ABSTRACT

Though the maize leaf has a large proportion (more than 30%) of its volume occupied by largely non-photosynthetic (yet probably respiring) epidermal tissue it has been reported that no carbon dioxide (CO_2) escapes from it in the light, even into a CO_2-free air stream. The aim of this investigation was to determine if the epidermis was active and if it was to discover the fate of the CO_2 produced in it by respiration.

Light and electron microscopy revealed that all epidermal cells had an apparently viable cytoplasm containing a full complement of organelles. The long epidermal cells, despite their large size, contained less cytoplasm than the cells of the stomata, an arrangement suggesting an uneven distribution of respiratory activity. Respirometry, despite considerable problems involved in the isolation of viable tissue, tended to confirm this view. Appreciable respiration was recorded in preparations containing only active stomatal cells and little or no increase in respiratory gas exchange could be detected in epidermal strips having a larger proportion of intact cells. Respiration was apparently unaffected by light.

Since CO_2 was released from isolated epidermal tissue in both the light and dark it was thought necessary to investigate potential mechanisms by which it could be retained in the light. A number of carboxylating and associated enzymes were assayed in extracts prepared from the lower epidermis of maize leaves. Evidence for appreciable activities of both phosphoenolpyruvate (PEP) carboxylase and malic enzyme were obtained, both were localised in the stomata. Some evidence for the presence of ribulosebisphosphate (RuBP)

carboxylase in long epidermal cells was also found, though it was felt to be too circumstantial for a definite conclusion to be reached.

The cuticular resistance to water vapour loss indicated that the resistance to CO_2 escape was probably very high. However, whilst a high cuticular resistance would reduce direct CO_2 escape it would also increase the potential advantage to the leaf of preventing the release of gaseous CO_2 within it.

Overall it was felt that though there was no single mechanism for the retention of CO_2 in the epidermis of maize several factors acting together reduced its release to a level undetectable by the techniques used up to now. These factors included a reduced respiration rate in long epidermal cells, a bunching of mesophyll chloroplasts close to the epidermal cells and an appreciable carboxylase activity in the active stomatal cells.

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CHAPTER 1

INTRODUCTION

One of the major problems faced by mankind today is the provision of an adequate food supply for a large and exponentially increasing world population. At the moment shortages can be largely overcome by improved agricultural techniques and selective breeding of high yielding varieties. However as demand increases the potential value of directed modification of edible plants and animals will become greater, and this can only be achieved if the mechanisms of the yield limiting steps are fully understood and any modifications of them recorded.

All foods are ultimately derived from the energy of the sun by the process of photosynthesis occurring in the green plant. Thus the elucidation of the steps of this process and the search for any modifications of it that may exist in different species is potentially of great importance as any increase in photosynthetic efficiency that may be bred into crop plants could result in proportional increases in yield at all levels of food production.

Photosynthesis in maize

Maize (*Zea mays* L.) is the third most important crop in the world (Duncan, 1975). It is thought to have originated in Central America and grows most successfully in areas having a combination of high ambient temperatures and moderate rainfall.

Until 1965 it was thought that all plants, including maize had the same pathway of photosynthetic carbon dioxide (CO_2) fixation; the photosynthetic carbon reduction (PCR) cycle first described by

Calvin and Benson (1948). This pathway is also called the C_3 pathway because the first stable produce of CO_2 fixation is a three carbon compound, phosphoglyceric acid (PGA). Any differences in photosynthetic efficiency or yield between different species of plant growing under comparable conditions were ascribed to variations in anatomy or respiratory activity.

However in 1965 Kortschak, Hartt and Burr reported experiments which indicated that the first stable products of CO_2 fixation in sugar cane were malate and aspartate and not PGA. This finding was confirmed by Hatch and Slack (1966) who then went on to describe the reactions involved more fully (Hatch and Slack, 1967, 1968, 1969a-c, 1970; Slack, 1968, 1969; Slack and Hatch, 1967; Slack, Hatch and Goodchild, 1969). This work drew attention to this subject and as a result the findings of Tarchevski and Karpilov (1963), who had earlier reported the same behaviour in maize, became more widely known.

This series of reactions is called the C_4 pathway because fixed carbon from CO_2 first appears in four carbon compounds. It has since been shown to operate in a wide variety of species, both mono- and dicotyledons (Downton, 1975), having a predominantly tropical distribution.

In addition to a different carboxylation mechanism C_3 and C_4 species differ in a number of other ways (Table 1.1). Perhaps the most striking is the characteristic leaf structure of C_4 species called Kranz-type anatomy which has two concentric layers of chlorophyllous cells surrounding the vascular bundles. The outer layer, known as the mesophyll, contains chloroplasts which are apparently similar to those of C_3 species. It is in this layer that CO_2 is first thought to be carboxylated to form four carbon compounds

Table 1.1: Principal differences between C_3 and C_4 species (after Black, 1973; Coombs, 1973; Zelitch, 1971).

	C_3 species	C_4 species
Maximum rate of net photosynthesis (mg CO_2 dm^2 h^{-1})	15-40	40-80
Optimum temperature for growth (°C)	15-25	30-47
CO_2 compensation point (p.p.m.)	30-70	0-10
Number of photosynthetic types of cell	1	2
Carboxylating enzymes present in significant amounts	RuBP carboxylase	RuBP carboxylase PEP carboxylase
Photorespiratory CO_2 escape	yes	no
Effect of light intensity	saturated at $\frac{1}{4}$-$\frac{1}{2}$ full sunlight	not saturated at full sunlight
Dry matter production under optimum conditions (tons/ hectare/year)	22.0 (±3.3)	38.6 (±16.9)
Dimorphic chloroplasts	no	yes
First stable product of CO_2 fixation	phosphoglyceric acid	malate or aspartate
Leaf structure	variable	tightly packed bundle sheath surrounded by mesophyll

which are then transferred to the inner layer of cells which is known as the bundle sheath. Bundle sheath cells are very tightly packed with few if any air spaces between them and often have chloroplasts with reduced grana and (compared to the mesophyll cells) large amounts of starch. In the bundle sheath it is thought that the four carbon compounds produced in the initial carboxylation are decarboxylated and the CO_2 produced refixed by the enzymes of the PCR cycle to produce PGA. Thus even in C_4 species apparently all CO_2 is ultimately assimilated via the C_3 pathway, the C_4 pathway acting as a transfer mechanism to bring CO_2 to the PCR cycle.

All investigations into photosynthesis in maize have shown it to be a typical C_4 plant having Kranz-type leaf structure, strongly dimorphic chloroplasts and producing predominantly malate in the initial carboxylation, this is then decarboxylated in the bundle sheath by the enzyme malic enzyme, one of three decarboxylases which occur in C_4 species.

C_3 and C_4 photosynthesis

When the behaviour of C_3 and C_4 species growing under similar environmental conditions are compared, certain differences in photosynthetic and growth rates become apparent. Whilst these differences often are not large at low temperatures and low light intensities at elevated ones C_4 plants typically show much higher rates of photosynthesis and growth, often two or three times greater than the majority of C_3 species. It is this high growth rate at high temperatures that has made many C_4 species important crop plants (e.g. maize, sugar cane) or weeds (e.g. *Amaranthus* sp.) in tropical or sub-tropical regions. The elucidation of the mechanisms responsible for this increased production are obviously of great potential importance in future crop development, particularly of

C_3 species, and as a result a great deal of work has been undertaken in recent years with this aim.

Early work suggested that the principal reason for the comparative success of C_4 species was the much greater affinity for carbon dioxide of its principal carboxylase, phosphoenolpyruvate (PEP) carboxylase. The carboxylase of the C_3 pathway, ribulose-bisphosphate (RuBP) carboxylase was believed to have a lower one. This was thought to result in C_4 plants competing more successfully for the available CO_2, concentrating it at the site of the PCR cycle by an initial carboxylation using PEP carboxylase followed by a malic enzyme mediated decarboxylation. Recent work (Walker and Lilley, 1976) has shown, however, that RuBP carboxylase has a much higher affinity than was first thought and as a result this hypothesis is no longer tenable.

Other work has tended to concentrate on the differing responses of C_3 and C_4 species to atmospheres having differing oxygen concentrations. This has revealed that as the oxygen concentration in the atmosphere is decreased net CO_2 assimilation in C_3 species increases whilst in C_4 species it stays relatively constant. This effect is thought to be mainly due to the inhibition of photorespiration, a CO_2 evolution in light requiring the presence of appreciable levels of oxygen in the atmosphere (dark respiration functions normally even if the oxygen concentration is as low as 2-3%). In C_3 species photorespiration is particularly important and may account for the loss of from 30 - 50% of photosynthate (Zelitch, 1971). C_4 species were thought to lack photorespiration (Forrester, Krotkov and Nelson, 1966) but more recent work (Volk and Jackson, 1972; Zelitch, 1968) has shown that this is not the case and that in fact they apparently reassimilate all photorespiratory CO_2. This reassimilation is clearly of great potential importance in increasing the efficiency of C_4 species.

Further work has also revealed that oxygen concentration also affects the rate of CO_2 assimilation directly because RuBP carboxylase can also act as an oxygenase producing PGA and phosphoglycollate from RuBP and oxygen. Low concentrations of oxygen and high concentrations of CO_2 tend to prevent this and C_4 species are probably more capable of attaining this condition because they can concentrate CO_2 at the site of PCR cycle activity. RuBP oxygenase activity is probably further inhibited by the small amounts of oxygen produced in the bundle sheath cells as a consequence of the low photosystem II activity present (Ku et al., 1974b). A low oxygen content may also result in a reduced rate of photorespiration in the tissue.

In addition to their ability to conserve photosynthate C_4 species are more successful, particularly in tropical climates for two other reasons. Firstly PEP carboxylase has a higher optimum temperature than RuBP carboxylase which means that C_4 plants can maintain large rates of photosynthesis at high temperatures. Secondly they have a much greater water use efficiency than C_3 plants i.e. for a certain transpirational water loss they are able to fix more CO_2; hence they are less subject to water stress. This is the result of two factors, firstly because no CO_2 is lost by photorespiration less CO_2 has to enter the plant to give the same dry weight increase, therefore the stomata can maintain a smaller aperture and water loss is reduced (the smaller aperture also means a higher leaf temperature, again favouring carboxylation by PEP carboxylase). Secondly, as CO_2 is predominantly fixed in the mesophyll there is no need for the air space system to penetrate the bundle sheath. Therefore the leaf can maintain a smaller air space system (Byott, 1976) reducing the volume of air that has to be saturated within it and since CO_2 enters the bundle sheath by active transport in the form of 4-carbon organic acids the outer walls of the bundle sheath can be suberised (O'Brien and Carr, 1970) and water loss through them reduced.

The CO_2 Compensation Point

Prior to the first description of the C_4 pathway it was known that some plants, including maize had CO_2 compensation points of close to zero (Meidner, 1962; Moss, 1962) i.e. they were capable of maintaining a CO_2 concentration of zero in a closed atmosphere in the light. Following the discovery of the C_4 pathway the association of it with species having low compensation points was shown. Typically C_3 species were found to have much higher compensation points in the region of 50-100 ppm.

The CO_2 compensation point of maize has been measured by a number of authors and values ranging from 0-10 ppm have been reported by most though Heichel and Musgrave (1969) have reported a variety with one of 27 ppm (a value which Moss, Willmer and Crookston, 1970, were unable to confirm). Some of these authors have also attempted to measure CO_2 efflux from an illuminated maize leaf (Forrester, Krotkov and Nelson, 1966; Hew, Krotkov and Canvin, 1969; Berry, Downton and Tregunna, 1969; Troughton, 1971; Volk and Jackson, 1972; Meidner, pers. comm.). A variety of techniques were used, most passed CO_2 free air over or through the leaf and then attempted to detect CO_2 in the outgoing air using either infra-red gas analysis, release of $^{14}CO_2$ or $^{12}CO_2$ from labelled leaves and measurement of its activity or collection of released gas in barium hydroxide solution followed by titration. Hew et al. also measured apparent photosynthesis at different concentrations of carbon dioxide and extrapolated the graph to zero CO_2 to obtain a value for CO_2 release at that concentration.

Volk and Jackson were the only workers to report any efflux of CO_2 from an illuminated leaf and they only detected it under very artificial conditions. A leaf was allowed to photosynthetise in an atmosphere containing $^{12}CO_2$ and then the atmosphere was quickly changed

to one with a high concentration of 96 atom $^{13}CO_2$. This effectively saturated the fixation sites with $^{13}CO_2$ and any $^{12}CO_2$ produced by photorespiration and dark respiration escaped to the outside where it was detected by mass spectrometry. The fact that such extreme measures were necessary to detect CO_2 evolution in illuminated maize stresses the great ability of the plant to retain CO_2 in the light under normal conditions.

The finding that no CO_2 escapes from an illuminated maize leaf was to be expected on the basis of its CO_2 compensation point of zero but if the anatomy of the leaf is considered then the absence of any efflux becomes difficult to explain.

If a section of a maize leaf is examined (plate 1.1) it is found that over 30% of its volume is accounted for by epidermal tissue. The epidermis is essentially chlorophyll free, the only chloroplasts being present in the guard cells, yet the other epidermal cells appear to be alive and must therefore have a source of energy; respiration which involves the release of CO_2 is the only one available to non-photosynthetic cells.

Under normal conditions in the light there is a CO_2 concentration gradient from the external atmosphere (300 ppm CO_2) to the carboxylation site in the mesophyll (potentially zero CO_2). Carbon dioxide produced by the epidermis under such conditions will tend to move down the concentration gradient into the mesophyll and be refixed there, though one might also expect some to enter the leaf spaces and escape into the external atmosphere. However the experiments quoted above involved the passage of CO_2 free air over the leaf therefore the intercellular spaces must also have had a CO_2 concentration of zero. Under such conditions CO_2 produced in the epidermis faces a

Plate 1.1: A typical transverse section of a maize leaf (mag x 350) showing the prominent epidermis on both leaf surfaces. A group of bulliform cells (►) can be seen on the upper surface and a stoma (→) is visible in the lower. the stomatal cells are clearly much smaller than the long epidermal cells.

similar concentration gradient in all directions, indeed if any CO_2 is being produced by either form of respiration in the mesophyll or bundle sheath the steepest gradient would be to the external atmosphere, either through the cuticle or into the leaf air-space system and out via the stomata and so some efflux would be expected. In these experiments this did not seem to occur and so it is possible that maize, and perhaps C_4 plants in general, have evolved a mechanism to prevent CO_2 loss from their epidermis. The aim of the present investigation was to determine if such a mechanism exists and assess its importance in relation to C_4 photosynthesis as a whole.

The Epidermis

The epidermis is the outer layer of cells on the plant body. It is therefore intimately concerned with the interaction between the plant and its environment and is specialised to permit this interaction yet avoid any damage that might result from unfavourable external conditions.

The leaf epidermis of maize is typical of the Gramineae: a single layer of cells consisting of five cell types, long epidermal cells, short epidermal cells (cork and silica cells), interstomatal cells, subsidiary cells and guard cells, (nomenclature after Metcalfe, 1960). In addition there are a variety of dermal appendages of varying distribution and frequency.

The cells of the epidermis are arranged in files running parallel to the longitudinal axis of the leaf. The stomatal cells are only present in certain files (usually about one in three) where they alternate with interstomatal cells, the other files contain solely long and short epidermal cells. The adaxial epidermis has a proportion of modified long epidermal cells having comparatively thin lateral

walls and a much greater volume. These cells, the bulliform cells, are concerned with the rolling and unrolling of the leaf both during development and during periods of water stress.

The external surface of the epidermis is covered with a cuticle which, particularly in young leaves, is overlain by a waxy layer. The cuticle is probably virtually impermeable to water and serves to reduce water loss from the leaves, impede invasion by pathogens and has a high contact angle which means that any water drops falling on it tend to run off, usually down the stem. This latter phenomenon, known as stem flow, is of great importance in maize, concentrating any rainfall into the soil in the vicinity of the roots.

The principal function of the epidermis is the control of water loss by the leaf coupled with the maintenance of adequate pathways for the exchange of carbon dioxide and oxygen. Clearly the stomata are the principal sites of control for this interchange, other epidermal cells can only modify the resistance to water loss and gaseous exchange very slowly by long-term changes in the cuticle and epicuticular waxes induced by alteration of the external environment. Thus the stomata must be regarded as the major site of energy expenditure in the epidermis and so their cells are probably the most metabolically active.

Though the functions of the rest of the epidermis such as acting as a store for water and as a path for its movement (Meidner, 1975); as a path for the translocation of photosynthate (Outlaw and Fisher, 1975) and as a support for the cuticle are essentially non-energy requiring the tissue still has a demand for some metabolic energy. This is needed to permit the laying down of new cell wall material during cell expansion and also for the synthesis of cuticle and epicuticular waxes. In addition long epidermal cells must have the

ability to respond to attack by invading pathogens. Contreras and Boothroyd (1974) have demonstrated that the nuclei of long epidermal cells of maize move to the site of germ tube penetration of Helminthosporium maydis spores and numerous other authors have shown elevated enzyme activities in maize leaves infected with H. maydis. It is probable that some of these enzymes are synthetised in the epidermis. This response must require an increased level of activity analogous to that reported in a variety of other species (see Scott, 1972). Thus in addition to basal activity concerned with growth and maintenance it is probable that long epidermal cells have the potential to sustain appreciable levels of metabolic activity during fungal attack. However, since they apparently lack the ability to photosynthetise and as there is no evidence for the transfer of high-energy molecules such as ATP between cells this energy must be derived by respiration and consequent CO_2 production within the cell.

Potential mechanisms of carbon dioxide retention

There are strong arguments indicating that carbon dioxide is produced in the leaf epidermis of maize. However, extensive research has failed to detect any escaping from the leaf in the light. There are several possible reasons for this:

(i) Despite the circumstantial evidence presented above the long epidermal cells are in fact dead like those of Commelina cyanea (Pearson and Milthorpe, 1974).

(ii) The epidermal cells are alive but their respiratory activity is so low as to be undetectable in the experiments which attempted to measure carbon dioxide efflux from the whole leaf in the light.

(iii) Enzymes are present in the epidermis that fix respiratory carbon dioxide before it can escape from the leaf.

(v) The cell walls of the epidermis and the cuticle are impermeable to CO_2 and hence it can only escape to the mesophyll where it is refixed.

The aim of this investigation was to determine if maize epidermis was respiring and if it was, which, if any, of these mechanisms were acting to prevent carbon dioxide escape.

General Methods

All experiments were carried out on plants of hybrid maize (Zea mays L.) var. Kelvedon 33, greenhouse grown under long days using a combination of natural and supplemented illumination. Temperatures were maintained at above 20°C.

Since it was also advisable to investigate the conditions prevailing in the epidermis of a C_3 species, which of course do lose carbon dioxide into a CO_2-free air stream, comparative experiments were also carried out on the leaves of Commelina communis L. (day flower) at certain points of the investigation. C. communis was selected because it could be easily grown in the same conditions as maize, its epidermis could be removed very easily and a great deal of published data was available on the physiology and anatomy of its epidermis.

It was difficult to isolate maize epidermis in large quantities and only a small proportion of intact cells were found in normally peeled epidermal strips. It was therefore necessary to investigate the suitability of the different techniques available for the isolation of epidermal tissue; full details of this investigation can be found in Chapter 3.

CHAPTER 2

STRUCTURE OF THE MAIZE LEAF

Introduction

The maize leaf displays a structure typical of most C_4 species with prominent bundle sheaths of tightly packed starch containing cells surrounded by a more diffuse mesophyll. The photosynthetic tissues are separated from the external atmosphere by a prominent epidermis consisting of a variety of cell types.

Microscopic examination of maize leaf epidermis has been limited to a few studies on the development and ultrastructure of the stomatal apparatus, there have been no reports concerned with long epidermal cells and their contents. It was therefore decided to examine the contents of these cells and assess their importance in the epidermis as a whole prior to any physiological or biochemical examination.

Methods

Preliminary investigations indicated that there was little difference in epidermal cell size and frequency between plants of varying age or between leaves on the same plant. Tissue for examination was taken from the mid-region of the upper leaf of a 6-8 week old plant.

(i) Light Microscopy:

Sizes of epidermal cells were measured from freshly prepared epidermal strips removed from the abaxial leaf surface and from previously prepared and mounted transverse leaf sections. Cell dimensions were recorded with the aid of a calibrated eye-piece

graticule. Cell volumes were estimated on the basis that long and short epidermal cells and guard cells were cuboid in form whilst subsidiary cells were treated as triangular prisms.

Attempts were made to measure cuticular thickness following staining of fresh material with a variety of lipid stains (Jensen, 1962: pp.264-265) such as Nile Blue and Sudan III.

