomatal Activity. Planta 130, 291-294.

Phillips, I.D.J., 1971: Introduction to the Biochemistry and Physiology of Plant Growth Hormones. McGraw Hill Book Co., New York.

Pincus, G., Thimann, K., 1948: The Hormones. Academic Press, New York.

Pitman, M.G., Lüttge, U., Läuchli, A., Ball, E., 1974a: Action of Abscisic Acid on Ion Transport as Affected by Root Temperature and Nutrient Status. J. Exp. Bot. 25, 147-155.

Pitmann, M.G., Lüttge, U., Läuchli, A., Ball, E., 1974b: Effect of Previous Water Stress on Ion Uptake and Transport in Barley Seedlings. Aust. J. Plant Physiol. 1, 377-385.

Pustovoitova, T.N., 1967*: In Russian. Fiziol. Rast. 14, 90.

Pustovoitova, T.N., 1970*: In Russian. Fiziol. Rast. 17, 575.

Pustovoitova, T.N., 1971: Effect of Wilting and Soil Drought on Endogenous Growth Regulators of Mesophytes. Fiziol. Rast., 19, 622-628. (Trans. by Consultants Bureau).

Quarrie, S.A., 1978: A Rapid and Sensitive Assay for ABA using Ethyl Abscisate as an Internal Standard. Anal. Bioch. 87, 141-156.

Quarrie, S.A., Jones, H.G., 1977: Effects of Abscisic Acid and Water Stress on Development and Morphology of Wheat. J. Exp. Bot. 28, 192-203.

Railton, I.D., Reid, D.M., Gaskin, P., MacMillan, J., 1974: Characterisation of Abscisic Acid in Chloroplasts of *Pisium sativum L. cv.* Alaska by Combined Gas Chromatography - Mass Spectrometry. Planta 117, 179-182.

Raschke, K., 1975a: Stomatal Action. Ann. Rev. Pl. Physiol. 26, 309-340.

Raschke, K., 1975b: Simultaneous Requirement of Carbon Dioxide and Abscisic Acid for Stomatal Closure in Xanthium strumanium L. Planta 125, 243-259.

Raschke, K., 1976: in: Transport and Transfer Processes in Plants. Eds. Wardlaw, I.F., Passioura, J.B. Academic Press, New York.

Raschke, K., 1977: in: Water Relations in Membrane Transport in Plants and Animals. eds. Jungreis, A.M., Hodges, T.K., Kleinzelter, A., Schultz, S.G. Academic Press, New York.

Raschke, K., Dittrich, P., 1977: |^{14}C| Carbon-dioxide Fixation by Isolated Leaf Epidermis with Stomata Closed or Open. Planta 134, 69-75.

Raschke, K., Fellows, M.P., 1971: Stomatal Movement in *Zea Mays:* Shuttle of Potassium and Chloride Between Guard Cells and Subsidiary Cells. Planta 101, 296-316.

Raschke, K., Firn, R.D., Pierce, M., 1975: Stomatal Closure in Response to Xanthoxin and Abscisic Acid. Planta 125, 149-160.

Raschke, K., Humble, G.D., 1973: No Uptake of Anions Required by Opening Stomata of *Vicia faba* : Guard Cells Release Hydrogen Ions. Planta 115, 47-57.

Raschke, K., Zeevaart, A.D., 1976: Abscisic Acid Contents, Transpiration, and Stomatal Conductance as Related to Leaf Age in Plants of *Xanthium strumarium* L. Plant Physiol. 58, 169-174.

Rasmussen, O.S., 1976: Water Stress in Plants: 1. Abscisic Acid Level in Tomato Leaves After a Long Period of Wilting. Physiol. Plant. 36, 208-212.

Reeve, D.R., Crozier, A., (In Press). in: Encyclopedia of Plant Physiology (New Edn. Vol.9). Gen. Eds. Pirson, A., Zimmermann, M.H., Springer-Verlag, Berlin.

Robards, A.W., 1976: in: Intercellular Communication in Plants: Studies on Plasmodesmata. Eds. Gunning, B.E.S., Robards, A.W. Springer-Verlag, Berlin.

Robinson, P.M., Wareing, P.F., 1964: Chemical Nature and Biological Properties of the Inhibitor Varying with Photopenod in Sycamore (*Acer pseudoplatanus).* Physiol. Plant 17, 314-323.

Robinson, G.A., Butcher, R.W., Sutherland, E.W., 1971: in: Cyclic AMP. Academic Press, New York.

Rogers, A.W., 1973: in: Techniques of Autoradiography, 2nd Edn. Elsevier Scientific Publishing Co., Amsterdam.