(ii) Electron Microscopy:

Electronmicrographs were taken from previously prepared leaf sections which had been fixed in glutaraldehyde and osmium tetroxide and double stained in uranyl acetate followed by lead citrate (Reynolds, 1963). Measurements were made from the micrographs and cytoplasmic volumes calculated on the basis of the same shapes used in light microscopy, except for guard cells in which the cytoplasm was treated as two triangular prisms joining at the middle of the cell.

(iii) Weight Determination:

The lower epidermis of 1 cm^2 leaf sections was removed. Both lower epidermis and mesophyll with attached upper epidermis were weighed, oven dried at 110°C and weighed again (after cooling in a dessicator). All weighings were carried out on a Beckman LM-500 Microbalance. The same procedure was carried out in the case of Commelina communis in order to obtain comparative measurements for a C_3 species.

Results and Discussion

(i) Light Microscopy:

A typical transverse section of a leaf and an epidermal peel of maize are shown in plates 1.1 and 2.1 respectively. It is apparent from the former that a considerable proportion of the leaf is occupied by

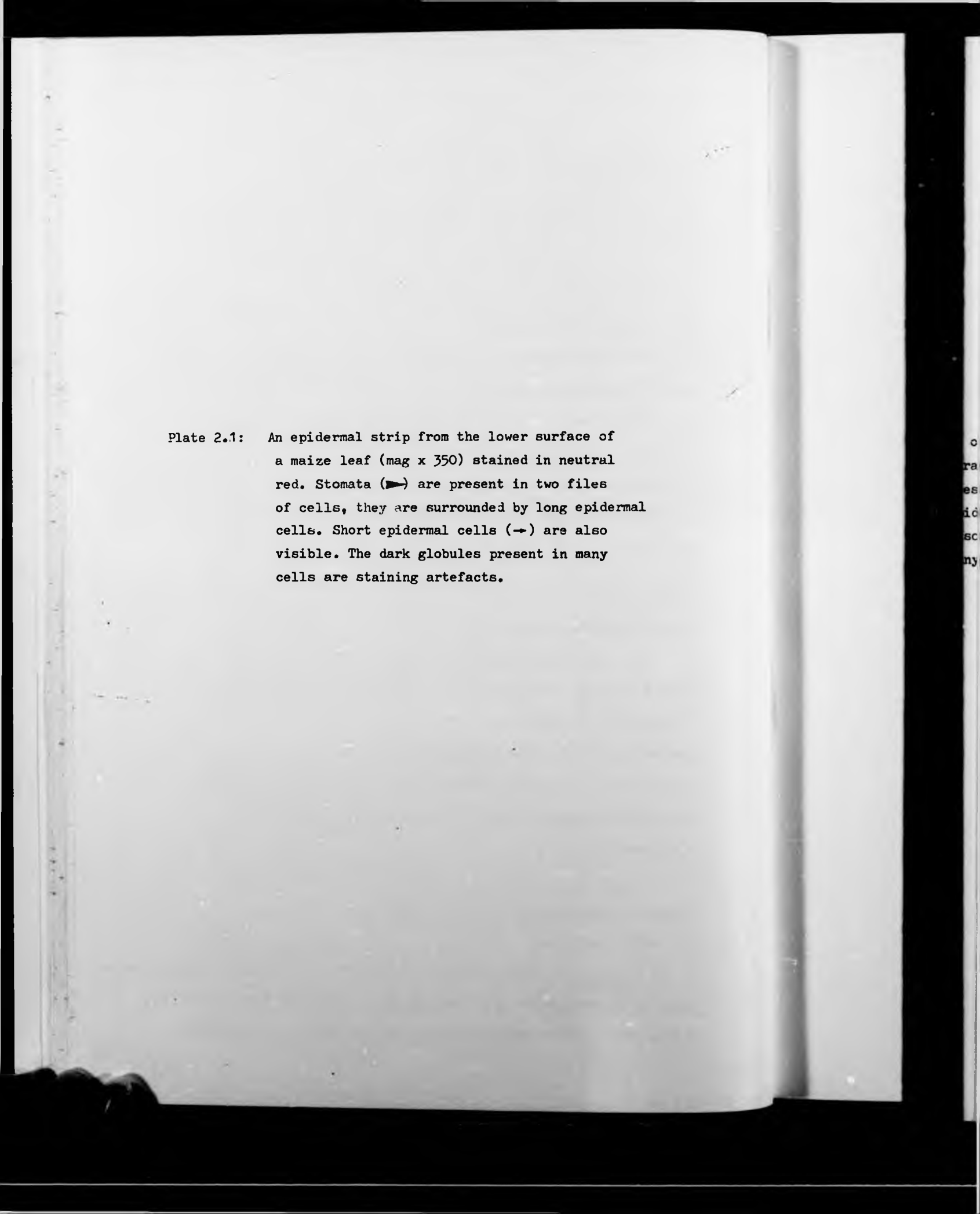
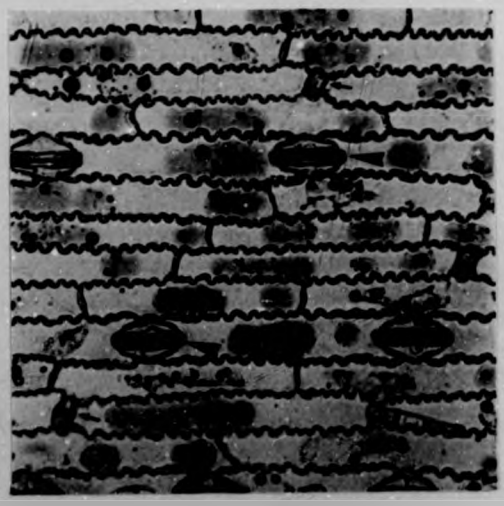


Plate 2.1: An epidermal strip from the lower surface of a maize leaf (mag x 350) stained in neutral red. Stomata (►) are present in two files of cells, they are surrounded by long epidermal cells. Short epidermal cells (→) are also visible. The dark globules present in many cells are staining artefacts.



epidermal tissue whilst the latter shows the majority of the epidermis to be non-photosynthetic long epidermal cells. This conclusion is confirmed by the data presented in Table 2.1. Over 35% of the leaf, expressed on a volume, fresh weight or dry weight basis, consists of epidermis. The proportion occupied on a volume basis is greater than that shown because bulliform cells were not considered in the estimate and these increase the volume of the adaxial epidermis considerably. If the volume of the leaf occupied by air spaces is taken into account (10.25%, Byott, 1976) then it is apparent thatas much as 50% of leaf tissue is non-photosynthetic epidermis. In addition there is a considerable volume of non-chlorophyllous tissue in the rigid mid-rib.

Leaves of C. communis have an epidermis of a similar size but because of the much greater thickness of the leaf lamina its proportional volume and weight is much less.

Table 2.2 shows the frequency and size of the various cell types in the lower epidermis of maize leaves. It is clear that long epidermal cells are dominant, their large size and frequency mean that they account for over 95% of the epidermis. In the adaxial epidermis they are still more important because of their slightly greater overall thickness (29μm) and the presence of bulliform cells which are modified long epidermal cells (plate 1.1) these are of varying depth with a mean of about 57μm and a maximum of nearly 100μm in some cases. In other respects the adaxial epidermis is similar to the abaxial and has the same stomatal density.

Metcalfe (1960) differentiates long epidermal cells and interstomatal cells but for the purposes of this investigation this distinction has been ignored. There was some variation in size of

Table 2.1 Characteristics of the gross leaf structures of maize and C. communis.

	Maize	C. communis
Mean thickness of leaf lamina (μm)	128 (±5.7)	237 (±15.3)
Mean thickness of long epidermal cells (lower epidermis - μm)	22 (±1.6)	27 (±2.8)
Mean thickness of long epidermal cells (upper epidermis - μm)	29 (±3.5)	31 (±1.7)
% of leaf volume occupied by epidermes	39.8	24.5
Dry weight of lamina (mg cm^{-2})	1.38 (±0.27)	1.95 (±1.09)
Dry weight of lower epidermis (mg cm^{-2})	0.26 (±0.08)	0.19 (±0.22)
% of leaf dry weight accounted for by epidermes	37.6	19.5
Fresh weight of lamina (mg cm^{-2})	9.56 (±0.97)	13.60 (±6.57)
Fresh weight of lower epidermis (mg cm^{-2})	1.88 (±0.31)	1.96 (±0.97)
% of total leaf fresh weight accounted for by epidermes	38.5	28.8

Table 2.2: Distribution of the different cell types in the lower epidermis of the maize leaf.

	Long epidermal cells	Short epidermal cells	Guard cells	Subsidiary cells
Mean cell length (μm)	140 (±10.0)	8 (±2.5)	39 (±4.5)	33 (±5.6)
Mean cell width (μm)	30 (±1.8)	15 (±13.3)	5 (±1.3)	14 (±1.4)
Mean cell depth (μm)	22 (±1.6)	22 (±1.6)*	15 (±1.6)	15 (±1.2)
Cell volume (μm^3)	92.4×10^3	2.64×10^3	2.93×10^3	3.47×10^3
Frequency of cells (per mm^2)	226 (±18.6)	3(±2.7)	114 (±37.1)	114 (±37.1)
% of lower epidermal area occupied by cell	95	0.1	2.2	2.7
% of lower epidermal volume occupied by cell	96.60	0.04	1.54	1.83

* Indistinguishable in transverse sections of leaf tissue from long epidermal cells.

long epidermal cells across the leaf, those adjacent to veins were typically somewhat shorter and wider but as can be seen from the confidence limits the differences were small. Hairs were present on both surfaces in limited numbers, on the abaxial, where they were most frequent (5 per mm^2), they were up to 60µm long. Short epidermal cells (both cork and silica cells, not differentiated in this study) were very infrequent on both leaf surfaces and so were disregarded for the rest of the investigation. Thus three types of cell, long, subsidiary and guard were considered to be of importance.

The stomatal density recorded (57 per mm^2) was less than the 108 reported by Meidner and Manfield (1968) and the 66 reported by Turner and Begg (1973), though the stomatal dimensions were similar. C. communis had a slightly lower density (47 mm^2) which compared favourably with values reported by Willmer et al. (1973) and Pearson and Milthorpe (1974).

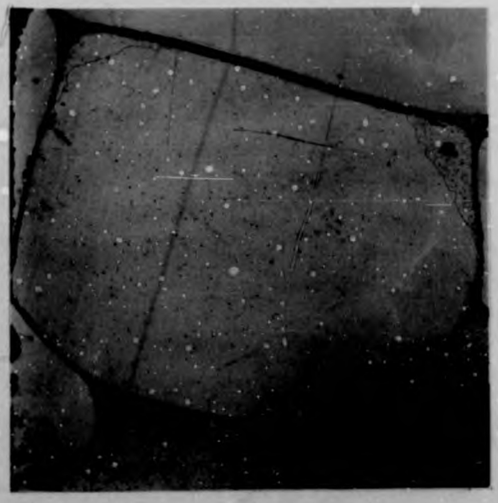
It proved impossible to measure cuticular thickness by light microscopy, staining only revealed a continuous very thin cuticle over the whole leaf surface which was not thick enough to measure. The inner surface of the guard and subsidiary cells appeared to be similarly cutinised but there was no evidence for cuticle deposition on the internal surface of the other epidermal cells.

(ii) Electron Microscopy:

Cell dimensions recorded from electronmicrographs were comparable to those from light microscopy.

The most striking feature of the epidermis was the large, virtually empty long epidermal cells. The cytoplasm was only present as a thin layer lining the cell wall (plate 2.2) and

Plate 2.2: An electronmicrograph of a transversely sectioned long epidermal cell (mag x 2000). The cytoplasm is visible as a thin layer adjacent to the cell wall (►) though the upper right hand corner (a) does contain a thicker layer in this case. Some mitochondria are visible (→). The clumping of mesophyll chloroplasts adjacent to the long epidermal cell wall is apparent.



accounted for less than 1% of the cell's volume (Table 2.3). It was more diffuse than that of other leaf cells but did contain a limited number of mitochondria and some endoplasmic reticulum (plate 2.3). The nucleus was the largest body in the cell (plate 2.4) and its volume may have exceeded that of the cytoplasm. No plastids were noted though Williams (1974) reported the presence of rudimentary ones having a small prolamellar body. The apparently functional cytoplasm present in long epidermal cells is indicative of at least some metabolic activity within them.

However, it is certain that this activity is considerably less than that found in the cells of the stomatal complex. Both subsidiary and guard cells contained a much greater proportion of cytoplasm (indeed the guard cells contained no apparent vacuole) and had a much greater density of mitochondria and other organelles (plate 2.5). Mitochondria were also appreciably larger.

Starch containing chloroplasts were very prominent in the swollen ends of the guard cells and indeed they and the nuclei apparently occupied a large proportion of the cytoplasmic volume. The chloroplasts had well developed lamallae though the granal stacks were less prominent than those of the mesophyll cells (plate 2.6). The overall structure of the guard cells was comparable to that described by Srivastava and Singh (1972) and Ziegler, Schmueli and Lange (1974).

The mesophyll and bundle sheath cells appeared similar to those described previously in maize (Laetsch 1974) though it is interesting to note the tendency of mesophyll chloroplasts to be positioned along cell walls adjacent to epidermal cells (plate 2.2).

Cytoplasmic volumes were estimated from electronmicrographs, (Table 2.3)

Table 2.3: Ultrastructural characteristics of the principal cell types in the lower epidermis of the maize leaf.

	Long epidermal cells	Guard cells	Subsidiary cells
Volume of cytoplasm per cell (μm^3)	555	506	1018
% of total cell volume occupied by cytoplasm	0.6	100	31
Volume of cytoplasm ($\mu m^3\ mm^{-2}$)	1.25×10^5	0.57×10^5	1.16×10^5
% of total epidermal cytoplasm	41.9	19.3	38.8
% of cell surface area in contact with:			
(i) External atmosphere	26.4	44.7	14.4
(ii) Internal atmosphere	9.0		14.4
(iii) Mesophyll	17.4	-	-
(iv) Long epidermal cells	47.1	8.8	40.4
(v) Subsidiary cells	- -	34.2	-
(vi) Guard cells	-	12.3	30.8
Thickness of inner cell wall (μm)	0.2 ($\pm$0.04)	Variable	0.2 ($\pm$0.04)
Thickness of outer cell wall and cuticle (μm)	0.74 ($\pm$0.2)	Variable	0.74 ($\pm$0.2)

Plate 2.3 : Mitochondria (►) and endoplasmic reticulum (e)
in the cytoplasm of long epidermal cells.

A: Mag x 40,000

B: Mag x 60,000

A

B

Plate 2.4: Transverse section of a long epidermal cell (mag x 4,000) showing the prominent nucleus (►). Mitochondria are also visible (→).

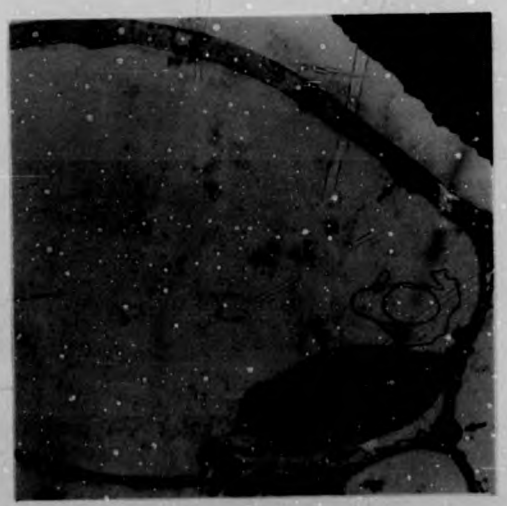
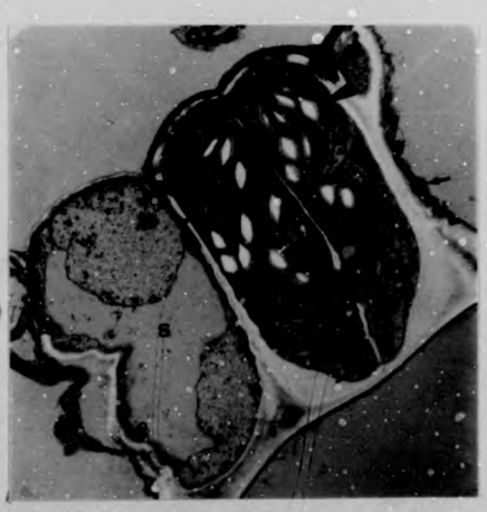


Plate 2.5: Transverse sections across the stomatal complex. Chloroplasts (►) and mitochondria (→) are prominent in the guard cells (g), only the latter are present in the subsidiary cells (s).

A : Section across the end of a stoma (mag x 5000). Note the absence of a membrane between the two guard cells and the apparent lack of cytoplasmic connection between guard and subsidiary cells.

B : Transverse section across the stomatal complex at the pore (mag x 8000). The cytoplasm of the guard cells is greatly reduced and the cell walls much thickened.

A



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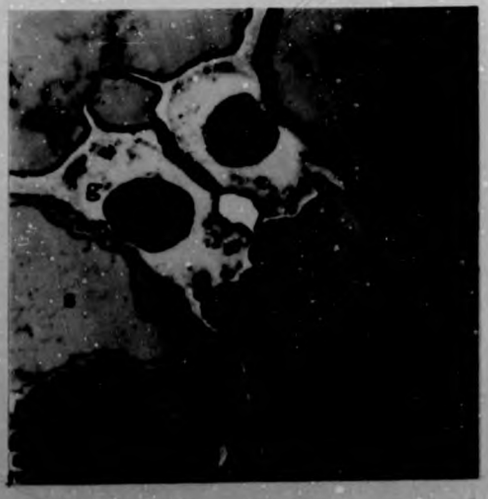

Plate 2.6: A mesophyll cell (m) containing prominent granal chloroplasts (►). A bundle sheath cell with its typical agranal chloroplasts (►) is visible in the top right-hand corner (mag x 12000).

Plate 2.7: A high power (mag x 90,000) view of the outer cuticularised wall of a long epidermal cell. The cell lumen can be seen in the upper left-hand corner. There is little discernable structure in the wall.

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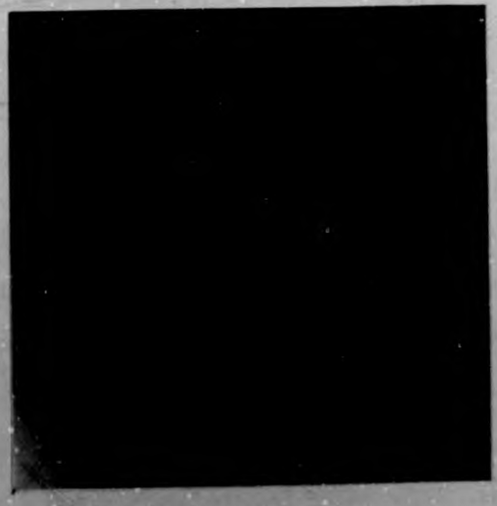

It is clear that despite the large overall volume of long epidermal cells their cytoplasmic content was very low, possibly less than the considerably smaller stomatal cells. The cytoplasm appeared to be fairly evenly distributed between the three cell types when their relative frequencies were taken into account and it was apparent that at least 50% of it was present in the stomatal cells as a whole.

The proportion of cell surfaces adjoining other cell types was estimated theoretically. Contact between the inner surface of the epidermis and the mesophyll was estimated from electronmicrographs. The major source of error in this procedure was the presence of wavy anticlinal walls in the long epidermal cells which meant that the proportion adjoining other epidermal cells was probably underestimated.

It is apparent that a major part of the typical long epidermal cell surface was in contact with other epidermal cells or the external atmosphere. A large proportion (close to 70%) of the rest was attached to mesophyll cells, a reflection of the small air space system of the maize leaf (Byott, 1976). No cytoplasmic connections between mesophyll and epidermis were observed.

Cuticular thickness was recorded from the electronmicrographs. The value recorded is probably conservative because of the loss of the waxy outer layers of the cuticle during fixation in non-polar solvents, however it does also include the thickness of the outer cell wall as it was impossible to observe a clear cut boundary between the two (plate 2.7). The cuticularised wall was clearly thicker than the inner wall (0.74µm compared to 0.2µm) though it was considerably less than that recorded in many other species.

This does not mean that it is necessarily more permeable as Kamp (1930) has shown that cuticular structure and composition are more important than thickness in controlling overall permeability.

CHAPTER 3

THE ISOLATION OF EPIDERMAL TISSUE

Introduction

Epidermal strips have been used in the study of stomatal behaviour for many years but it is only recently that they have been isolated in large quantities to permit biochemical studies to be undertaken. At first investigations were concerned with the determination of chlorophyll content of guard cells (Freeland, 1951; Shaw and MacLachlan, 1954) but more recent work has been concerned with the metabolism of the tissue, in particular of the stomatal cells (Allaway, 1973; Allaway and Hsiao, 1973; Pallas and Wright, 1973; Willmer et al., 1973; Willmer, Pallas and Black, 1973; Willmer and Dittrich, 1974). These studies have demanded the isolation of large quantities of uncontaminated epidermal strips having a large proportion of intact stomatal cells. Only a few species can satisfy these criteria and therefore experiments have been restricted to a small number of plants, principally Vicia faba, Tulipa gesnariana and species of Commelina.

The current investigation differed from previous ones in two respects:

(i) Experiments had to be carried out on the epidermis of a particular species, maize, rather than on a species that could be selected for its ease of peeling. While other C_4 species can probably prevent CO_2 efflux in the light in a similar way to maize there are none which have been so thoroughly investigated. Furthermore it is unlikely that any C_4 species has an epidermis that can be removed any more easily than that of maize.

(ii) Epidermal tissue had to be isolated with a high proportion of intact epidermal cells, previous workers, only concerned with stomatal cells, could accept the loss of a proportion of epidermal cell contents during peeling (Willmer et al., 1973).

Though the lower epidermis of maize leaves can be removed in large pieces by normal longitudinal epidermal peeling the yield of intact long epidermal cells is low (less than 1%) making the analysis of their contents and metabolism very difficult. Attempts were therefore made to improve upon normal longitudinal peeling techniques so that strips having a high proportion of intact long epidermal cells and very little mesophyll contamination could be isolated.

There are two factors which are responsible for the difficulty in isolating intact maize epidermis having a large proportion of viable cells:

(i) The epidermal cells are arranged in files with their long axes running parallel with the longitudinal axis of the leaf. Therefore any attempt at the removal of the epidermis by longitudinal peeling places a strain on the cells when they bend through an obtuse angle at the point of separation of mesophyll from epidermis.

(ii) The epidermis of maize is more firmly attached to the mesophyll then the epidermis of many C_3 species such as C. communis and V. faba. This means that any attempt at peeling is much more likely to damage the cells.

Pallaghy (1971) reported that epidermal strips of maize could be isolated with physiologically active stomata by careful transverse peeling. This had the advantage that the cells were rotated about their long axes during peeling and were therefore put under less stress. Pallaghy did not indicate the number of epidermal cells

remaining intact but Raschke and Fellows (1971) using a similar technique claimed that they all did. Neither paper gave any indication of the potential of the method for the large scale isolation of tissue.

Transverse peeling overcame the problem presented by the shape and orientation of the cells by changing the direction of peeling, however it was felt that it might also be solved by modifying the cell contents so that they were less susceptible to the damage inflicted during longitudinal peeling. The infiltration of a leaf with a plasmolysing solution prior to peeling would, it was hoped, reduce the damage because the plasmolysed protoplasts would be placed under less strain during peeling.

As will be shown the previous two methods were unsuccessful in providing suitable epidermal strips, presumably because they only attacked the problem caused by the arrangement of the cells. The difficulty presented by the tight union between epidermis and mesophyll was harder to overcome; however two methods developed for studying the distribution of photosynthetic enzymes in C_4 plants offered potential solutions.

Progressive grinding of leaf tissue to yield separate extracts of mesophyll and bundle sheath cells was first described by Björkman and Gauhl (1969) who investigated the distribution of carboxylating enzymes in the leaf of Atriplex rosea. Pieces of leaf tissue were gently ground in buffer resulting in the release of mesophyll cell contents, following filtration the extract was used as a mesophyll extract, meanwhile the remaining pieces of leaf tissue were resuspended in fresh buffer and ground vigorously to yield a bundle sheath extract. The method was further modified by Bucke and Long (1971 a, b) to a four stage procedure yielding separate extracts of colourless cells,

mesophyll cells, bundle sheath cells and bundle sheath cells plus some vascular tissue. The technique has been widely used to study the distribution of a variety of enzymes in the leaves of C_4 species. (Andrews et al., 1971; Poincelot, 1972; Hatch and Kagawa, 1973; Rathnam and Das, 1975 a, b). Epidermal extracts prepared by progresssive grinding have been used for enzyme assays by Bucke and Long (1971 a, b) and Rathnam and Das (1975) and it was felt that the technique could be of some use in this investigation though, of course, it could not be used in experiments requiring intact cells. Experiments were therefore carried out to determine which cell types were ruptured during progressive grinding and the overall purity of the extracts prepared using this technique. Progressive grinding has two disadvantages when investigating enzyme distribution in whole leaf tissue, firstly it is impossible to be certain that only one cell type is being ruptured and also it is very difficult to isolate replicate extracts having the same composition. As a result of this virtually all research into the distribution of enzymes in C_4 leaves is now done on extracts of cells that have been separated by enzymic digestion of their cell walls.

The isolation of plant material by enzymic digestion with wall degrading enzymes was first described by Chayen (1952) who isolated cells from the root meristems of Vicia faba using pectinase. Yields of intact cells were very low and it was not until Cocking (1960) isolated protoplasts from tomato root tips with 5% cellulase that the method was widely accepted. The technique has since been modified many times to permit the isolation of large numbers of viable protoplasts from a wide range of tissues and species (Gregory and Cocking, 1965; Ruesink and Thimann, 1966; Takebe, Otsuki and Aoki, 1968; Power and Cocking, 1970; Kanai and Edwards, 1973; Chen et al., 1974; Landova and Landa, 1975; Shephard and Totten, 1975). The precise

conditions and solutions used to isolate protoplasts is dependent on the species and tissue used (Otsuki and Takebe, 1969) but the basic procedure is the same in all cases. Pieces of tissue are vacuum infiltrated with a solution containing both an osmoticum and cell wall degrading enzyme (usually pectinase or cellulase or both), following several hours incubation protoplasts can be isolated by a variety of techniques such as flotation, centrifugation and filtration.

The only report of the isolation of epidermal tissue by enzyme digestion is from nutsedge (Cyperus rotundus L.) by Chen et al. (1974). However because they separated all the principal cell types present in the leaf they were forced to use small (1 - 2mm^2) pieces of tissue. The different cell types were then isolated by sieving through various sizes of nylon mesh. The current investigation only required epidermal cells and it was thought that these could be obtained in better yields if large pieces of tissue were taken, infiltrated, incubated and then normal epidermal peels taken. Experiments were carried out to ascertain if this was the case and to determine the optimum concentrations of osmoticum and wall degrading enzymes.

Previous workers have used plants which have yielded epidermal strips essentially free of contamination from mesophyll cells so the cleaning of tissue has been a minor problem. Willmer et al. (1973) washed strips of T. gesnariana in running tap water; Freeland (1951) scraped strips of Hymenocallis littoralis clean with a blade and brush and Pallas and Dilley (1972) used sonication to remove clinging chloroplasts from strips of V. faba. Unfortunately epidermal strips of maize are very liable to contamination because of the firm attachment between mesophyll and epidermis. Pallaghy (1971) reported that such contamination could be removed by dipping the strips once in 2.5% Triton X-100 or several times in distilled water.

The success of each of the methods used to isolate epidermal tissue was judged on the basis of three criteria:

(i) The ease with which adequate amounts of tissue could be isolated.

(ii) The viability of the isolated epidermis.

(iii) The amount of contamination present and the ease with which it could be removed.

The methods that best satisfied these criteria were used in further experiments.

Methods

(i) Preparation of material:

The sixth or seventh leaf of a 75 - 100 cm tall (about 8 weeks old) maize plant was excised and its cut end kept immersed in water. Sections of lamina about 0.5 × 3.0 cm were removed and a cut made through the upper epidermis and mesophyll with a sharp blade. The cut was perpendicular to the veins for longitudinal peels, parallel with them for transverse. The lower epidermis was then peeled off, either immediately (normal peels), after vacuum infiltration with osmoticum (peeling after plasmolysis) or after infiltration and incubation with osmoticum containing enzyme (wall digestion). Osmotica used were sucrose, sorbitol, ethylene glycol, potassium chloride and calcium chloride. Pectinase (Sigma Ltd.) and cellulase (Onozuka ss) were used to digest and weaken cell walls.

It was essential to make the initial cut prior to infiltration as the leaf would not remain sufficiently rigid to do it after. Vacuum infiltration was most complete if the leaf sections were floated one deep in the solution to be infiltrated and held just beneath the

surface with 'Netlon' (0.5 cm mesh). The vacuum was applied for about twenty minutes and during this time the flask was frequently agitated.

(ii) Progressive grinding:

It was found preferable to use larger leaf sections (2 × 3 cm) when preparing extracts by this technique as much of the final contamination originated from the cut edges of the leaf pieces. Because large pieces had a larger area to perimeter ratio this source of contamination was proportionally reduced. The leaf sections were gently abraded in a mortar and pestle containing 10 ml distilled water and 3 ml acid washed glass beads (150 - 200 µm diameter, Sigma Ltd.). As soon as traces of chlorophyll started to appear the leaf pieces were removed and replaced with fresh ones and grinding continued. The solution was finally filtered through a 30 µm nylon mesh.

(iii) Evaluation of the techniques investigated:

The three criteria described in the introduction were used. They were examined as follows:

(a) Ease of isolation could only be judged subjectively.

(b) The viability of the isolated epidermis should ideally have been monitored by assaying the activity of a suitable marker enzyme. However the length of time required to isolate sufficient tissue for enzyme assays made this impractical.

Instead a variety of microscopic techniques were used. Epidermal strips were stained in either neutral red (1 : 10,000) or 0.05% Evans Blue (Gaff and Okong'o-Ogola, 1971). Both stains were made up in boiled tap water or, for enzyme treated tissue, in the incubating osmoticum. Cell viability was evaluated on the ability of the vacuole of the protoplast to take up neutral red; the ability of the whole protoplast to exclude Evans Blue and its ability to undergo osmotic shrinkage and swelling. Neutral

red is a vital stain taken up into the vacuoles of living cells at pHs greater than 6.5 (Stadelman and Kinzel, 1972) whilst Evans Blue is a non-permeating pigment incapable of crossing intact membranes and is therefore excluded from living cells; since it does not enter the cell and therefore does not damage them, it is particularly useful for testing the integrity of protoplasts which are required for other experiments. The ability of a protoplast to shrink or swell under osmotic stress was the best indicator of the condition of the membranes of the protoplast. However, careful observation was required to ensure that it was the whole protoplast that was changing volume. It was possible for the plasmalemma but not the tonoplast to rupture under some conditions and when this occurred the vacuole changed volume with the dead cytoplasm from around it collected together at a point on its surface.

The yield of intact epidermal cells was expressed as a percentage of the total number of that cell type present per microscope field. At least ten fields on each of three strips were sampled for each treatment.

(c) The amount of contamination present was evaluated subjectively on the prepared strips and recorded as light (scattered separate clinging chloroplasts and protoplasts), moderate (some groups of clinging material but still less than 25% of the epidermal cells obscured) or heavy. The most successful preparations were collected in bulk, ground with 5 ml distilled water and 2 ml acid washed glass beads, filtered and their protein and chlorophyll contents determined. Extracts prepared by progressive grinding were treated in the same way.

The protein concentration of the extracts was determined with Folin's reagent (Lowry et al., 1951). Chlorophyll was determined in 96% ethanol by the method of Wintermans and DeMots (1965). Total

chlorophyll concentration and concentration of chlorophylls a and b were determined using their equations (equations 3.1 - 3.3)

$$\text{Chlorophyll a } (\mu g/ml) = 13.7\ A_{665} - 5.76\ A_{649} \quad \text{(Eqn. 3.1)}$$

$$\text{Chlorophyll b } (\mu g/ml) = 25.8\ A_{645} - 7.6\ A_{665} \quad \text{(Eqn. 3.2)}$$

$$\text{Chlorophyll a + b } (\mu g/ml) = 6.1\ A_{665} + 20.04\ A_{649} \quad \text{(Eqn. 3.3)}$$

A_x - Absorbance at x nm

Results

Normal Epidermal Peels:

Transverse epidermal peels prepared by the method of Pallaghy (1971) yielded only a small number of intact long epidermal cells that were capable of taking up neutral red and excluding Evans Blue (Table 3.1). The yield of intact stomatal cells was good. The difference in the yield from that of Pallaghy or Raschke and Fellows (1971) was possibly caused by the use of a different cultivar and different growth conditions. Longitudinal epidermal peels gave a lower yield of intact cells (Table 3.1) but the yield of apparently viable stomatal cells was still high.

During peeling transverse strips invariably broke at the first major vein encountered and as a result only small pieces (maximum 0.5 × 1.0 cm) of tissue could be isolated. Much larger pieces (up to 1.0 × 3.0 cm) could be obtained by longitudinal peeling and therefore this method was used in further investigations because it was felt that the greater speed of isolation balanced the decrease in the number of intact cells.

A typical longitudinal peel is shown in Plate 3.1. No intact long epidermal cells were present and what remained of the cell contents was gathered together in the cell lumen. The guard and subsidiary cells appeared normal and took up neutral red and plasmolysed. Pallaghy, however, reported a loss of ions from such cells.

Table 3.1: Yields of intact cells in epidermal strips following normal peeling and peeling after infiltration with various osmotica.

Treatment	Strips removed transversely			Strips removed longitudinally		
	Guard cells intact (%)	Subsidiary cells intact (%)	Long epidermal cells intact (%)	Guard cells intact (%)	Subsidiary cells intact (%)	Long epidermal cells intact (%)
Normal peel	93	85	<10	90	74	0
Peel removed after infiltration with water	*	*	<10	*	*	0
Peel removed after infiltration with 0.6M sorbitol	*	*	42	*	*	16
Peel removed after infiltration with 0.6M sucrose	*	*	38	*	*	14
Peel removed after infiltration with 1.2M sucrose	*	*	53	*	*	20

* Precise yield not determined but similar to that recorded in epidermal peels isolated by normal peeling.

Plate 3.1 : A normal longitudinal epidermal peel removed from the lower surface of a maize leaf (mag x 400). Cells were plasmolysed in 1.2M sucrose and stained in neutral red to demonstrate their integrity. Clearly the majority of guard and subsidiary cells were intact (they could also be deplasmolysed). All other cells were damaged; they did not take up neutral red and their contents were gathered together in the cell lumen (►).

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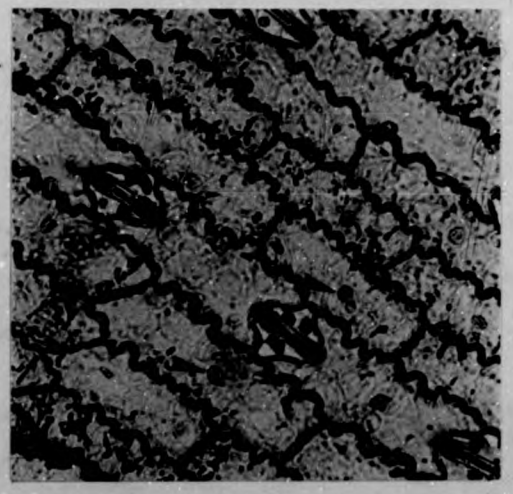

Contamination was variable in both longitudinal and transverse epidermal peels though it was seldom heavy. Pallaghy reported two methods to remove it involving either the dipping of strips once in 2.5% Triton X-100 or twenty times in distilled water. The former proved most satisfactory (Plate 3.2) whilst the latter was found to be very ineffective and time-consuming when large amounts of tissue had to be treated. Triton X-100 had no apparent effect on the ability of the intact cells to take up neutral red or plasmolyse.

Protein and chlorophyll contents of extracts prepared from Triton washed longitudinal peels and whole leaf tissue were determined (Table 3.2). The protein : chlorophyll ratio was over six times higher in the epidermal extract than in the extract of the whole leaf. The value for the epidermal extract was probably lower than the true figure for the intact epidermis because of the loss of protein from the chlorophyll free long epidermal cells which were ruptured during peeling. The value for the whole leaf, 21, was similar to that found in nutsedge, 16.7 (Chen et al., 1974).

The distributions of chlorophylls a and b are different in C_4 plants (Kanai and Edwards, 1973; Edwards and Black, 1971; Woo, Pyliotis and Downton 1971; Chen et al., 1974). Bundle sheath cells are depleted in chlorophyll b and therefore have a high chlorophyll a/b ratio (Kanai and Edwards report a value of about 6 in maize). Mesophyll cells have a much higher proportion of chlorophyll b and therefore a lower a/b ratio of about 3.0. Kanai and Edwards have shown the leaf as a whole to have a ratio of about 4.0, in good agreement with the value obtained in this study.

There has been little data published concerning the chlorophyll a : b ratios of leaf epidermal tissue. Willmer, Pallas and Black (1973) reported values of about 2.4 in the lower epidermis of C.communis

Plate 3.2 : Fluorescence micrographs (mag x 400) of normal longitudinal epidermal peels of maize before (A) and after (B) washing in 2.5% Triton X-100. Chlorophyll fluoresced red in the wavelength used. The removal of contaminating chloroplasts by the detergent is very apparent. No guard cell chlorophyll appears to be lost however.

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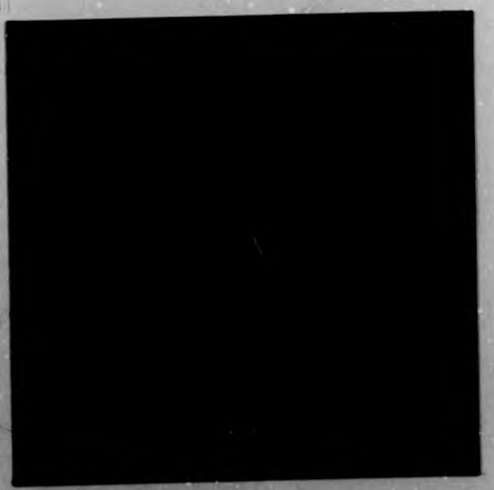

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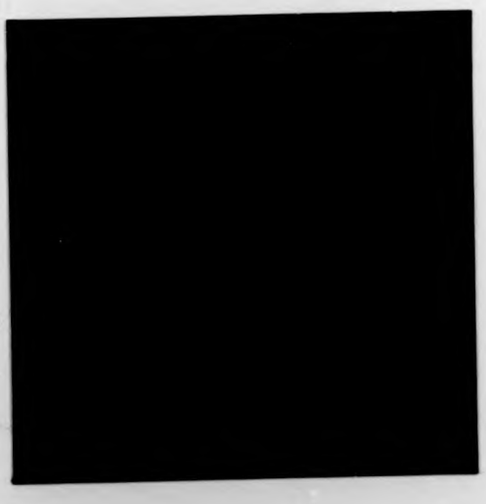


Table 3.2: Relative quantities of protein and chlorophylls in extracts of maize epidermal tissue and maize leaf tissue prepared from material isolated with different techniques.

Extract prepared from:	Protein :chlorophyll ratio	Ratio of chlorophyll a to chlorophyll b
Normal longitudinal epidermal strip	129	2.9
Enzyme treated epidermal strip	146	3.1
First stage of progressive grinding	44	2.4
Maize leaf lamina	21	3.9
Maize leaf lamina following enzyme treatment	40	4.3

and T. gesnariana. Pallas and Dilley (1972) reported one of 4.5 in the epidermis of V. faba. The difference between these reported values is surprising since all three species are C_3 plants. Extracts prepared from epidermal strips of maize (Table 3.2) had a chlorophyll a : b ratio of 2.9, intermediate between the values reported for C_3 plants and quite close to that reported for maize leaf mesophyll tissue (Kanai and Edwards, 1973).

Peels isolated immediately after plasmolysis:

A slight increase in the yield of intact cells was achieved when leaf tissue was infiltrated with 0.6M sorbitol, particularly if strips were peeled transversely (Table 3.1), 0.6M sucrose gave similar yields. Over 50% of the long epidermal cells could be obtained intact using 1.2M sucrose as osmoticum and peeling transversely. The higher yield was caused by the lower osmotic potential of the solution inducing a greater degree of plasmolysis in the cells and therefore making the protoplasts less liable to strain damage during peeling. As mentioned before the transversely peeled strips invariably broke at the first vein, therefore only small pieces of tissue could be obtained by this method making it unsuitable for use in the large scale isolation of tissue. The yield with infiltrated longitudinal peels was not sufficiently increased however to justify their use.