Saftner, R., Raschke, K., 1978: Donnan and Membrane Potentials of Guard Cells of *Commelina communis*. Plant Physiol. Supp. 61, 86.

Saunders, P.F., 1978: in: Isolation of Plant Growth Substances. Ed. Hillman, J.R. Cambridge University Press, London.

Schlenk, H., Gellerman, J., 1960: Esterification of Fatty Acids with Diazomethane on a Small Scale. Anal. Chem. 32, 1412-1414.

Schnabl, H., 1978: Compensation of the Potassium Charge by Chloride Ions during Stomatal Movement in *Allium cepa* Fed. Eur. Soc. Pl. Physiol. Inaug. Meeting Edinburgh Abs. 472-473.

Schnabl, H., Bornman, C.H., Ziegler, H., 1978: Studies on Isolated Starch - Containing (*Vicia faba*) and Starch-deficient (*Allium cepa)* Guard Cell Protoplasts. Planta 143, 33-40.

Schnabl, H., Ziegler, H., 1977: The Mechanism of Stomatal Movement in *Allium cepa L.* Planta 136, 37-43.

Seeley, S.D., Powell, L.E., 1970: Electron-Capture-Gas-Chromatography for Sensitive Assay of Abscisic Acid. Anal. Biochem. 35, 530-533.

Šesták, Z., Čatský, Jarvis, P.G., 1971: Plant Photosynthetic Production Manual of Methods. Dr. W. Junk N.V. The Hague.

Shaw, M., MacLachlan, G.A., 1954: The Physiology of Stomata: 1. Carbon Dioxide Fixation in Guard Cells. Can. J. Bot. 32, 784-794.

Sheriff, D.W., 1977: Evaporation Sites and Distillation in Leaves. Ann. Bot. 41, 1081-1082.

Sibaoka, T., 1969: Physiology of Rapid Movements in Higher Plants. Ann. Rev. Pl. Physiol. 20, 165-184.

Simpson, G.M., Saunders, P.F., 1972: Abscisic Acid Associated with Wilting in Dwarf and Tall *Pisum sativum*. Planta 102, 272-276.

Smith, F.A., Raven, J.A., 1976: in: Encyclopedia of Plant Physiology (New Edn. Vol.2a) Gen. Eds. Pirson, A., Zimmermann, M.H. Springer-Verlag, Berlin.

Squire, G.R., Mansfield, T.A., 1972: A Simple Method of Isolating Stomata on Detached Epidermis by Low pH Treatment: Observations of the Importance of Subsidiary Cells. New Phytol. 71, 1033.

Stålfelt, M.G., 1961: The Effect of the Water Deficit on the Stomatal Movements in a Carbon Dioxide-free Atmosphere. Physiol. Plant. 14, 826-843.

Starling, E.H., 1905: The Chemical Correlations of the Function of the Body. The Lancet 2, 1905**, 339-341.

Stevens, R.A., Martin, E.S., 1977: New Structure Associated with Stomatal Complex of the Fern *Polypodium vulgare*. Nature 265, 331-334.

Steward, F.C., Krikorian, A.D., 1971: Plants, Chemicals and Growth. Academic Press, New York.

Stewart, G.R., 1969: Abscisic Acid and Morphogenisis in *Lemna polyrhiza L.* Nature 221, 61-62.

Sweetser, P.B., Vatvars, A., 1976: High performance Liquid Chromatographic Analysis of Abscisic Acid in Plant Extracts. Analyt. Biochem. 71, 68-78.

Tal, M., 1966: Abnormal Stomatal Behaviour in Wilty Mutants of Tomato. Plant Physiol. 41, 1387-1391.

Tal, M., Imber, D., 1970: Abnormal Stomatal Behaviour and Hormonal Imbalance in *flacca*, a Wilty Mutant of Tomato II. Auxin- and Abscisic Acid- Like Activity. Plant Physiol. 46, 373-377.

Tal, M., Imber, D., 1971: Abnormal Stomatal Behaviour and Hormonal Imbalance in *flacca*, a Wilty Mutant of Tomato III. Hormonal Effects on the Water Status in the Plant. Plant Physiol. 47, 849-850.

Tal, M., Imber, D., Itai, C., 1970: Abnormal Stomatal Behaviour and Hormonal Imbalance in *flacca* , a Wilty Mutant of Tomato: 1. Root Effect and Kinetin-like Activity. Plant Physiol. 46, 367-373.