It was found that the viscosity of the osmotica presented a major problem in this technique. Solutions of sucrose and sorbitol of high enough concentration to cause an adequate amount of plasmolysis had a high viscosity. This meant that their penetration into the leaf following vacuum infiltration was very slow and somewhat irregular which resulted in quite a high proportion of strips, because of incomplete infiltration, having to be rejected. To overcome this problem the suitability of less viscous osmotica having similar osmotic

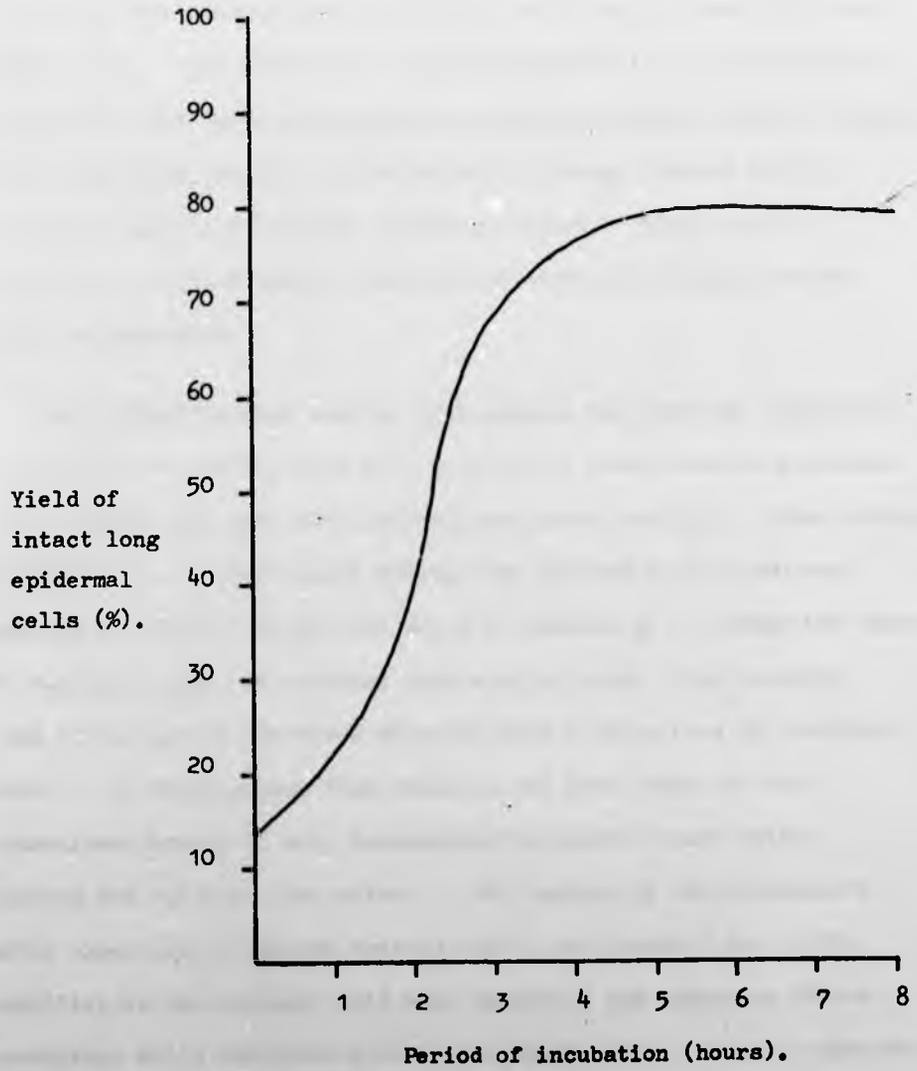
potentials to 1.2M sucrose were examined. 1.6M ethylene glycol, though it penetrated the tissue very quickly did not improve the yield above that obtained with normal, non-plasmolysed tissue. This was probably a result of its low reflection coefficient which meant that it could pass through the cell membrane into the cell to an appreciable extent therefore reducing plasmolysis (Greenway and Leahy 1970; Nobel, 1974). A mixture of 0.4M KCl and 0.4M $CaCl_2$ (Ruesink and Thimann, 1966) induced sufficient plasmolysis to increase the yield slightly but it was still lower than that obtained with 0.6M sorbitol and the treatment also affected the ability of the stomatal cells to take up neutral red. Further studies therefore used solutions of sucrose or sorbitol despite the proportion of strips that had to be rejected because of poor infiltration.

Enzyme Digestion:

Experiments using 0.6M sorbitol, 1% cellulase and 0.5% pectinase as incubation medium (Hall pers. comm., Kanai and Edwards, 1973) indicated that a high proportion of the long epidermal cells could be kept intact if the cell walls were weakened prior to peeling. In addition the epidermal strip seldom broke as it sometimes did with normal peels because the attachment between mesophyll and epidermal cells was weakened proportionally more during enzyme treatment than the close,cuticularised union between neighbouring epidermal cells.

A series of experiments was therefore carried out to find the optimum enzyme treatment to give the best yield of intact cells. The effect of incubation time on yield was investigated using the same combination of osmotica and enzyme as above (Fig. 3.1). Yield increased for up to four hours and then remained constant until after at least eight hours incubation when it started to decline again due to a loss of epidermal protoplasts during peeling. However it was also found that contamination increased during incubation so it was

Fig. 3.1 Effect of period of incubation upon yield of intact long epidermal cells of maize leaf epidermis. Epidermal strips were removed after incubation in a solution of 0.6M sorbitol containing 1% cellulase and 0.5% pectinase.


100
90
80
70
60
50
40
30
20
10
Yield of intact long epidermal cells (%).
1
2
3
4
5
6
7
8
Period of incubation (hours).

necessary to use the shortest incubation time concomitant with maximum yield, i.e. four hours. Shaking had no apparent effect on the yield of intact cells.

The effects of differing osmotica and osmotic concentration were then investigated. Solutions of sucrose or sorbitol at a range of concentrations containing 1% cellulase and 0.5% pectinase were used (Table 3.3). The yield of intact long epidermal cells was closely correlated with the concentration of osmoticum used; probably because of the differing degrees of protoplast shrinkage induced by the different osmotic potentials of the solutions. There was no difference in yield between sucrose and sorbitol solutions of the same concentration.

When attempts were made to deplasmolyse the sorbitol incubated protoplasts by placing them in a solution of lower osmotic potential it was found that they burst without any prior swelling. When protoplasts incubated in a sucrose based medium were treated in the same way, some burst immediately but the majority swelled up to occupy the whole of the cell lumen and ruptured some minutes later, this occurred even if the tissue was moved straight from 1.2M sucrose to distilled water. It would appear that sorbitol had some effect on the plasmalemma making it very susceptible to osmotic shock whilst sucrose did not have this effect. The rupture of the protoplasts after expansion in sucrose treated tissue was probably due to the inability of the weakened cell wall to resist the expansion of the protoplast which therefore tended to keep expanding until it ruptured.

Tissue incubated in a sorbitol based medium was also slower to take up neutral red and appeared less normal than that incubated in a sucrose medium and therefore it was decided to use sucrose as osmoticum in all further experiments. Despite is high concentration even

Table 3.3: The effect of different osmotica and various osmotic concentrations upon the yield of intact epidermal cells obtained after four hours incubation in solutions of the osmcticum, 1% cellulase and 0.5% pectinase.

Osmoticum	Yield of intact subsidiary cells (%)	Yield of intact guard cells (%)	Yield of intact long epidermal cells (%)
1.2M Sucrose	96	98	72
0.6M Sucrose	*	*	60
0.3M Sucrose	*	*	21
0.6M Sorbitol	97	98	67
0.4M Sorbitol	*	*	28
0.3M Sorbitol	86	94	17
0.15M Sorbitol	*	*	0
Filtered tap water	75	90	0

* Not determined

incubating solutions containing 1.2M sucrose had no effect on the ability of the protoplasts to take up neutral red, exclude Evans Blue or deplasmolyse. It was decided to use this concentration in further experiments, higher concentrations of sucrose were too viscous to permit satisfactory vacuum infiltration.

The optimum concentration of wall degrading enzymes was then determined. Previous workers have used a variety of enzymes at many different concentrations, some relying on pectinase or cellulase alone, others pectinase followed by cellulase and others a mixture of both. Because of the large pieces of tissue used in this investigation it was not possible to change the enzyme solutions during the incubation. Therefore a range of enzyme concentrations and mixtures were used in single phase treatments (Table 3.4).

Though the use of cellulase alone in the incubation medium permitted the isolation of strips having over 50% of their long epidermal cells intact a mixture of the two enzymes increased the yield still further. Pectinase alone gave poor yields. Maximum yields were obtained with 4% cellulase and 0.4% pectinase (as used by Power and Cocking, 1970). The improved yields with high concentrations was possibly caused by either adsorption of the enzyme onto the cell wall (and hence its inactivation) or by its end product inhibition (Halliwell, 1963; Selby and Maitland, 1965; Ruesink and Thimann, 1966). It was therefore decided to use an incubating medium containing 1.2M sucrose, 4% cellulase and 0.4% pectinase in further experiments. A typical strip prepared using this combination of osmotica and enzyme and incubated for four hours is shown in Plate 3.3

Contamination and enzyme treated tissue:

Because the enzyme treatment had also weakened the union between the mesophyll cells contamination proved a considerable problem in

Table 3.4: Effect of enzyme concentration upon the yield of intact long epidermal cells in epidermal strips removed after four hours incubation in enzyme solution. The osmoticum in all cases was 1.2M sucrose.

Concentration of cellulase (%)	Concentration of pectinase (%)	Yield of intact long epidermal cells (%)
4	-	73
2	-	65
1	-	50
-	0.4	25
-	0.1	20
2	1	77
4	0.4	84
1	0.5	72

Plate 3.3 : Typical epidermal strips removed after enzyme digestion and stained in neutral red. Virtually all of the protoplasts appeared intact and capable of accumulating neutral red.

A : Mag x 100

B : Mag x 500

enzy

Virt ly

and pable

A

B

enzyme treated tissue. It was apparently unaffected by the type of enzyme treatment used. There were two factors that were of importance in controlling it however:

(i) Length of incubation period:

Since contamination increased with longer incubation periods it was necessary to use the shortest incubation time possible.

(ii) Angle of peeling:

This presented a complex problem because it was also related to yield of intact cells. In untreated tissue it was necessary to pull the epidermis back at an angle of about 160° to obtain a reasonable epidermal strip, if a smaller angle was used the strip broke. Using this technique all epidermal cells were ruptured but contamination was light. Prior plasmolysis permitted slightly greater yields because less strain was placed on the protoplasts but the same peeling angle was necessary because epidermal-mesophyll connections had not been weakened, therefore contamination was about the same.

Because enzyme treatment weakened the connection between cells it was possible to peel strips at much smaller angles (70 - 80°). However this resulted in much more contamination since no lateral force was applied to the epidermal-mesophyll connections and therefore mesophyll cells could be simply lifted out with the strip, particularly since the union between the mesophyll cells had also been weakened. This problem could only be overcome by peeling at a more acute angle but then epidermal cells were ruptured. A compromise was therefore necessary and a peeling angle of between 90 and 120° was used which gave a reduced (though still appreciable) level of contamination and yields of over 80% intact long epidermal cells.

A number of methods were available to remove the remaining contamination. Though Triton X-100 had been suitable for washing normal peels it was found to cause the rupture of all intact long epidermal cells even if it was present in only minute quantities. Washing in osmoticum alone had no apparent effect whilst washing and a short incubation in fresh enzyme solution resulted in protoplast rupture. Sonication (Pallas and Dilley, 1972) was also unsuccessful, all epidermal cell protoplasts ruptured before removal of contamination was complete.

The technique used by Freeland (1951) proved the only one suitable for use with this tissue. Isolated strips with large areas of contamination were rejected whilst the others had small areas of contamination removed by gently scraping with a sharp blade followed by a washing in osmoticum. Contaminating vascular tissue could easily be removed with a pair of fine forceps.

Protein and chlorophyll concentrations in enzyme treated tissue:

Tissue was incubated for four hours, peeled, cleaned and ground up in 5ml of water. Protein and chlorophyll levels were determined in the extract (Table 3.2). A major problem was the high protein contents recorded, a result of the protein present in the incubating medium, which caused protein : chlorophyll ratios to be very high. The error was greatest in extracts prepared from enzyme treated leaf tissue because it was impossible to wash the enzyme solution from the air spaces of the leaf. Isolated epidermal strips could be washed more thoroughly but there was probably still an error as cellulase is adsorbed onto the walls (Halliwell, 1963) and so cannot be completely removed by washing. However in view of the contamination present in extracts prepared from enzyme treated epidermal strips it is probable that the high protein : chlorophyll ratio recorded was

also a function of the increased protein content of the extract derived from the intact long epidermal cells of the strips.

The value of chlorophyll a : b ratios in extracts prepared by enzyme digestion was similar to that recorded in normal extracts though that for the whole leaf was slightly greater, probably a result of the selective loss of mesophyll protoplasts during the enzyme treatment.

The use of enzyme digestion to isolate other epidermal tissue from more refractory leaves was also briefly investigated with moderate success. With a lower concentration of sucrose (to improve infiltration) the lower epidermis of leaves of sugar cane, rice and palm was removed with at least 50% of their cells intact. Further modification of the incubating medium would probably result in still further increased yields (Otsuki and Takebe, 1969).

Extracts prepared by progressive grinding:

Microscopic examination of tissue pieces that had undergone the first stage of progressive grinding revealed that the method was not successful at releasing the contents of large numbers of long epidermal cells free from contamination. Because of the resilient nature of the cuticle it was found that the glass beads tended to penetrate through the cuticle at one point and then tear right through the epidermis to release both epidermal and mesophyll cell contents. Thus, instead of the leaf surface being gently abraded away to leave the ruptured long epidermal cells on the surface just a few of these cells were ruptured before mesophyll cell contents started to escape. The only way in which an extract with a reasonable concentration of epidermal cell contents could be isolated was by removing the leaf pieces as soon as chlorophyll started to escape and replacing them with fresh pieces. Microscopic examination also revealed that a large

majority of the cells ruptured were long epidermal cells. Comparatively few stomatal cells were damaged; presumably because of their much thicker walls. Thus the final extract was comparatively enriched with long epidermal cell contents.

The final epidermal extract looked more highly contaminated than those obtained by the other techniques and chlorophyll and protein determinations confirmed this (Table 3.2). The protein : chlorophyll ratio was considerably less than half that of epidermal extracts obtained by the other methods. This was because of the greatly increased mesophyll contamination. However it was still much higher than that obtained in extracts of the pure leaf indicating that the protein level was supplemented by protein from the chlorophyll-free long epidermal cells. Since the epidermal extract prepared by progressive grinding contained little material from stomatal cells its chlorophyll a : b ratio was a reflection of the ratio in the mesophyll. The value obtained was slightly lower than that of the other epidermal extracts.

Discussion

It is apparent that all the methods attempted had both advantages and disadvantages when tissue was to be isolated for further analysis of epidermal activity and contents.

(i) Normal longitudinal peeling followed by dipping in 2.5% Triton X-100 yielded tissue that was very low in contamination. The method was also easy and yielded large amounts of tissue quite quickly. However virtually no long epidermal cells were left intact and an unknown proportion of their contents were lost during washing.

(ii) Normal longitudinal peeling following infiltration with a plasmolysing solution was little more successful than normal peels without infiltration. However infiltration took a considerable time

and it was felt that the slight increase in yield was not justified by the extra time required to isolate sufficient tissue and therefore the method was abandoned.

(iii) Normal longitudinal peeling following treatment with cell wall weakening enzymes increased the yield of cells by a large amount and virtually all the cells of the epidermis where left intact. It had the disadvantages that contamination, which was greater than on normal peels, could only be partially removed and also that it took a lot longer to isolate a satisfactory amount of tissue. Four hours of treatment with a plasmolysing solution containing possibly toxic enzymes (Basham, 1975) also possibly had unknown effects on the metabolism of the cells.

(iv) The major advantage of progressive grinding was the speed at which extracts could be prepared. However the amount of contamination from mesophyll cells was considerable.

Since none of the methods gave a completely satisfactory extract it was decided to use extracts prepared by each of the three most successful, normal longitudinal peeling, enzyme treatment followed by peeling and the first stage of progressive grinding. The use of peels removed transversely or after plasmolysis was abandoned. In addition to avoiding the problems of using just one extract this approach had the advantage that it was possible to examine the distribution of enzymes in the different cells of the epidermis. This was possible because of the selectivity of two of the methods for different cell types, normal epidermal peeling providing extracts rich in stomatal cells, progressive grinding extracts containing the contents of long epidermal cells but few stomatal cells.

The effects of Triton X-100 on the membranes of epidermal cells was of interest. Triton is a non ionic detergent commonly used for

the selective solubilisation of biological materials in particular chloroplasts (Deamer and Crofts, 1967; Bottomly, 1970). Pallaghy (1971) reported its use as a washing agent to remove contaminating chloroplasts from epidermal strips of maize but was unable to explain why its use caused the stomata of the strip to open slightly. The reason is apparent from these results showing that long epidermal but not stomatal cells are ruptured by Triton. In an epidermal peel with all its cells intact the guard cells tend to be pressed together by the turgor of the surrounding epidermal cells, when this turgor is lost the cells will tend to move apart slightly and so the stomatal pore opens slightly. Thus in the strips used by Pallaghy washing ruptured the epidermal cells and the stomata opened slightly.

Further experiments involving the washing of epidermal strips with dilute Triton were carried out on strips of Commelina communis and Vicia faba. The same effect was noted with normal epidermal cells rupturing and stomatal cells remaining intact though in C. communis there was a tendency for the subsidiary cells to rupture as well, particularly if washing was continued for a long time. Thus it appears that Triton (at a concentration of about 2%) causes a selective rupturing of epidermal cells in isolated epidermal strips making possible the removal of their contents (by washing) and leaving the subsidiary and guard cells intact for further study. This technique would seem potentially easier than the low pH treatment of Squire (1972), the use of rollers (Allaway 1973) or ultrasonic irradiation (Durbin and Graniti, 1975).

Triton at concentrations greater than 0.1% is thought to affect membranes by disrupting their structure and forming hydrophobic shells about the particles formed and thus solubilising them. Its differential effect on stomatal and normal epidermal cells could therefore indicate

that they have differing membranes. This seems unlikely however because electron microscopy has shown them to both have similar, apparently normal, cell membranes and it is unlikely that small differences could affect their susceptibility to a general attack such as that by Triton. The reason for the differential effect therefore probably lies in differences in the wall structure of the cells, either physical or chemical, resulting in the stomatal cell walls having a lower permeability.

CHAPTER 4

THE MEASUREMENT OF RESPIRATORY ACTIVITY IN EPIDERMAL TISSUE

Introduction

A discussion of the physiology and role of the maize leaf epidermis (Chapter 1) led to the conclusion that the tissue probably respires. This was further confirmed by electron microscopy (Chapter 2) which revealed that all epidermal cells contained apparently normal cytoplasm with mitochondria. However the absence of CO_2 efflux from an illuminated leaf indicated that, unless respiratory activity was so low as to be insignificant, conditions in the epidermis must be such as to prevent its escape. The primary aim of this section of the investigation was to confirm this respiratory activity, obtain, if possible, an estimate of its magnitude and examine any effects of light upon it. Respiratory activity of the isolated lower epidermis of Commelina communis was also recorded for comparative purposes.

The measurement of epidermal respiration:

Epidermal respiration should ideally have been measured in vivo however this was clearly impractical and so it was necessary to remove the tissue and measure its respiratory activity following peeling. This led to large changes in the physiological state of the tissue and consequent modification of respiratory activity. These changes were expected to originate from several sources:

(i) Rupturing and death of cells during isolation

(ii) Mechanical stimulation of respiration due to peeling (Audus, 1936)

(iii) An increase in respiration due to wounding (MacNicol, 1976)

(iv) Polyphenol oxidase activity in the damaged tissue resulting in a falsely elevated oxygen uptake.

The last of these could have been avoided by measuring the carbon dioxide exchange of the tissue but this proved impossible as none of the available techniques were suitable. Furthermore if CO_2 exchange had been measured and CO_2 efflux was restricted in the isolated tissue by non-photosynthetic CO_2 fixation then the recorded respiration rates would have been below the actual values.

It was hoped that the effects of the factors listed above could be quantified, at least approximately and estimates of epidermal respiration obtained.

The effects of light upon respiration:

Many attempts have been made to elucidate the effects of light upon dark respiration in both photosynthetic and non-photosynthetic tissues. The former are particularly difficult to investigate because of the interactions that occur between dark respiration, photosynthesis and photorespiration. Mangat, Levin and Bidwell (1974) have presented evidence for a 75% suppression of dark respiration in illuminated leaves of Phaseolus vulgaris, possibly mediated by ATP produced by photophosphorylation. However Raven (1972) has reported that since the metabolic pathways of dark respiration are also central to the provision of substrates for biosynthetic pathways very similar rates of CO_2 evolution are required in the light or dark though it is probable that oxidative phosphorylation is partially uncoupled in illuminated leaves.

The effects of light on dark respiration in non-photosynthetic tissues, despite the absence of photosynthesis and photorespiration is still confused. Hew and Krotkov (1968) indicated that respiration in chlorophyll free leaves is unaffected by light. Kowallik and Gaffron (1967) have shown that blue light at low intensities stimulates respiration in a yellow mutant of Chlorella, perhaps by increasing

membrane permeability. Ninneman and French (1973) have confirmed this in yeast but have also shown appreciable inhibition at high light intensities, probably as a result of the breakdown of cytochromes.

The categorisation of the epidermis as either photosynthetic or non-photosynthetic is impossible. The guard cells contain apparently active chloroplasts and since it is probable that they do not have a high biosynthetic load (their function being predominantly mechanical) there is no reason why respiratory activity, or at least oxygen uptake should not be reduced in the light by the same mechanisms that appear to function in the mesophyll. Other epidermal cells, which are concerned with the biosynthesis of cuticular and cell wall materials, are apparently non-photosynthetic however. Therefore respiration, if it is controlled, must be controlled by other means, either by a direct light effect or by products of the mesophyll or guard cells. The finding by Outlaw and Fisher (1975) that the epidermis of Vicia faba acts as a pathway for the translocation of photosynthates also raises the possibility that epidermal respiration is stimulated in the light in vivo by increased substrate availability.

Methods

(i) Plant Material:

Plants were greenhouse grown under natural lighting supplemented to 16 hours daylength. No special prior treatment was supplied.

(ii) Manometry:

Initial experiments indicated that constant volume (Warburg) manometry was more suitable for this investigation than constant pressure (Gilson) manometry. The latter was found unsatisfactory because only one flask could be prepared at a time and this meant that any small changes of temperature during the run had comparatively large

effects (for a further discussion of this problem see Carver and Gloyne, 1971). Since each Warburg manometer only utilises one flask this problem was minimised and any changes of temperature were reliably compensated for with a thermobarometer.

Maize epidermal tissue was isolated in two ways, normal epidermal peeling and peeling following incubation in an enzyme solution. The latter would, it was hoped, reduce errors due to rupturing of cells. With both techniques tissue was collected for fixed time periods, normal peels for 40 minutes, enzyme treated ones for 30. Time was used as the basis of tissue collection rather than absolute sample size because it was felt preferable to have samples having a similar age structure. As the strips were collected they were floated on either 0.1M (normal peels) or 1.2M (enzyme treated peels) ice-cold sucrose.

At the end of the collection period the isolated strips and 1 ml of the bathing medium were transferred to 20 ml Warburg flasks. The flasks had previously been calibrated using mercury (Purvis, Collier and Walls, 1966). 0.2 ml 6N KOH was introduced into the centre well of the flasks and they were sealed onto the manometers and placed in a shaking water bath at 25°C. Following 15 minutes equilibriation readings were taken at 15 minute intervals for 90 minutes. The tissue was then removed, surface moisture removed, weighed and then dried to constant weight. A similar procedure was followed to determine respiratory activity in maize leaf tissue and epidermal or leaf tissue of C. communis. Light regime was modified by using either blackened flasks or normal ones illuminated with two 100 watt incandescent lights.

(iii) Cartesian Diver Microrespirometry:

The great sensitivity of this technique meant that only one small

piece of tissue was required in each experiment. Therefore the piece of tissue could be isolated by transverse peeling, which, though it did not give any more intact cells than longitudinal peeling, probably caused less damage so that the significance of wound respiration in longitudinal peels could be assessed.

Maize epidermal tissue was removed and placed in the head of a Cartesian diver (about 2µl gas volume) with 0.1M sucrose. The sucrose was isolated from the 0.1N NaOH in the rest of the diver and the flotation vessel by an air bubble. The diver was stoppered, placed in the flotation vessel at 20°C and after 15 minutes equilibration readings continued for 90 minutes. Such a system is a constant volume, changing pressure system, the diver being returned to the same position for each reading by altering the pressure in the system. (For further details of the method see Klekowski, 1971). The dry weight of the tissue was recorded using Beckmann LM-500 microbalance.

I acknowledge with gratitude the assistance of Dr. V. Bryant with this technique and the temporary use of her apparatus and divers.

(iv) Polyphenol oxidase activity:

Polyphenol oxidase activity was determined in extracts of whole maize leaf tissue and enzyme treated epidermal tissue by polarography (Baldry et al., 1970). Its optimum pH was determined in whole leaf extracts prepared by grinding pieces of leaf tissue (about 1 cm^2) first in liquid nitrogen and then in 15 ml 0.05M phosphate buffer pH 7.0 followed by filtration through a 30µm mesh nylon net. The extract was kept on ice until used. 0.5 ml was added to 2.5 ml of one of a range of buffers (0.4M acetate, pH 4.0, 4.6 and 5.0; 0.2M citrate - 0.4M phosphate pH 5.4; 0.4M phosphate pH 6.0, 6.5, 7.0, 7.6 and 8.0 as used by Wilson, Nuovo and Darby, 1973) in a

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darkened Rank oxygen electrode at 25°C and the stopper inserted. The endogenous rate of oxygen uptake was recorded and 0.1 ml of 10mM substrate introduced into the chamber. Substrates used were, chlorogenic acid, caffeic acid (introduced as a suspension), catechol (o-diphenol), p-cresol and L- β -3-4 dihydroxyphenylalanine (L-DOPA). When the optimum pH had been determined extracts were prepared in that buffer and the enzyme activities determined on the basis of protein and chlorophyll concentrations. Protein was determined by the method of Lowry et al. (1951), chlorophyll (in 96% ethanol) by the method of Wintermans and De Mots (1965) and oxygen solubility from the equation given by Umbreit, Burris and Stauffer (1972, p.132).

Epidermal polyphenol oxidases were assumed to have the same pH optimum as those of the whole leaf. Extracts were prepared from peels isolated after enzyme treatment and stored in liquid nitrogen until required. The tissue was ground in 10 ml of buffer and filtered and assayed within 30 minutes. Because of the low rates of oxygen uptake encountered 1 ml of extract was introduced into the chamber without additional buffer.

To facilitate the conversion of enzyme activities to a dry weight basis the fresh weight : protein content and freshweight : dry weight ratios of the tissue were determined.

(iv) Estimation of epidermal pH:

Epidermis was isolated by normal longitudinal peeling and frozen in liquid nitrogen. When sufficient had been collected it was packed into the upper half of a polythene tube which was partially closed at its mid-point, this was placed in a centrifuge tube and centrifuged for 10 minutes at 10,000g (Sorval RC2-B centrifuge). The pH of the extruded drop which collected at the base of the centrifuge tube was measured, (Cohen and Atsmon, 1970).

Results

(i) Warburg Manometry:

The respiration rates recorded are shown in Table 4.1. Results are expressed on a dry weight basis since it was not possible to measure the area of tissue used and fresh weights were too variable because of the difficulty of removing all incubating medium. The rates were converted to an area basis using the dry weight : area relationship given in Chapter 2 and an R.Q. of 1.0 was assumed when values of oxygen uptake were converted to CO_2 output (Zelitch, 1971 p.131).

The values shown for each treatment are the means of the rates obtained by linear regression for each replicate. Since the intercepts of each individual regression did not pass through the origin it was not possible to quantify the variation as a single value reflecting scatter about the mean slope. Instead two values were calculated, the standard deviation of the rates of the individual replicates about the treatment mean and the mean standard deviation of the readings about the individual regression lines i.e. the amount of variation between replicates and within replicates.

Whole maize leaf yielded results much as expected, a fairly high respiration rate in the dark (1815µl O_2 g^{-1}DW h^{-1}; 0.49 mg CO_2 dm^{-2} h^{-1}) and none in the light. Previous workers have reported somewhat higher rates of dark respiration in the range of 0.8 - 3.0 mg CO_2 dm^{-2} h^{-1} (El - Sharkawy and Hesketh, 1965; El - Sharkawy, Loomis and Williams, 1967; Hew, Krotkov and Canvin, 1969; Hostra and Hesketh, 1969; Heichel, 1971; Luttge, Ball and Von Willert, 1971; Dube et al., 1975). The difference was probably due to the use of a different cultivar, the absence of a previous high light intensity treatment which would have stimulated dark respiration (Heichel, 1970) and errors in the conversion of oxygen uptake on a dry weight basis to CO_2 release on an area basis.

Table 4.1 ; Respiration rates of maize and C. communis leaf tissues measured at 25°C by Warburg manometry.

The figures in brackets (a,b) after each rate are: a - Standard deviation of the rates about the mean rate.
b - Mean standard deviation of the observations about each individual rate.

Source of tissue and its means of isolation	No. of replicates	Rate of oxygen uptake ($\mu l\ O_2\ g^{-1}\ DW\ h^{-1}$)		Dark respiration rate ($mg\ CO_2\ dm^{-2}\ h^{-1}$)
		Light	Dark	
Maize leaf lamina	10	0	1815 (439 , 142)	0.49
Enzyme treated maize leaf lamina	5	-	109 (37 , 10)	0.029
Normal epidermal strips of maize leaf	12	438 (193 , 115)	384 (223 , 124)	0.02
Normal epidermal strips of maize floated upon 1.2M sucrose	5	-	24 (15 , 10)	0.001
Enzyme treated epidermal strips of maize leaf	5	-	30 (19 , 8)	0.002
C. communis leaf lamina	5	-	3134 (587 ,332)	1.065
Normal epidermal strips of C. communis leaf	5	1189 (214 , 112)	1284 (70 , 102)	0.048

Respiratory activity in maize epidermal tissue isolated by longitudinal peeling was clearly lower than in the whole leaf. The only intact cells in this preparation were the guard and subsidiary cells so the rate measured was probably an indication of their activity, though phenol oxidation and wounding responses may have elevated it. The great variability enccuntered both within and between runs meant that the use of respiratory inhibitors to elucidate the importance of the former would have been very difficult.

Comparison of the respiratory activity of epidermal tissue in the light and dark using a Students t-test (there was no significant difference between the variances of the two means) revealed that there was no significant difference ($P > 0.05$) between the two measured rates.

Commelina communis whole leaf tissue showed the highest respiration rate of the tissues investigated, no other published rates for the tissue could be traced but it was comparable to that found in other C_3 species (Zelitch, 1971).

Epidermal tissue of C. communis had a considerably greater respiratory activity than that of maize epidermis on a dry weight basis though on an area basis the difference was somewhat less because of the greater weight of maize epidermis. It is apparent that the variation between and within replicates was proportionally less with this tissue, probably a result of the smaller amount of damage caused by peeling. A t-test was again carried out to compare the respiration rates in the light and dark and again there was no significant difference between them ($P > 0.05$).

An appreciable inhibition of respiration was to be expected in enzyme treated tissues, an effect of the high osmotic concentration of

the incubating medium (Greenway, 1970). However it was hoped that the inhibition would be small enough to permit a viable correction to be made, Greenway reported an inhibition of 50-60% in maize root tissue and this would probably have been acceptable. However as can be seen an actual inhibition of nearly 95% was encountered in both whole leaf and normal epidermal peels. Clearly the degree of correction required in this case prevents any viable conclusions being made concerning the activity of those cells ruptured during normal peeling. The most that can be said is that the corrected rate (514µl O_2 g^{-1}DW h^{-1}) was only slightly more than the respiratory activity of normal epidermal strips so that the activity of cells ruptured in the latter preparation was probably small. (The correction factor was derived from the degree of inhibition noted in enzyme treated whole leaf tissue and in normal epidermal strips floated on 1.2M sucrose). The large effect of enzyme treatment on respiratory activity was unexpected and could have been caused by a variety of factors, the lower water potential of the solution used (-45.4 bar instead of -20.8 bar), the presence of toxins in the enzyme solution, an anomalous increase in dry weight due to sucrose remaining in the tissue during drying (the solution used was equivalent to 40% sucrose) and perhaps a differential effect of osmotica on the respiratory activity of leaf and root tissues (Greenway used the latter). The recent report (Taylor and Hall, 1976) of elevated respiratory activity in isolated maize protoplasts would seem to indicate that the high osmotic concentration of sucrose used in the present study was the critical factor since in other respects there was little difference between the two techniques used to isolate protoplasts.

(ii) Cartesian Diver Microrespirometry:

A mean respiration rate of 758µl O_2 g^{-1}DW h^{-1} was recorded in transversely peeled sections of maize leaf epidermis. This is

appreciably greater than that measured in normal longitudinal peels by Warburg manometry and from it two conclusions may be drawn:

(a) longitudinal epidermal peels were probably damaged and their full respiratory potential not achieved.

(b) Since transverse peels were probably less damaged it might be expected that the contribution of wound and mechanically stimulated respiration to the recorded rate would be small. The higher respiration rate recorded in such peels therefore probably indicates the insignificance of wound respiration in normal longitudinal peels.

(iii) Polyphenol Oxidase Determinations:

The conclusions that may be drawn from studies on polyphenol oxidase activity in crude extracts using the oxygen electrode are very limited (Brown, 1967). The complexity of the reactions involved and their unknown stoichiometries make conclusions other than about the presence or absence of polyphenol oxidases unwise. However it was felt that the use of more complex assay procedures was not justified in this investigation in view of other errors involved in the measurement of respiratory activity in this tissue.

Extracts of maize leaf were found to have appreciable polyphenol oxidase activity, (Table 4.2). The three most suitable substrates, catechol, caffeic acid and chlorogenic acid all showed a similar response to pH (Fig. 4.1) with a broad peak of activity and an optimum pH of about 5.5. All further experiments were therefore carried out in extracts prepared in 0.2M citrate - 0.4M phosphate buffer pH 5.5. Earlier investigations into polyphenol oxidase activity (Ku, Gutierrez and Edwards, 1974a; Baldry et al., 1970) have used higher pHs when assaying the enzyme polarographically, probably because the

Table 4.2: Activity of polyphenol oxidase in extracts of maize leaf tissue and maize leaf epidermis. All values are expressed in μmoles oxygen taken up. All experiments were carried out at pH 5.5.

Substrate	Activity in whole leaf tissue		Activity in enzyme treated leaf tissue		Activity in enzyme treated epidermal tissue	
	mg^{-1}chl. h^{-1}	mg^{-1}pro. h^{-1}	mg^{-1}chl. h^{-1}	mg^{-1}pro. h^{-1}	mg^{-1}chl. h^{-1}	mg^{-1}pro. h^{-1}
Chlorogenic acid	117	10.6	107	3.8	105	1.2
Caffeic acid	52	3.2	38	1.1	53	0.5
Catechol	21	1.5	15	0.5	81	0.6
p -Cresol	7	0.3	0	0.0	26	0.3
L - DOPA	6	0.4	5	0.2	8.1	0.1

Fig. 4.1 pH curve for polyphenol oxidase activity from a crude extract of maize leaf tissue. Substrates showing appreciable activity were chlorogenic acid (⊙), caffeic acid (×), and catechol (□). Conditions used were as described in the Method.

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work has been concerned with the inhibition of other enzymes at these pH's. Neither paper gave any indication of the optimum pH of the polyphenol oxidase assayed. Macri, Di Lenna and Vianello (1974) used a pH of 6.5 when assaying polyphenol oxidases of maize leaves spectrophotometrically.

Extracts of whole maize leaf showed most activity towards chlorogenic acid, followed by caffeic acid and catechol (Table 4.2). There was little additional oxygen uptake with L-DOPA and p-Cresol as substrates. The results are similar to those reported by Baldry et al. and Ku et al. though the latter reports oxidation of L-DOPA as well.

Enzyme treated whole maize leaf showed a similar activity to normal maize leaf on a chlorophyll basis indicating that there was no inhibition of polyphenol oxidase activity by the enzyme solutions used. The activity on a protein basis was greatly reduced because of the falsely high protein concentration of the enzyme treated extract as a result of the preserce of adsorbed cellulase and pectinase. The same problem applied to extracts of enzyme treated epidermis.

The epidermal extract showed a slightly lower activity towards chlorogenic and caffeic acids than the leaf as a whole (shown by the lower activities than macerated leaf on a protein basis and the similar activities, despite the reduction in chlorophyll content, on a chlorophyll basis). Catechol and p-cresol oxidases showed a slightly higher activity, though still much lower than chlorogenic acid oxidase activity.

Using the following ratios it was possible to convert enzyme activities on a protein basis to a dry weight basis:

Epidermal fresh weight : protein ratio	:	104
Epidermal fresh weight : dry weight ratio	:	7.1
Leaf fresh weight : protein ratio	:	28.2
Leaf fresh weight : dry weight ratio	:	6.9

The epidermal activity of polyphenol oxidase, using chlorogenic acid as substrate, was 1848µl O_2 g^{-1}DW h^{-1} considerably greater than the uptake recorded by Warburg manometry.

A mean epidermal pH of 5.5 was recorded, slightly lower than that of the whole leaf (pH 5.7). This value is close to the optimum for polyphenol oxidase and though it probably represents the vacuolar rather than cytoplasmic pH it is likely to be the pH at which the enzyme would function in a damaged cell.

Thus it would appear that all oxygen uptake recorded was possibly due to phenol oxidation, particularly as the pH of the epidermis was close to the optimum pH of the enzyme. However there are several considerations which indicate that this was not likely:

(i) Polyphenol oxidase activity was recorded with excess substrate (2 mM) whilst in the leaf it is almost certainly lower, Macri et al. (1974) reported a total phenol content of about 2.1 mg g^{-1} FW in maize leaves whilst Baldry et al. (1970) report about the same concentration of o-diphenols in sugar cane leaves. Baldry also reported a stoichiometry of 2 µmoles O_2 consumed per µmole o-diphenol present and if this is used to convert phenol content to total expected oxygen uptake a value of about 1800µl O_2 g^{-1}DW is obtained. Since less than half of the total leaf content of o-diphenols are present in the epidermis of maize (Bucke and Long, 1971b) the maximal rate of phenol oxidation could only be maintained for a short period of time.

(ii) The isolated epidermal strips were floated on sucrose for an appreciable length of time prior to being placed in the flask and during this time an appreciable proportion of the phenols could have been oxidised or escaped into the sucrose.

(iii) Normal epidermal peels that were floated on 1.2M sucrose showed a similar reduction in oxygen uptake to enzyme treated leaf tissue yet polyphenol oxidase was not inhibited by the enzyme treatment. Therefore if polyphenols were being oxidised in the floated strips a smaller reduction in oxygen uptake was to be expected.

It would therefore appear that though polyphenol oxidase was present in the tissue, its influence in increasing the measured respiration rate was probably small, particularly if its products tended to inhibit respiration in any way.

Discussion

It is clear that of the factors mentioned in the Introduction which were thought might modify the respiratory activity of isolated epidermal strips the only one of critical importance was the rupturing and death of cells. Wound respiration, mechanical stimulation and polyphenol oxidase activity were probably insignificant in changing the apparent respiratory activity. Unfortunately it proved impossible to quantify the error due to cell death but in view of the distribution of cytoplasm (see Chapter 2) it is likely that the activity of the ruptured cells was comparatively low and the error therefore small.

In addition to changes in respiratory activity induced by the isolation procedures there were also perhaps changes caused by the experimental conditions, in particular the absence of carbon dioxide in the atmosphere in the Warburg flasks, which though it does not

affect respiration directly (Zelitch, 1971), could affect respiratory activity in two ways:

(i) There could be no net photosynthetic carbon fixation in the tissue resulting in a possible depletion of respiratory substrates. Though the presence of sucrose in the bathing solution probably reduced this problem substrate availability would certainly differ from the normal.

(ii) Stomata respond to low ambient CO_2 concentrations by opening and remaining open, even in the dark (Meidner and Mansfield, 1968). In functional epidermal strips this would result in a continued output of metabolic energy in both the light and dark to maintain stomatal opening and consequently an elevated respiratory activity. In whole leaf tissue open stomata would result in reduced diffusion resistances and a consequent increase in respiratory activity (Begg and Jarvis, 1968).

Though the consequences of these effects, particularly the latter, were large they were of minor importance in this investigation because the experiments described in Chapter 1 which demonstrated the absence of CO_2 escape from illuminated maize leaves were carried out in similar conditions of low ambient CO_2 concentrations. Thus though the results obtained in this investigation were not directly comparable to those recorded in normal field grown plants they were meaningful in terms of the problem being examined.

From the foregoing discussion it is clear that the measurement of respiratory activity of isolated maize epidermis was open to many errors which decrease the significance of any results obtained. However it is possible to draw certain qualitative conclusions from the results.

Maize epidermal tissue repeatedly showed a significant oxygen uptake following its isolation by a variety of techniques. Most experiments were carried out on normal longitudinal peels which had a high proportion of ruptured long epidermal cells. The uptake measured in this preparation was therefore principally by the cells of the stomatal complex (with perhaps some phenol oxidation in the ruptured cells) and I feel that it is evidence for respiratory activity in those cells. As discussed earlier the large correction factor employed to obtain comparable rates for enzyme treated tissue make an estimate of respiratory activity in intact long epidermal cells of doubtful value, however the small difference noted between corrected respiration rates in enzyme treated tissue and normal epidermal peels indicated that the activity was probably low.

The results obtained by Cartesian diver microrespirometry cast some doubt on the reliability of the data obtained by Warburg manometry. Though the higher rate recorded using the former technique could be explained by the higher proportion of long epidermal cells intact it seems unlikely in view of the small increase in transversely peeled tissue (probably still less than 10%). A more probable explanation was that, despite their ability to accumulate neutral red, stomatal cells in normal longitudinally peeled strips were damaged and their respiratory activity reduced as a result. Pallaghy (1971) reported that longitudinally peeled strips were unable to respond to changes in environmental conditions and showed a loss of potassium whilst transversely peeled strips appeared normal. If this was the case then the respiration rate recorded with Cartesian divers was a better indication of respiratory activity within the tissue. Cartesian diver microrespirometry is certainly potentially a very useful technique for measuring respiratory activity of small amounts of plant tissue and certainly deserves further examination.