Tal, M., Nevo, Y., 1973: Abnormal Stomatal Behaviour and Root Resistance and Hormonal Imbalance in Three Wilty Mutants of Tomato. Biochem. Genet. 8, 291-300.

Taylor, H.F., Burdon, R.S., 1970: Identification of Plant Growth Inhibitors Produced by Photolysis of Violaxanthin. Phytochem. 9, 2217.

Taylor, H.F., Burdon, R.S., 1973: Preparation and Metabolism of 2-[^{14}C]-cis, Trans-xanthoxin. J. Exp. Bot. 24, 873-880.

Taylor, H.F., Smith, T.A., 1967: Production of Plant Growth Inhibitors from Xanthophylls: A Possible Source of Dormin. Nature 215, 1513-1514.

Thimann, K., 1948: in: The Hormones. Eds. Pincus, G., Thimann, K., Academic Press, New York.

** No volume nos.

Thomas, D.A., 1975: in: Ion Transport in Plant Cells and Tissues. Eds. Baker, D.A., Hall, J.L. North-Holland Pub. Co., Amsterdam.

Tucker, D.J., Mansfield, T.A., 1971: A Simple Bioassay for Detecting Antitranspirant Activity of Naturally Occurring Compounds such as Abscisic Acid. Planta 98, 157-163.

Tukey, H.B., Went, F.W., Muir, R.M., Van Overbeck, J., 1954: Nomenclature of Chemical Plant Regulators. Plant Physiol. 29, 307-308.

Tyree, M.T., 1970: The Symplast Concept. A General Theory of Symplastic Transport According to the Thermodynamics of Irreversible Processes. J. Theor. Biol. 26, 181-214.

Vaadia, Y., 1976: Plant Hormones and Water Stress. Phil. Trans. R. Soc. Lond. B. 273, 513-522.

Van Die, J., Tammes, P.M.L., 1975: in: Encyclopedia of Plant Physiology (New Edn., Vol.1). Gen. Eds. Pirson, A., Zimmermann, M.H. Springer-Verlag, Berlin.

Van Kirk, C.A., Raschke, K., 1978a: Presence of Chloride Reduces Malate Production in Epidermis during Stomatal Opening. Plant Physiol. 61, 361-364.

Van Kirk, C.A., Raschke, K., 1978b: Release of Malate from Epidermal Strips during Stomatal Closure. Plant Physiol. 61, 474-475.

Van Overbeek, J., 1950: in: Agricultural Chemistry Vol.1. Ed. Frear, D.E.H. Van Nostrand, London.

Varga, M.B., Ferenczy, L., 1957: Paper-chromatographic Examination of Inhibiting Substances in Fleshy Fruits. Naturwissenschaften 44, 398-399.

Wade, N., 1978: Guillermin and Schally: The Years in the Wilderness. Science 200, 279-282.

Wain, R.L., 1977: in: Plant Growth Regulation. Ed. Pilet, P.E. Springer-Verlag, Berlin.

Walton, D.C., Dorn, B., Fey, J., 1973: The Isolation of an Abscisic Acid Metabolite 4-dehydrophaseic Acid from Non-imbibed *Phaseolus vulgaris* seed. Planta 112, 87-90.

Walton, D.C., Galson, E., Harrison, M.A., 1977: The Relationship between Stomatal REsistance and Abscisic-acid Levels in Leaves of Water-stressed Bean Plants. Planta 133, 145-148.

Walton, D.C., Harrison, M.A., Cote, P., 1976: The Effects of Water Stress on Abscisic-acid Levels and Metabolism in Roots of *Phasedus vulgaris L.* and Other Plants. Planta 131, 141-144.

Walton, D.C., Sondheimer, E., 1972a: Metabolism of 2-^{14}C-(±)-Abscisic Acid in Excised Bean Axes. Plant Physiol. 49, 285-289.

Walton, D.C., Sondheimer, E., 1972b: Activity and Metabolism of ^{14}C-(±)-Abscisic Acid Analogues. Plant Physiol. 49, 290-292.

Wareing, P.F., 1977: Growth Substances and Integration in the Whole Plant. Symp. Soc. Exp. Biol. 31, 337-365.

Wareing, P.F., Saunders, P.F., 1971: Hormones and Dormancy. Ann. Rev. Pl. Physiol. 22, 261-288.

Webster's Dictionary, 1961: 3rd Edn. Bell and Sons, London.

Wellburn, A.R., Ogunkanmi, A.B., Fenton, R., 1974: All-Trans-Farnesol: A Naturally Occurring Antitranspirant? Planta 120, 255-263.