Thus though the results obtained from maize epidermis are very poor they do provide evidence of respiratory activity in the tissue though it is probably largely restricted to the cells of the stomatal complex. However it has proved impossible to quantify it with any confidence and the rates recorded could be either greater or less than the normal rate *in vivo*, though the latter would seem the most probable. It is therefore impossible to be certain that the rate of CO_2 production by respiration would be sufficient to be detected in the experiments described in Chapter 1 which failed to detect CO_2 efflux from the intact leaf in the light.

Probably because of the much greater proportion of intact cells obtained the epidermis of *C. communis* proved to be considerably more suitable for measuring the respiratory activity of epidermal tissue and as a result the data obtained are much more reliable. The much greater rates recorded were perhaps unexpected, particularly in view of the higher stomatal density in maize epidermis. Though the difference could have been caused by a respiration rate in maize epidermis lowered to reduce CO_2 loss there are several other, perhaps more likely, causes, for example the greater proportion of intact cells, a large wound response and stomata demanding a higher energy expenditure to maintain opening. If the rate recorded in isolated epidermal strips of *C. communis* was the same as that occurring in the epidermis when attached to the leaf then epidermal respiration and consequent CO_2 escape could be of importance in the establishment of the CO_2 compensation point of that species.

The absence of any effect of light on measured oxygen uptake of both *C. communis* and maize epidermis was unexpected. The guard cells of both species contain apparently active, starch containing chloroplasts (see Chapter 2 and Pearson and Milthorpe, 1974) and both show

fluorescence typical of chlorophyll. It might be expected that, though full photosynthesis was suppressed by the absence of carbon dioxide, the apparent oxygen uptake in the light might be lowered as a result of the reassimilation of respiratory CO_2 and consequent photosynthetic release of oxygen. Clearly this was not the case.

This finding was particularly surprising in the case of maize because of the ability of maize leaves to retain CO_2 in the light. A possible mechanism for this retention was thought to be the inhibition of respiratory activity in the light and whilst this could occur in the long epidermal cells (the methods used were not sufficiently accurate to decide) it is clear that it did not occur in the cells of the stomata, the most active cells of the epidermis where, in addition to any inhibition of respiratory activity, photosynthetic CO_2 fixation and oxygen release would also be expected to reduce apparent oxygen uptake.

The findings by Das and Raghavendra (1974) that the guard cells of C. benghalensis and Petunia hybrida have low rates of the Hill reaction and noncyclic photophosphorylation but high rates of cyclic photophosphorylation may partially explain the lack of an apparent light effect. If a similar situation occurs in the guard cells of maize then photosynthetic reassimilation of respiratory CO_2 could occur without a stoichiometric release of oxygen (though in the absence of appreciable non cyclic photophosphorylation the source of NADPH required must remain in doubt). The techniques used in this study measured oxygen exchange and therefore such a refixation would not be noted.

The absence of any light effect on respiratory exchange in C. communis indicates that photosynthetic fixation is also probably comparatively unimportant in the stomata of that species. Das and

Raghavendra did detect some Hill reaction activity in the tissue so some oxygen production, and consequent reduction of apparent respiration in the light was to be expected, clearly this did not occur.

Pearson and Milthorpe (1974) have reported that the epidermis of C. cyanea shows only autotrophic CO_2 fixation and that dark fixation was minimal. A light fixation rate of 140ng CO_2 mg^{-1} min^{-1} was reported, equivalent to about 4000µl CO_2 g^{-1} h^{-1}, clearly much greater than the respiration recorded. However an appreciable quantity of reductant would have been required to sustain this fixation - if it was derived from non-cyclic photosphosphorylation then a reduction in apparent oxygen uptake was to be expected. As this did not happen it was more likely that the reductant was derived from respiratory activity in which case dark fixation as was described by Willmer and Dittrich (1974) would appear to be the more likely mechanism of CO_2 capture.

CHAPTER 5

THE ASSAY OF ENZYMES CONCERNED WITH CARBON DIOXIDE METABOLISM IN MAIZE LEAF EPIDERMIS

Introduction

Respirometry indicated that isolated maize epidermis released carbon dioxide in the light. However no CO_2 could be detected escaping from the intact leaf. A possible reason for this was that it was refixed by carboxylating enzymes in the epidermis when it was attached to the leaf and the products transferred either to the guard cells or mesophyll where they were further metabolised. It was therefore necessary to assay the activity of these enzymes in the epidermis.

The reassimilation of respiratory CO_2 by plants in the light has been recognised for many years and was first quantified by Audus (1947). It has since been demonstrated in the photosynthetic tissues of many species, including maize (El-Sharkawy, Loomis and Williams, 1967; Luttge, 1973). Such reassimilation is dependent upon the photosynthetic activity of the leaf. Its efficiency is a function of the diffusion resistances between the mitochondria, chloroplasts and the external atmosphere and also their relative distribution and frequency.

Many authors (see Chollet and Ogren, 1975, for a recent discussion) have discussed the potential advantages of the Kranz type anatomy found in the leaves of C_4 species in relation to respiratory reassimilation. The respiring bundle sheath cells are surrounded by a mesophyll containing high carboxylase activities and any CO_2 released must pass through the latter in order to reach the external atmosphere; therefore most, if not all respiratory CO_2 can be reassimilated.

The potential value of such photosynthetic reassimilation in preventing CO_2 escape from the epidermis of maize is very limited however. The epidermal cells are in direct contact with the leaf air space system as well as the mesophyll and so it is probable that gases could escape without passing through photosynthetic cells, particularly if the cuticle is permeable to CO_2. Therefore non-photosynthetic reassimilation of respiratory CO_2 is the only means of preventing CO_2 efflux from the chlorophyll-free epidermal cells.

Reports of significant non-photosynthetic CO_2 fixation are more limited though it is probable that all plants have the ability to a limited extent. Perhaps the most well known is Crassulacean acid metabolism (CAM) which is found in a variety of desert adapted species. CAM involves the dark fixation of CO_2 by the enzyme phosphoenolpyruvate (PEP) carboxylase to form malate which is stored until the light period when it is broken down by malic enzyme to liberate CO_2 which is then refixed by ribulose bisphospate (RuBP) carboxylase. The hypothesis of Bradbeer et al. (1958) that RuBP carboxylase is also involved in the dark fixation has recently been discounted (Cockburn and McAulay, 1975; Bradbeer, 1975).

Non-photosynthetic CO_2 fixation, or the potential for it, has also been reported in a variety of C_3 species, either in the leaves in the dark (Kunitake, Stitt and Saltman, 1959; Hall et al., 1959; Zelawski, Reich and Stanley, 1970) or in a variety of essentially non-photosynthetic tissues in the light e.g. bean pods (Crookston, O'Toole and Ozbun, 1974) the trunk of Robinia pseudoacacia (Höll, 1974), Gladiolus cormel (Ginzberg, 1975), pea carpel (Hedley, Harvey and Keely, 1975), soybean pods (Quebedeaux and Chollet, 1975) and apple seedlings (Ryc and Lewak, 1975). The mechanism of non-photosynthetic CO_2 fixation in these cases has not been fully resolved but most reports

implicate PEP carboxylase as the enzyme responsible for the initial fixation. When the properties of plant carboxylases are considered then the reasons for the involvement of this enzyme become apparent.

Plant Carboxylases:

Green plants contain two carboxylases in significant quantities and also an enzyme capable of facilitating the transport of CO_2. RuBP carboxylase (previously known as carboxydismutase or ribulose diphosphate carboxylase; E.C. 4. 1. 1. 39) is the most widespread and is the fundamental enzyme of the photosynthetic carbon reduction (PCR) cycle (Calvin and Benson, 1948; Bassham and Calvin, 1957); the ultimate means of CO_2 fixation in all green plants. The PCR cycle involves the expenditure of a considerable amount of energy (three molecules of ATP and two of NADPH to fix one of CO_2) and since respiration liberates only 6.3 molecules of ATP for each molecule of CO_2 released (calculated on the basis of 38 molecules of ATP released per molecule of glucose completely respired) it is apparent that the only viable source of this energy is photophosphorylation.

For this reason RuBP carboxylase could not mediate purely non-photosynthetic reassimilation of CO_2. However it could do so if part of the PCR cycle was located in photosynthetising cells so that a transfer of substrates and products took place between the site of RuBP carboxylase-mediated CO_2 fixation and the other energy demanding steps of the cycle. Thus it might be possible for RuBP carboxylase to assist CO_2 retention in a tissue such as maize leaf epidermis in the light and it was therefore decided to investigate its distribution.

PEP carboxylase (E.C. 4. 1. 1. 31) is usually present in smaller quantities then RuBP carboxylase in plant cells but it appears to be universally distributed and has several properties which make it suitable for assisting in the reassimilation of respiratory CO_2:

(i) Unlike RuBP carboxylase it appears to be a cytoplasmic enzyme (Hatch and Kagawa, 1973), it can therefore function close to the sites of CO_2 release and independent of active chloroplasts.

(ii) Each molecule of CO_2 fixed by PEP carboxylase requires the conversion of one ATP to AMP during the synthesis of PEP from pyruvate by the enzyme pyruvate phosphate dikinase and the reduction of one molecule of NADPH during the reaction converting oxalacetate to malate. Therefore a maximum of 5ATP equivalents are required at the site of fixation - less than is produced by respiration yielding a single molecule of CO_2. The energy required for reassimilation could be further reduced if aspartate was the first stable product (for which no NADPH has to be reduced) or by the synthesis of PEP in a photosynthetic cell followed by its transfer to the site of respiration. The latter would seem unlikely however, in view of the instability of PEP. It must also be recalled that though the maximum net loss to the respiring cell would be five ATP equivalents the total energy loss to the tissue would be only two ATP equivalents because a molecule of NADPH is produced upon decarboxylation of malate by malic enzyme.

PEP carboxylase exists in several forms (Ting and Osmond, 1973 a, b; Mukerji and Ting, 1971) differing in their functions and their values of K_m and V_{max}. However it is apparent that all can function in either the light or dark, in photosynthetic or non-photosynthetic cells using substrates derived either from pyruvate by the light activated enzyme pyruvate phosphate dikinase (Slack, 1968) or by the glycolytic pathway from PGA, an intermediate in both respiration and PCR cycle carbon dioxide fixation, (Sutton, 1975; Huber and Edwards, 1975a).

Green plants contain a third enzyme which, though it is not a carboxylase, could facilitate the transport of carbon dioxide between

the epidermis and mesophyll of maize leaves and therefore assist in its retention. Carbonic anhydrase (E.C. 4. 2. 1. 1.) catalyses the interconversion of CO_2 and bicarbonate in solution. Under normal conditions this equilibrium is attained comparatively slowly because of the rearrangement of bonds that is necessary (Edsall, 1969); the enzyme greatly increases the speed at which this equilibrium is attained.

Carbonic anhydrase was thought to aid photosynthesis in the leaf either by facilitating the diffusion of CO_2 (permitting rapid equilibrium with bicarbonate and therefore diffusion of two molecular species) or by locally increasing the concentration of CO_2 at the carboxylation site. However evidence has been presented (Jacobsen, Fong and Heath, 1975) that photosynthetic reactions are not affected by inhibitors of carbonic anhydrase and it is now postulated that the enzyme functions in the plant cell either by preventing transient pH changes in the chloroplast (which might denature proteins) or by hydrating other compounds than CO_2.

In addition to increasing effective concentration carbonic anhydrase can also provide transport of CO_2 across membranes (Longmuir, Forster and Woo, 1966; Enns, 1967). In animal cells this is one of its major functions (Maren, 1967).

In the epidermis it could fulfil either or both of these roles since the retention of CO_2 requires both its solubilisation and its transport. However in view of the low overall pH of the epidermis (5.5) the ability of the enzyme to increase the effective concentration of CO_2 must be doubted. At pH 5.0 less than 4% of dissolved CO_2 is present as bicarbonate, 96% is present as the dissolved gas (Jarvis, 1971), obviously carbonic anhydrase could only slightly increase the short term capacity for CO_2. However since the cytoplasm has a higher

pH, probably close to neutrality its capacity for bicarbonate is potentially greater and carbonic anhydrase could possibly aid its formation and transfer across the plasmalemma to the mesophyll.

Enzyme Distribution in the C_4 plant:

In recent years much research has been concerned with the elucidation of the distribution of photosynthetic enzymes in the leaves of C_4 plants. The findings of this work are of considerable significance when data obtained from epidermal extracts are to be considered. Two types of enzyme distribution in the C_4 leaf have been proposed with supporting evidence. That of Coombs and co-workers (see Coombs et al., 1976 for the most recent discussion) suggests that RuBP carboxylase is present in the chloroplasts of both bundle sheath and mesophyll cells whilst PEP carboxylase is restricted to the cytoplasm of the mesophyll cells. Thus they visualise the C_4 cycle occurring between the cytoplasm and chloroplasts of the mesophyll cells. Their results conflict with those of the supporters of the other hypothesis first proposed by Hatch and Slack (1971), which suggests that PEP carboxylase is located solely in the mesophyll cells and RuBP carboxylase largely in the bundle sheath. Thus they propose that the C_4 cycle acts as a shuttle for CO_2 between the two cell types rather than between cytoplasm and chloroplast of the same cell.

Most of the early work in these studies involved a variety of progressive grinding techniques with a variety of species and data satisfactorily demonstrating either hypothesis was not obtained because of the considerable impurities present. More recently enzyme digestion techniques have been evolved which permit a much better separation of the different cell types. Using this technique Edwards and co-workers have investigated enzyme distribution in a variety of C_4 species producing data that favours the hypothesis of Hatch and Slack (Kanai and Edwards, 1973; Ku, Gutierrez and Edwards

1974; Huber and Edwards, 1975a). Coombs et al. have attempted to explain these findings in terms of the distribution of the enzyme polyphenol oxidase which they believe to be concentrated in the epidermis and mesophyll (Bucke and Long, 1971b; Coombs et al., 1976) and responsible for the inhibition of RuBP carboxylase activity in extracts of those tissues. However Ku, Gutierrez and Edwards (1974a) have produced conflicting data showing polyphenol oxidase activity in both mesophyll and bundle sheath cells. These data, coupled with the much better isolation techniques employed by Edwards' group and the much greater carboxylase activities reported by them have meant that the hypothesis of Hatch and Slack is now generally accepted.

Comparatively little work has been carried out on the distribution of carbonic anhydrase in the C_4 leaf though Poincelot (1972) has presented evidence indicating that it is concentrated in the mesophyll of maize leaves.

Enzyme Distribution in the Epidermis:

Shaw and Maclachlan (1954) first presented evidence that stomatal cells have the ability to fix CO_2 from the atmosphere. It was thought that this occurred via the PCR cycle. However recent work by Willmer and co-workers (1973 a, b; 1974) has shown that PEP carboxylase is probably the active enzyme. The work was carried out on extracts prepared from the lower epidermis of two C_3 species, Tulipa gesnariana and Commelina communis and showed PEP carboxylase activities as high, if not higher, than those reported in whole leaf extracts of many C_4 species.

Since all evidence to date (Willmer and Pallas, 1973; Meidner and Mansfield, 1968) has indicated a similar stomatal mechanism in all plant species the presence of PEP carboxylase in the stomata of maize was also perhaps to be expected.

It was therefore clear that though PEP carboxylase probably could function in maize epidermis to prevent CO_2 escape (and was therefore assayed) care had to be taken in evaluating the significance of any found in view of its presence in the epidermal tissue of C_3 species which of course lose CO_2 in the light in a CO_2-free atmosphere.

In addition to PEP carboxylase Willmer also detected considerable quantities of malic enzyme (E.C. 1. 1. 1. 40) in the tissue he investigated. This enzyme usually functions as a decarboxylase and so any CO_2 fixed in the stomata by PEP carboxylase was probably either rereleased elsewhere in the epidermis or in a different phase of stomatal movement. The presence or absence of malic enzyme was therefore potentially of importance in assessing the distribution of any PEP carboxylase found in maize leaf epidermis and it consequently wasnecessary to determine its activity as well.

Methods

(i) Preparation of Extracts:

6 - 8 week old maize plants, greenhouse grown with supplemented lighting were used in all experiments. Three epidermal extracts prepared using tissue isolated by the following methods (described in Chapter 3) were investigated -

(a) Normal longitudinal peels of lower epidermis washed in 2.5% Triton X-100.

(b) Longitudinal peels removed after four hours incubation in 1.2M sucrose containing 4% cellulase and 0.4% pectinase.

(c) The first stage of progressive grinding.

Isolated epidermis was keptin liquid nitrogen until required.

Extracts were also prepared by grinding whole maize leaf tissue (excluding the midrib) either fresh from the plant or following enzyme

treatment identical to that given the epidermis.

Tissue was ground up in a cold pestle and mortar containing 5 ml grinding medium (50 mM Tris-HCl, pH 8.3; 5 mM $MgCl_2$; 5 mM dithiothreitol (DTT) and 1 mM ethylenediamine tetracetic acid (EDTA)). Grinding medium was made up and stored at 5°C until required, DTT was added just before use. Cell rupture was increased during maceration by the addition of 2 ml glass beads (150 - 200 µm dia.). After grinding the extract was filtered through a 30 µm nylon net and stored on ice until required.

(ii) Assay of PEP Carboxylase:

Initial experiments indicated that the spectrophotometric method of Lane, Maruyama and Easterday (1969) was unsuitable and the method using ^{14}C labelled bicarbonate described by Willmer, Pallas and Black (1973) was used.

Solutions of 0.5M Tris-HCl, pH 8.3; 0.2M $MgCl_2$; 0.1M DTT; 0.05M PEP and 0.05M $NaH^{14}CO_3$ (Radiochemical Centre, Amersham) were prepared and stored (the latter three frozen) until required. All assays contained 10 µl $MgCl_2$ (final concentration 10 mM); 10 µl DTT (5 mM) and 20 µl PEP (5 mM). Extract and buffer were added to a total volume of 180 µl (40 µl buffer and 100 µl extract or 140 µl epidermal extract) and after 1 minute preincubation 20 µl $NaH^{14}CO_3$ (5 mM) added.

Samples (30 µl leaf assays, 50 µl epidermal) were withdrawn at timed intervals (at 1, 2, 3 and 4 minutes during assays on leaf extracts, at 2, 5 and 10 minutes with epidermal extracts) and added to excess saturated 2, 4 dinitrophenyl hydrazine in 4NHCl in a scintillation vial to drive off excess bicarbonate. 10 ml of scintillation fluid (700 ml toluene, 300 ml 2-methoxyethanol, 100 mg POPOP and 4g 2, 5-Diphenyloxazole (PPO)) was added and activity determined in a Packard Tri-Carb liquid scintillation spectrometer (Model 2425).

Quenching was determined using an external standard and comparison with a quench curve produced using standard ^{14}C-n hexadecane and varying quantities of 2, 4 dinitriphenylhydrazine (Fig. 5.1). Controls containing no PEP were carried out. Enzyme activity was expressed on both a chlorophyll and protein basis; chlorophyll was determined in 96% ethanol (Wintermans and DeMots, 1965), protein using Folins reagent (see Appendix 1).

(iii) Assay of RuBP carboxylase:

0.2M HEPES-NaOH buffer pH 7.8 and extract were added to 15 µl 0.1M DTT; 15 µl $MgCl_2$ and 30 µl 0.2M $NaH^{14}CO_3$ to give a total volume of 295 µl (100 µl leaf extract and 135 µl buffer or 160 µl epidermal extract and 75 µl buffer). After 3.5 min incubation the first aliquot was withdrawn and 5 µl 0.04M RuBP introduced. Further aliquots were withdrawn after a further 1, 3 and 6 minutes. All aliquots (50 µl epidermal assays, 30 µl leaf) were added to excess 2,4 dinitrophenyl hydrazine and counted as described before.

(iv) Assay of Malic Enzyme:

Activity was measured spectrophotometrically using 1 cm path length microcells (capacity 1 cm^3). Each cell contained 50 µl 0.5M Tris-HCl buffer pH 8.0; 25 µl 0.1M DTT; 20 µl 0.005M NADP, 5 µl 0.1M EDTA, 50 µl 0.05M L-malate and cell extract and water to a total volume of 475 µl. The endogenous rate of NADP reduction was determined at 340 nm using a Cecil CE 272 U.V. spectrophotometer linked to a pen recorder. 25 µl 0.1M $MgCl_2$ was then introduced and the rate of enzyme mediated NADP reduction measured. Activity was calculated from the extinction coefficient of NADP ($E^{mM}_{360} = 6.2$).