Went, F.W., 1926*: On Growth-accelerating Substances in the Coleoptile of *Avena sativa*. Proc. K. Ned. Akad. Wet. 30, 10-19.

Went, F.W., 1927*: Wuchsstoff und Wachstum. Recl. Trav. Bot. Néerl. 25, 1-116.

Went, F.W., Thimann, K.V., 1937: Phytohormones. The MacMillan Co., New York.

Weyers, J.D.B., 1975: B.Sc. Thesis, University of Glasgow Botany Department.

White, J.C., 1973: Ph.D. Thesis, University of Glasgow.

Wieringa, K.T., Bakhuis, J.A., 1957: Chromatography as a Means of Selecting Effective Strains of *Rhizobia*. Plant and Soil 8, 254-262.

Wilkins, M.B., 1977: in: Plant Growth Regulation. Ed. Pilet, P.E. Springer-Verlag, Berlin.

Williams, W.T., Shipton, M.E., 1950: Stomatal Behaviour in Buffer Solutions. Physiol. Plant 3, 479-486.

Willmer, C.M., Beattie, L.N., 1978: Cellular Osmotic Phenomena During Stomatal Movements of *Commelina communis* I. Limitations of the Incipient Plasmolysis Technique for Determining Osmotic Pressure. Protoplasma 95, 321-332.

Willmer, C.M., Don, R., Parker, W., 1978: Levels of Short-chain Fatty Acids and of Abscisic Acid in Water-stressed and Non-stressed Leaves and their Effects on Stomata in Epidermal Strips and Excised Leaves. Planta, 139, 281-287.

Willmer, C.M., Mansfield, T.A., 1969: A Critical Examination of the Use of Detached Epidermis in Studies of Stomatal Physiology. New Phytol. 68, 363-375.

Willmer, C.M., Pallas, J.E. Jr., Black, C.C. Jr., 1973: Carbon Dioxide Metabolism in Leaf Epidermal Tissue. Plant Physiol. 52, 448-452.

Willmer, C.M., Rutter, J.C., 1977: Guard Cell Malic Acid Metabolism during Stomatal Movements. Nature 269, 327-328.

Wright, S.T.C., 1969: An Increase in the "Inhibitor-β" Content of Detached Wheat Leaves Following a Period of Wilting. Planta 86, 10-20.

Wright, S.T.C., Hiron, R.W.P., 1969: (+)-Abscisic Acid, the Growth Inhibitor Induced in Detached Wheat Leaves by a Period of Wilting. Nature 224, 719-720.

Zabadal, T.J., 1974: A Water Potential Threshold for the Increase of Abscisic Acid in Leaves. Plant Physiol. 53, 125-127.

Zeevaart, J.A.D., 1971: (+)-Abscisic Acid Content of Spinach in Relation to Photoperiodand Water Stress. Plant Physiol. 48, 86-90.

Zeevaart, J.A.D., 1974: Levels of (+)-Abscisic Acid and Xanthoxin in Spinach under Different Environmental Conditions. Plant Physiol. 53, 644-648.

Zeevaart, J.A.D., 1976: Physiology of Flower Formation. Ann. Rev. Pl. Physiol. 27, 321-348.

Zeevaart, J.A.D., 1977: Sites of Abscisic Acid Synthesis and Metabolism in *Ricinus communis L.* Plant Physiol. 59, 788-791.

Zeiger, E., Hepler, P.K., 1976: Production of Guard Cell Protoplasts from Onion and Tobacco. Plant Physiol. 58, 492-498.

Zeiger, E., Heppler, P.K., 1977: Light and Stomatal Function: Blue Light Stimulates Swelling of Guard Cell Protoplasts. Science 196, 887-889.

Zeigler, H., 1975: in: Encyclopedia of Plant Physiology (New Edn. Vol.1) Gen. Eds. Pirson, A., Zimmermann, M.H., Springer-Verlag, Berlin.

ERRATA

Page	line	word	correction
iii	27	7	EXPERIMENTAL
vi	10	6	Detection
x	5	3	Pyrrolidone
3	7	4	*ergons*
12	14	3	Robison
51	29	6	nectaries
71	7	3	Ilford
76	11	2	pyrrolidone
84	16, 17	-	Invert Section headings
94	8	2	are
120	15	2	relationship
128	16	1	intracellular
131	9	5	have
144	21	1	β-emissions
169	3	8	microscopy
171	6	1	Polyvinylpyrrolidone
226	2	2	are a
230	31	1	Endogenous
243	1	1	Robison
243	33		add: Smith, J.A.C. (1978) Ph.D. Thesis, University of Glasgow

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