(v) Assay of Carbonic Anhydrase:

Activity was measured using bromothymol blue as described by Rickli et al.(1964). Because the grinding medium used for the other

Fig. 5.1 : Relationship between counting efficiency and the value of external standard ratio recorded by the Packard Tri-carb liquid scintillation spectrometer used in this study. The curve was prepared using standard ^{14}C-n hexadecane and varying quantities of 2, 4 dinitrophenylhydrazine.

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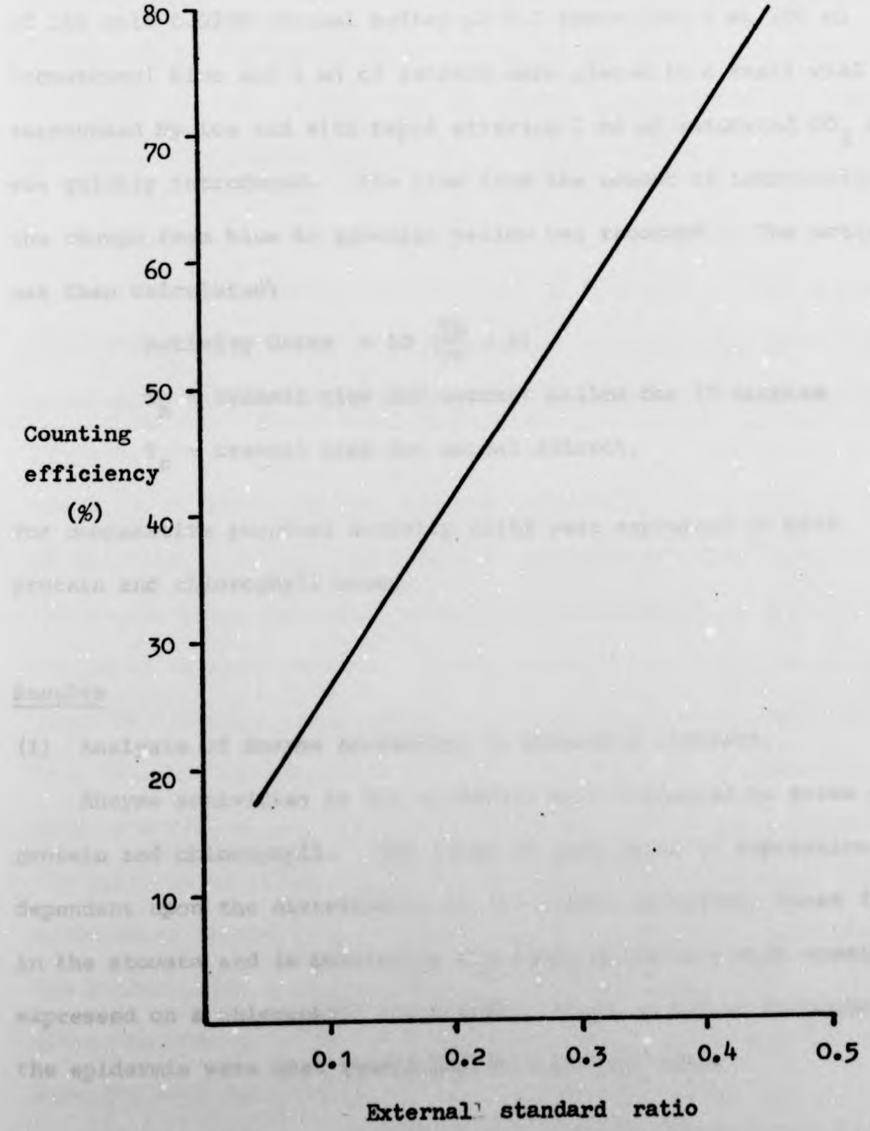


enzyme assays contained possibly inhibiting Cl^- (Verpoorte, Mehte and Edsall; 1967) it was necessary to use a different one lacking $MgCl_2$.

Leaf and epidermal tissue was ground up, filtered and stored in ice until required. Saturated CO_2 solution was prepared by bubbling CO_2 from dry ice through ice cold water for at least 30 minutes. 2 ml of ice cold 0.025M Veronal buffer pH 8.2 containing 2 mg/100 ml bromothymol blue and 1 ml of extract were placed in a small vial surrounded by ice and with rapid stirring 2 ml of saturated CO_2 solution was quickly introduced. The time from the moment of introduction to the change from blue to greenish yellow was recorded. The activity was then calculated:

$$\text{Activity Units} = 10 \left(\frac{Tb}{Tc} - 1\right)$$

T_b - transit time for extract boiled for 10 minutes

T_c - transit time for normal extract.

For comparative purposes activity units were expressed on both protein and chlorophyll bases.

Results

(i) Analysis of Enzyme Activities in Epidermal Extracts:

Enzyme activities in the epidermis were expressed in terms of both protein and chlorophyll. The value of each means of expression was dependent upon the distribution of the enzyme concerned, those found in the stomata and in particular the guard cells were most meaningfully expressed on a chlorophyll basis whilst those occurring throughout the epidermis were best quantified on a protein basis.

The activities of enzymes in the different extracts gave some indication of their distribution within the epidermis, though analysis was complicated as a result of contamination from the mesophyll and the large variation encountered in the data. Extracts prepared from

normal longitudinal peels which had been briefly washed in 2.5% Triton X-100 were the most contamination free (see Chapter 3) but all long epidermal cells had been ruptured during peeling and so an unknown proportion of their contents were lost. The enzymes present in the extract were therefore probably most representative of the stomata and so their activities were most meaningfully expressed on a chlorophyll basis; since membrane proteins probably remained in the long epidermal cells (plate 3.1) expression on a protein basis would have resulted in falsely low activities.

Extracts isolated by the first stage of progressive grinding were enriched in the contents of long epidermal cells and were therefore theoretically deficient in chlorophyll. However they were liable to considerable mesophyll contamination resulting in comparatively low protein : chlorophyll ratios (Table 3.2). As a consequence of the partition of photosynthetic enzymes between the two principal tissues of the maize leaf the importance of this contamination depended upon the enzyme being assayed. Because PEP carboxylase is present in the mesophyll in large amounts, it was to be expected that the extract would be strongly contaminated with it giving falsely high activities. This could be circumvented because of the absence of chlorophyll in the long epidermal cells; if PEP carboxylase was present in these cells its activity on a chlorophyll basis would have been greater than that encountered in the leaf as a whole (as a result of the enrichment of the extract with cytoplasm from non-chlorophyllous cells), particularly since the contaminating mesophyll had a higher PEP carboxylase activity than the bulk leaf tissue.

The other two enzymes assayed in extracts prepared by the first stage of progressive grinding, RuBP carboxylase and malic enzyme are localised in the bundle sheath cells and so were not present in the contamination. Thus the presence of either enzyme in the extract

probably represented enzyme from epidermal cells, though its measured activity would be lowered as a result of protein and chlorophyll present in the contamination. Expression of data on a chlorophyll basis is not very meaningful in this extract because of the absence of chlorophyll in long epidermal cells.

Enzyme treated epidermal tissue had a further source of error in addition to contamination. This was derived from the pectinase and cellulase adsorbed onto the cell walls which resulted in falsely high protein concentrations in the extracts. Protein could therefore only be used as a criterion for the expression of enzyme activity if a correction factor, derived by comparison of normal and enzyme treated leaf extracts, was applied. This could only be very approximate however because of the differing effects washing had on the two enzyme treated tissues and the large amount of correction required.

The contamination in enzyme treated extracts was similar to that found in extracts prepared by the first stage of progressive grinding and so expression of data also had problems similar to those encountered in that extract, though they also contained chlorophyll derived from the guard cells.

(ii) PEP Carboxylase Activities:

The activity measured in whole maize extracts (Table 5.1) was lower than most previous reports which range from 379 µmoles CO_2 mg^{-1} $chl.h^{-1}$ (Kanai and Edwards, 1973) to 1080 µmoles CO_2 $mg^{-1}chl.h^{-1}$ (Hatch, Slack and Bull, 1969). The low activity encountered in this work is probably a result of either varietal differences (as shown by Kanai and Edwards) or the comparatively low light intensities under which the plants were grown, PEP carboxylase activity being sensitive to light regime (Hatch, Slack and Bull).

It is clear that PEP carboxylase was strongly inhibited by enzyme

Table 5.1: Activity of PEP carboxylase in extracts of maize leaf lamina and maize leaf epidermis. Mean activities and 95% confidence limits are expressed in μmoles of CO_2 fixed.

Source of extract and its method of preparation	No. of replicates	Enzyme activity per mg^{-1}chl. h^{-1}	Enzyme activity per mg^{-1}pro. h^{-1}
Maize leaf lamina	9	354 (±104)	19.2 (±5.60)
Enzyme treated maize leaf lamina	5	34 (±26)	1.2 (±0.91)
Normal epidermal peels of maize leaf	7	485 (±276)	3.9 (±2.22)
Enzyme treated epidermal peels of maize leaf	5	48 (±74)	0.31 (±0.48)
First stage of progressive grinding	8	201 (±99)	5.1 (±2.50)

treatment used in isolating the tissue, activity in enzyme treated leaf tissue being less than 10% of that in normal leaf tissue. It is possible that some of this was due to the selective loss of mesophyll protoplasts during enzyme treatment.

Of the epidermal extracts, that prepared from normal epidermal peels had the highest activity (Table 5.1). On a chlorophyll basis it was higher than that found in the whole leaf, an indication of a high PEP carboxylase activity in the epidermis. The much lower activity in extracts prepared by the first stage of progressive grinding suggests that PEP carboxylase was localised in the stomatal cells.

The extracts prepared from enzyme treated epidermal tissue tended to confirm this finding, a value of 500 µmoles CO_2 mg^{-1}chl h^{-1} being obtained after correction. (The recorded epidermal activity was increased by the same factor that the activity in the whole leaf was depressed by enzyme treatment to give the corrected value.) Though the significance of this corrected value is low because of the large size of the correction applied its closeness to that recorded in normal epidermal peels must indicate a mainly stomatal distribution of the enzyme.

(iii) Malic Enzyme Activities:

Previous reports of malic enzyme activity in maize leaf tissue have ranged from about 500 µmoles NADP mg^{-1}chl.h^{-1} (Chen, Brown and Black, 1971; Downton and Hawker, 1973) to nearly 1,000 µmoles NADP mg^{-1} chl.h^{-1} (Kanai and Edwards, 1973). The activity recorded in this study was considerably lower (Table 5.2), this could be due to a lower activity of the enzyme in the cultivar used or to a loss of enzyme activity during isolation. The latter seems possible in view of the lower activity of malic enzyme than PEP carboxylase recorded.

Table 5.2: Activity of malic enzyme in extracts of maize leaf lamina and maize leaf epidermis. Mean activities and 95% confidence limits are expressed in μmoles of NADP reduced.

Source of extract and its method of preparation	No. of replicates	Enzyme activity per mg^{-1} chl. h^{-1}	Enzyme activity per mg^{-1} pro. h^{-1}
Maize leaf lamina	7	175 (±117)	5.9 (±3.95)
Enzyme treated maize leaf lamina	3	319 (±290)	9.6 (±8.74)
Normal epidermal peels of maize leaf	7	476 (±188)	4.4 (±1.72)
Enzyme treated epidermal peels of maize leaf	3	209 (±375)	1.5 (±2.69)
First stage of progressive grinding	6	128 (±40)	3.0 (±0.93)

The reverse was to be expected since maize is known to be predominantly a malate former (Johnson et al, and following papers in the same publication) and previous studies (Kanai and Edwards, 1973; Berry, Downton and Tregunna, 1969; Bucke and Long, 1971a) have reported higher activities of malic enzyme than PEP carboxylase.

The higher activity on a chlorophyll basis in enzyme treated leaf tissue (Table 5.2) was probably due to the loss of mesophyll protoplasts during the treatment resulting in a loss of chlorophyll but not malic enzyme. It is clear that the enzyme was not inhibited by the enzyme isolation procedure.

The activities recorded in the three epidermal extracts show that malic enzyme had a similar distribution to PEP carboxylase though it had a greater activity. It is clear that there is little, if any, malic enzyme activity in the long epidermal cells. The lower activity in extracts from enzyme treated tissue was probably a result of malic enzyme free mesophyll contamination increasing chlorophyll content.

(iv) RuBP Carboxylase Activities:

Previous workers (Ku, Gutierrez and Edwards, 1974; Kanai and Edwards, 1973; Chen, Brown and Black, 1971; Bjorkman and Gauhl, 1969; Poincelot, 1972) have reported RuBP carboxylase activities in maize leaf tissue ranging from 138 to 210 μmoles CO_2 $mg^{-1}chl.h^{-1}$, all somewhat lower than those found in whole maize leaf tissue in this study (Table 5.3). There was a clear inhibition of RuBP carboxylase activity in the enzyme treated leaf tissue. It was probably even greater than that indicated in the table because of the loss of mesophyll protoplasts that occurred during the enzyme treatment with consequent loss of chlorophyll and protein but not RuBP carboxylase.

All epidermal extracts contained lower activities of RuBP

Table 5.3: Activity of RuPB carboxylase in extracts of maize leaf lamina and maize leaf epidermis. Mean activities and 95% confidence limits are expressed in μmoles of CO_2 converted to acid stable products.

Source of extract and its method of preparation	No. of replicates	Enzyme activity per mg^{-1} chl. h^{-1}	Enzyme activity per mg^{-1} pro. h^{-1}
Maize leaf lamina	11	262 (±169)	12.50 (±8.13)
Enzyme treaed maize leaf lamina	7	40 (±17)	0.96 (±0.41)
Normal epidermal peels of maize leaf	8	142 (±48)	1.46 (±0.50)
Enzyme treated epidermal peels of maize leaf	5	197 (±292)	1.50 (±2.22)
First stage of progressive grinding	10	114 (±76)	2.91 (±1.95)

carboxylase than untreated leaf extracts (Table 5.3). The highest activity was surprisingly in extracts prepared from enzyme treated tissue and though the variation obtained was considerable it appears probable that some RuBP carboxylase activity was present in this extract. Indeed if the activity is corrected for inhibition by the same amount that whole leaf tissue activity was reduced then apparent activities of 1300 µmoles CO_2 $mg^{-1}chl.h^{-1}$ and 19 µmoles CO_2 $mg^{-1}pro.h^{-1}$ are obtained; these are very high indeed.

The lower activity recorded in tissue prepared by normal epidermal peeling indicated that the enzyme was probably largely concentrated in the long epidermal cells, though it is possible that some was present in the chloroplasts of the guard cells in view of their apparent PCR cycle activity as shown by their starch grains (Plate 2.5).

Extracts prepared by the first stage of progressive grinding also gave some indication of RuBP carboxylase activity in long epidermal cells of maize epidermis (Table 5.3). The overall activity recorded was the lowest found in the three epidermal extracts examined, but since the contamination in it was essentially RuBP carboxylase free (because it was from the mesophyll) much of that activity must have originated in the epidermis.

(v) Carbonic Anhydrase Activities:

The difficulty involved in the assay of this enzyme and its sensitivity to light regime during the growth of the plant (Everson, 1971) probably account for the variability of the data previously published concerning its distribution in maize; values have ranged from 350 units $mg^{-1}chl.$ (Everson and Slack, 1968) to 4780 units mg^{-} chl. (Poincelot, 1972). There was no apparent inhibition of activity in enzyme treated whole leaf extracts and the values obtained compared favourably with the published data (Table 5.4).

Table 5.4: Activity of carbonic anhydrase in extracts of maize leaf lamina and maize leaf epidermis. Mean activities and 95% confidence limits are expressed in activity units (after Rickli et al., 1964).

Source off extract and its method of preparation	No. of replicates	Enzyme activity per mg^{-1}chl. h^{-1}	per mg^{-1}pro. h^{-1}
Maize leaf lamina	5	1241 ($\pm$362)	41.6 ($\pm$12.06)
Enzyme treated maize leaf lamina	3	957 ($\pm$529)	31.9 ($\pm$17.55)
Enzyme treated epidermal peels of maize leaf	3	422 ($\pm$489)	4.17 ($\pm$4.84)

The activity in enzyme treated epidermal extracts was lower than that in the whole leaf and it is unlikely that the epidermis contained appreciable quantities of the enzyme, particularly since Poincelot reported a concentration of carbonic anhydrase activity in the mesophyll cells of the maize leaf.

Discussion

The reduction in the activity of certain enzymes in tissue that had been treated with wall degrading enzymes was unexpected in view of the apparent absence of such effects in the work of Kanai and Edwards (1973) and Ku, Gutierrez and Edwards (1974). There is considerable evidence that cell wall degrading enzymes, or contaminants in them, can impair the viability of the isolated protoplast (Fushtey, 1957; Evans, Keates and Cocking, 1972; Jensen, Fracki and Zaitlin, 1971; Tseng and Mount, 1974; Gigot et al., 1975; Wegmann and Mulbach, 1973) but most workers have implicated this effect with membrane modifications. Clearly this was not the case in this study.

Kanai and Edwards used a similar technique for the isolation of tissue but did not report any inhibition of enzyme activity. It is therefore probable that the inhibition originated from a factor by which the two methods differed; either the use of pectinase and sucrose (though both were used to isolate apparently viable protoplasts of tobacco by Power and Cocking) or the less effective washing procedures which had to be used in this study.

Clearly this reduction in enzyme activity has made the analysis of data difficult and it is apparent that in addition to the testing of protoplast integrity with staining techniques it is essential to assay certain marker enzymes when protoplast isolation procedures are to be developed. A recent review by Huber and Edwards (1975b) has drawn attention to the problems involved in this and the methods available to overcome them.

PEP carboxylase activity in maize leaf epidermis (Table 5.1) was less than that reported by Willmer, Pallas and Black (1973) in the lower epidermis of C. communis (751 μmoles CO_2 $mg^{-1}chl.h^{-1}$) or T. gesnariana (11,200 μmoles CO_2 $mg^{-1}chl.h^{-1}$). It is therefore unlikely that it was of importance in preventing the escape of CO_2 from the maize leaf particularly since it apparently had a similar distribution. This conclusion was confirmed by the presence of malic enzyme in the epidermis again with a similar distribution and having a greater activity (though it was less than the activity reported by Willmer in two C_3 species). Thus it would appear that unless the malic enzyme was inactive or acting as a carboxylase, (which it can do in the presence of excess pyruvate and bicarbonate; Black, 1973), the PEP carboxylase present in the epidermis could not be functioning to prevent the efflux of CO_2 produced by respiration.

Before the precise roles of malic enzyme and PEP carboxylase in leaf epidermis can be understood it will be necessary to identify their exact locations in order to elucidate their degree of separation and whether it is spatial (as in C_4 species) or temporal (as in CAM species). Willmer and Dittrich (1974) appear to favour the latter hypothesis, malic enzyme activity being controlled by either light of pH.

Meidner and Willmer (1975) stated that high activities of carbonic anhydrase in the lower epidermis of C. communis. Its activity in the lower epidermis of maize was apparently less, probably indicating that it was not important in aiding the retention of CO_2. However, like malic enzyme, until its distribution is fully clarified this cannot be certain; if it was associated with the plasmalemma of epidermal cells adjoining the mesophyll then it could assist the transport of CO_2 even if it was only present in small quantities. In view of its low activity and the low pH in the epidermis it is most improbable that it was increasing the overall solubility of CO_2 in the tissue.

The only carboxylating enzyme present in the epidermal extracts of maize in elevated quantities was RuBP carboxylase. Its apparent activity in enzyme treated tissue was greater than that in both the mesophyll of maize and the epidermis of C. communis and nearly as high as that reported in the epidermis of T. gesnariana (Willmer, 1973b) latter tissue had appreciably greater activities of PEP carboxylase and malic enzyme as well probably indicating a generally higher level of metabolic activity in that tissue.

Thus compared to the epidermis of both C_3 species maize epidermis was apparently enriched in RuBP carboxylase which appeared to be concentrated in the long epidermal cells. It is perhaps therefore involved in CO_2 retention by maize epidermis. This finding was unexpected for several reasons:

(i) There is no evidence that RuBP carboxylase occurs outside the chloroplast. To function in the long epidermal cells it would have to.

(ii) It is unlikely that the intermediates of the PCR cycle which would have to be transferred for the fixation to be energetically viable are sufficiently mobile to move between the chloroplasts of the mesophyll (or perhaps the bundle sheath) and the epidermis.

(iii) There is no evidence that RuBP carboxylase can function non-photosynthetically.

Kanai and Edwards (1972) have reported experiments providing some evidence of RuBP carboxylase in the epidermis of maize leaf tissue. In extracts of bundle sheath cells they found an activity of 235 µmoles CO_2 $mg^{-1}chl.h^{-1}$ whilst in extracts containing both bundle sheath and epidermal cells they reported one of 300 µmoles CO_2 $mg^{-1}chl.h^{-1}$.

This difference could be due to appreciable mesophyll contamination in the first extract or alternatively to RuBP carboxylase activity in the epidermis.

CHAPTER 6

DETERMINATION OF CUTICULAR RESISTANCE

Introduction

There are two possible pathways for gaseous exchange between the leaf and atmosphere; the cuticle and the stomata. The cuticle is specialised to reduce one type of exchange, the loss of water vapour and in fulfilling this function it presents a high resistance to the diffusion of other gases. The contribution of gaseous exchange via the cuticle is therefore probably insignificant compared to the gaseous exchange of the whole leaf. However its importance to epidermal cells, particularly those distant from stomata, is potentially greater. For example, cuticular resistance (r_c) to gaseous exchange, if low could permit appreciable epidermal respiration independent of stomatal aperture, if high it could restrict it. In a situation in which the leaf was actively photosynthetising so that oxygen was freely available to the epidermis a high value of r_c could be significant in reducing CO_2 loss through the cuticle.

The importance of cuticular resistance in preventing CO_2 loss from the epidermal cells of maize leaves is difficult to assess and depends upon the external conditions. At the CO_2 compensation point the concentration of CO_2 inside the leaf must be regarded as zero and so the concentration gradient from its site of production in the epidermis must be similar in all directions. In such a situation a high value of r_c would mean that more CO_2 would move towards the inside of the leaf than would diffuse directly through the cuticle. However this would not prevent the escape of CO_2 from the leaf as much of it would enter the intercellular air spaces and diffuse out from these via the stomata down the concentration gradient.

Under normal conditions however, with an external CO_2 concentration of about 300ppm, a high cuticular resistance to CO_2 escape movement could be very effective at preventing any CO_2 escape from the leaf because of the prevailing concentration gradient towards the mesophyll. For this reason it was thought valuable to measure the cuticular and stomatal resistances of maize leaves in order to assess their importance in assisting the retention of CO_2 by them.

The Measurement of Resistances:

It is well established that the stomata are the principal pathway for gaseous exchange by the leaf and since their importance was first quantified by Brown and Escombe (1905) they have been the subject of many papers, both experimental and theoretical (see Meidner and Mansfield, 1968; Monteith, 1973 and Raschke, 1975 for recent reviews). The significance of cuticular exchange has been subject to more disagreement however, much of it arising from the problems involved in its measurement.

The two resistances are in parallel and can be related by Ohms law:

$$\frac{1}{r_l} = \frac{1}{r_s} + \frac{1}{r_c}$$

r_l - total resistance of leaf surface

r_s - stomatal resistance

r_c - cuticular resistance

Clearly in order to quantify either r_c or r_s it is necessary to isolate one or the other from the experimental system. Most workers have made such determinations using hypostomatous leaves in which r_c can be determined from the adaxial surface and r_l from the abaxial. This approach clearly is not possible with amphistomatous leaves such as maize and as a result r_c can only be measured indirectly. The

usual method is to measure r_l with closed stomata and assume this value is equivalent to r_c. An important error involved with this technique is that any slightly open somtata tend to reduce the value of r_c recorded, therefore the figure obtained must be regarded as a minimum value of r_c.

Since the present study was concerned with the retention of CO_2 by the leaf ideally the resistance to CO_2 transfer should have been measured. However the measurement of any resistance requires the measurement of concentration at either end of the pathway and also the rate of flow along it. The external concentration of CO_2 may easily be monitored by methods such as infra-red gas analysis or absorption and titration and from the rate of change of concentration the rate of flow calculated. It is impossible, however, to measure internal CO_2 concentration; a known CO_2 concentration is only maintained in the air space system at the CO_2 compensation point and under these conditions stomata are open and there is no net flux of CO_2. Estimation of resistance by the measurement of CO_2 transfer across both leaf surfaces and the mesophyll was also unsuitable because of the unknown component due to mesophyll resistance and the possible production or assimilation of CO_2 within the leaf.

It was therefore necessary to measure resistances to water vapour transfer and use these to estimate resistances to carbon dioxide movement. The former may be measured by a variety of techniques all depending on the assumption that water loss from the cell wall surface is always equivalent to evaporation from a free water surface and that the atmosphere at the site of evaporation is always saturated. Though this assumption has been questioned by several authors present evidence seems to indicate that the water vapour pressure is only reduced below that of the saturated vapour pressure under water stress (Jarvis and Slatyer, 1970) and can be neglected under normal conditions.

Since the diffusion coefficient is a function of molecular weight and size, and the ratio of diffusion coefficients of water vapour and carbon dioxide stays constant, independent of temperature and pressure (Jarvis, 1971) it is possible to interconvert the resistances to diffusion of the two substances, providing they both follow the same path:

$$rCO_2 = rH_2O \ \frac{DH_2O}{DCO_2}$$

rH_2O - diffusive resistance to water vapour transfer

rCO_2 - diffusive resistance to carbon dioxide transfer

$\frac{DH_2O}{DCO_2}$ - ratio of molecule diffusion coefficients of water and carbon dioxide, equivalent to 1.605 (Fuller, Schettler and Giddings, 1966, quoted in Jarvis, 1971)

Two methods were used to obtain values of resistance to water vapour transfer. The simplest approach involved measuring the water loss from a leaf by weighing under controlled conditions with leaf temperature being monitored with a fine thermocouple (a method used by Williams and Amer, 1957 and Shepherd, 1964). Resistances were calculated from a derivation of Ohms law (Holmgren, Jarvis and Jarvis, 1965):

$$T = \frac{Wi - Wa}{\Sigma r}$$

T : transpiration flux (mg cm^{-2} s^{-1})

Wi : water vapour concentration inside leaf mg H_2O cm^{-3}

Wa : water vapour concentration outside leaf mg H_2O cm^{-3}

Σr : total resistance to water vapour transfer (s cm^{-1})

More recently the development of the diffusion porometer has provided a new technique for the measurement of rH_2O. A number of different designs of instrument are available (Stiles, 1970; Monteith and Bull, 1970; Meidner, 1970; Stigter et al., 1973; Byrne et al., 1970; Turner and Parlange, 1970) varying in the type of sensor used, the

chamber geometry and the method of calibration. All depend upon the measurement of the time required for the humidity of a chamber sealed against a leaf to change between two known values. From this time it is possible to calculate the resistance from a previously prepared calibration curve. A porometer of the type described by Meidner (1970) was used in this study.

The Diffusion Pathways:

The conversion of diffusive resistance to water vapour transfer to resistance to CO_2 transfer is only possible if both molecules have identical vapour diffusion pathways. In most plants they almost certainly do not.

When stomatal resistances have been measured it has always been assumed that the two pathways are similar - from the mesophyll cell wall, across the substomatal cavity and through the stomatal pore. However the recent findings of Meidner (1975) Aston and Jones (1976) and Burbano et al (1976a, b) indicate that this probably is not the case and that a principal site of evaporation, at least in Tradescantia virginiana and Avena sterilis is the internal walls of the epidermis including those of the guard and subsidiary cells. This has not been demonstrated in maize though it would certainly seem possible in view of the large volume of apparently water storing epidermal cells. If this was the case then the value of r_sH_2O recorded, whilst not related to the resistance faced by CO_2 diffusing to the mesophyll, would be indicative of the resistance faced by CO_2 diffusing from the epidermis into the sub-stomatal cavity.

The value of r_cH_2O is more difficult to relate to r_cCO_2. Carbon dioxide and water vapour probably have similar diffusion pathways from the liquid-air interface to the atmosphere. However it is likely that this interface lies close to the surface just beneath the waxy layer (Jarvis, 1971). If so then the vapour pathway is

very short compared to the liquid phase and it is here that differences between the water and carbon dioxide diffusion paths would lie.

Water reaches the liquid-air interface by mass flow, therefore the resistance is hyrdaulic rather than diffusive. Carbon dioxide on the other hand moves largely by diffusion (thcugh it will tend to flow with the water) and so faces a greater resistance, particularly since it is diffusing through a liquid phase which lowers its diffusion coefficient, and therefore increases the diffusive resistance by a factor of 10^4. The greater resistance to CO_2 transfer than H_2O transfer may be slightly lessened by the greater area available to CO_2 for diffusion, water is limited to small pores, CO_2 is able to move through the whole cuticle. Furthermore the greater solubility of CO_2 in hydrophobic solvents (Edsall, 1969) could mean that it is more concentrated in the waxy components of the cuticle (Dugger, 1952). Barrs (1968) has indicated that r_cH_2O is less than r_cCO_2, however, at least in cotton and pepper plants.

It is impossible to assign separate values for the resistances to water vapour transfer and hydraulic flow of water through the cuticle because of the difficulty of differentiating them. However experiments involving the removal of the outer waxy layer from the cuticle of several species (Hall and Jones, 1961; Horrocks, 1964) indicate that the former is probably the main component of the measured value.

Therefore the value of r_cH_2O recorded is probably applicable to the gaseous diffusion path of CO_2 but the CO_2 also faces an additional liquid phase resistance which is is impossible to quantify because of the factors discussed above.

Previous investigations into cuticular resistances:

Cuticular resistance to CO_2 exchange has been measured in a variety of species and a wide range of values have been obtained. The earliest report is that of Blackman (1895) who concluded that cuticular CO_2 uptake was minimal compared to that entering the leaf via the stomata. Brown and Escombe (1905) confirmed these findings. However since then other workers have produced conflicting data, Mitchell (1936) produced some evidence of cuticular photosynthesis in tomato and pelargonium; though it is uncertain that the stomata were fully closed. Freeland (1948) produced evidence of cuticular resistance differing between species and showed some correlation between cuticular thickness and r_cCO_2. Using Coleus he recorded a cuticular uptake of a similar magnitude to the stomatal. Dugger (1952) agreed with this finding. More recently Dorokhov (1963, reported in Martin and Juniper, 1970) showed appreciable CO_2 uptake by the upper surface of apple leaves and Antipov (1974) demonstrated CO_2 efflux through a variety of leaf cuticles in the dark.

Conversely Holmgren, Jarvis and Jarvis (1965) in a thorough investigation of cuticular exchange in a number of woody and herbaceous species could only detect minimal CO_2 uptake through the cuticle in all of them. Zelitch (1971) claims that the cuticle is almost totally impervious to CO_2 and O_2. Whilst the latter view is perhaps true in certain species the vast majority clearly show gaseous exchange at night or under conditions of water stress when the stomata are closed and so cuticular exchange must occur, though in most species a large concentration gradient is probably required before appreciable transfer occurs.

No attempts have been made to measure r_cCO_2 of maize leaves though Maynard et al. (1974) have presented data which may be interpreted as showing that it is considerable. Working with plants of

a mutant of maize completely lacking guard cells (having instead two appressed subsidiary cells with no apparent pore between them) they have demonstrated that such plants, whilst they usually die within three weeks under normal conditions, will live significantly longer in a CO_2 enriched atmosphere. This would appear to indicate that the cuticle has a sufficiently high resistance to CO_2 transfer to prevent photosynthesis under normal conditions.

Because of its comparative ease of measurement there is much more data available on cuticular resistance to water loss. A very large range of values have been reported, typically shade species which have poor drought tolerance, e.g. aspen, have low resistances (Jarvis and Jarvis, 1963) whilst xerophytes have much higher ones. Though increased cuticular resistance to water vapour transfer may be a result of cuticular thickening, greater waxiness and secondary thickening (obscuring the lumen of the epidermal cells), Kamp (1930) has demonstrated that changes in cuticular composition and structure can also have large effects so that species having the same cuticular thickness may have very different resistances.

Cuticular resistance to water loss is also very variable within species, differing between cultivars (Dube et al. 1975); between plants grown under differing conditions (Slavik, 1973; Barrs, 1973; Moreshet, 1970) and between leaves on the same plant - young leaves are typically considerably more waxy and therefore have a greater resistance.

Reports involving the simultaneous measurement of r_cCO_2 and r_cH_2O are very limited, Holmgren, Jarvis and Jarvis (1965) reported widely differing ratios between the two whilst Barrs (1968) showed that the permeability to water vapour was greater than that for CO_2 in both sunflower and pepper. However since both of these investi-

gations involved measurements of cuticular photosynthesis the findings were not useful for the current investigation which involved a much shorter path length of CO_2 diffusion; from the atmosphere to the epidermal cell instead of from the atmosphere to the mesophyll.

Methods

(i) Preparation of Material:

Plants were greenhouse grown as described previously. At an age of 6-8 weeks and four days prior to experiments they were transferred to constant environment growth rooms at 25°C (± 2°C) and kept in 16h light periods provided by high intensity fluorescent lighting (>12,000 Lux). Water vapour pressure deficit (WVPD) was maintained at about 25mbar. All experiments were carried out in the growth room.

(ii) Measurement of leaf resistances by weighing:

Maize leaves were excised under water from the upper part of the plant and sealed into filtered tap water filled 5 cm petri dishes. After removal of surface moisture they were weighed and then transferred to a perspex chamber (17 x 11 x 5 cm) containing drying agent.

Leaf temperature was monitored using a small thermocouple held against the abaxial leaf surface. Air temperature was measured with a similar thermocouple approximately 1 cm above the adaxial surface. Wet and dry thermocouples were used to record humidity.

The air in the chamber was stirred using a small fan which reduced the boundary layer resistance (measured with moist filter papers - Jarvis, 1971) to less than 1.5 sec cm^{-1} depending on leaf size. Light intensity was 12,000 Lux (measured with a Lambda Li170 Photometer) during the light period.

At thirty minute intervals the leaf and petri-dish were weighed and from the weight loss values of r_s or r_c calculated using the equation of Holmgren, Jarvis and Jarvis (1965) given earlier. r_s was recorded during the light period, r_c during the period of minimum transpiration in the dark period.

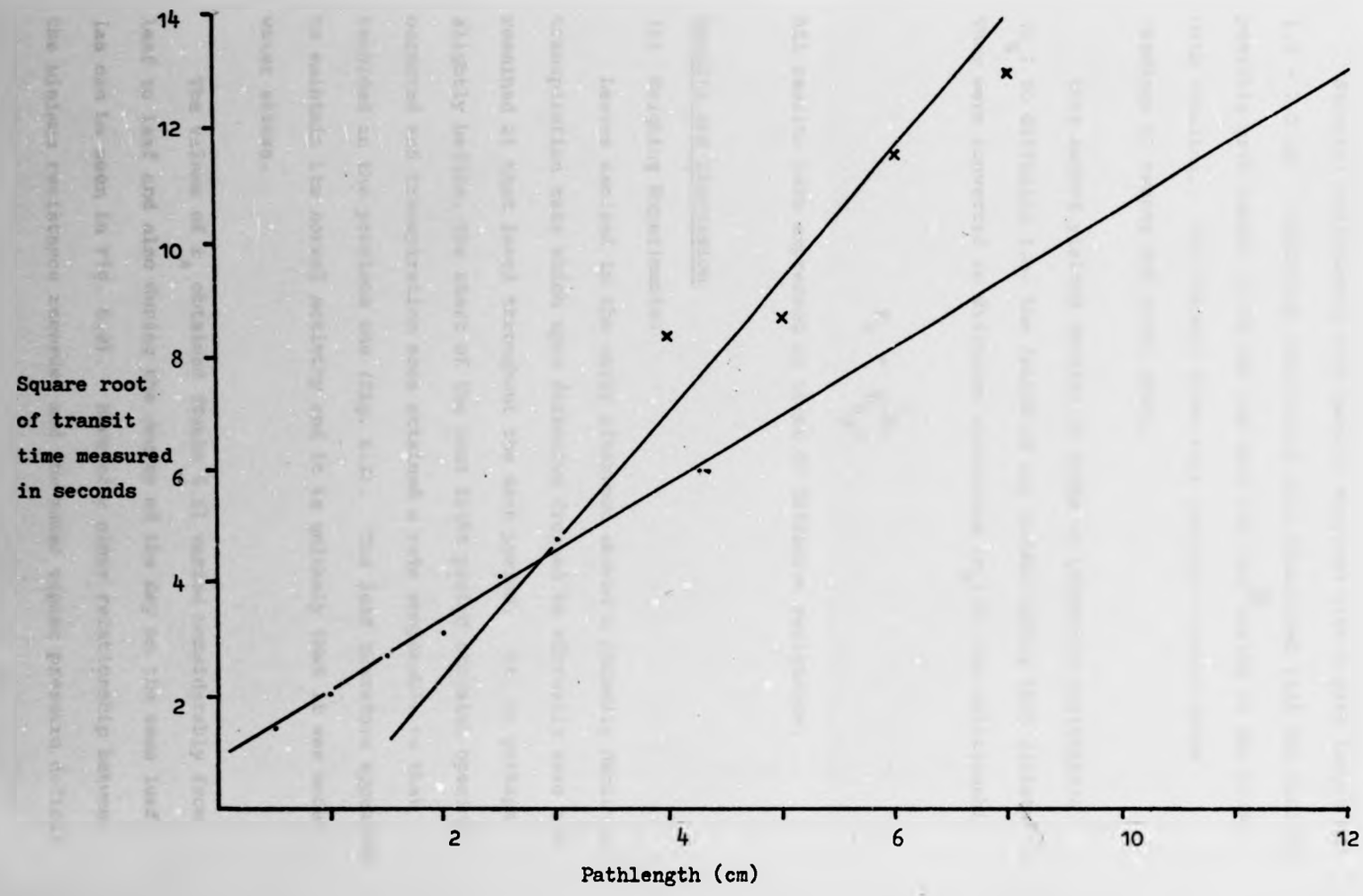
Humidity was modified by the use of two drying agents, silica gel which gave a WVPD of 19.0 mbar, and sulphuric acid (30% by weight) giving one of 3.0 mbar. Experiments were also carried out in the open growth room having a WVPD of 25.0 mbar. Whilst these methods of controlling humidity were not the most sensitive the humidites maintained were fairly stable under a given set of conditions (± 0.6 mbar) though there was a 5.0 mbar difference in humidity between the stomata when open and when they were closed. Leaf temperature behaved in a similar way, ± 0.5°C, with a difference of as much as 2°C between leaf and air temperature when the stomata were open.

Similar experiments were carried out with stems of C. communis bearing 3-5 leaves.

(ii) Diffusion Porometry:

A porometer identical to that described by Meidner (1970) was used. Calibration was carried out using moist filter papers and varying path lengths. The upper part of the clamp was sealed with a coverslip to prevent convectional cooling. Though calibrations derived with path lengths of up to 3 cm were linear (Fig 6.1) at greater lengths the points became more variable and the apparent slope of the line increased. This was probably due to three factors, drying out of the filter paper during the transit period; mass flow in the long porometer chamber due to convection currents and finally leakage from the chamber to the atmosphere becoming more prominent because of the long transit times involved. Because of this error in the long path length calibration all calibrations were derived

Fig. 6.1 Typical calibration curve for the diffusion porometer. Points obtained using longer pathlengths (x) clearly depart from the linearity of the points recorded with shorter pathlengths (•).


14
12
10
8
6
4
2
Square root of transit time measured in seconds
2
4
6
10
12
Pathlength (cm)

from extrapolated short path length ones. This was possible because the rate of diffusion is linearly related to path length.

Stomatal resistances were usually measured with a path length of 1.0 - 2.0 cm. Cuticular resistances were determined with the minimum possible path length (0.25 cm) and with the leaf sealed in the clamp with vaseline. All transit times were recorded between meter readings of twenty and sixty units.

This method yielded results in terms of geometric resistances (r_g) to diffusion i.e. the length of air column having that resistance. They were converted to diffusive resistance (r_u) by the relationship -

$$r_u = \frac{r_g}{D_{H_2O}}$$

All results were expressed in terms of diffusive resistance.

Results and Discussion

(i) Weighing Experiments:

Leaves excised in the early afternoon showed a steadily declining transpiration rate which upon darkening dropped to virtually zero and remained at that level throughout the dark period. At, or perhaps slightly before, the start of the next light period stomatal opening occurred and transpiration soon attained a rate comparable to that recorded in the previous one (fig. 6.2). The leaf therefore appeared to maintain its normal activity and it is unlikely that it was under water stress.

The values of r_s obtained (Table 6.1) varied considerably from leaf to leaf and also during the course of the day on the same leaf (as can be seen in Fig. 6.2). However a clear relationship between the minimum resistance recorded and the water vapour pressure deficit

Fig. 6.2 Changes in the rate of transpiration of an excised maize leaf during a 20h period. Darkness is indicated by the thickened section of the abscissa.

Rate of transpiration (g H_2O h^{-1})
0.5
0.4
0.3
0.2
0.1
2
4
6
8
10
12
14
16
18
20
Time after excision (hours)

Table 6.1: Diffusive resistances (s cm^{-1}) to water vapour loss from leaves of maize and *C. communis* measured by the weight loss from excised leaves. Humidity was varied as described in the text; temperature was 25°C (±2). The results shown are the mean of at least three replicates.

Tissue	Water vapour pressure deficit (mbar)			
	25	19	14	3.0
Maize leaf, open stomata (r_s)	13.0 (±2.4)	-	4.0 (±0.6)	1.3 (±0.5)
Maize leaf, closed stomata (r_c)	450 (±290)	182 (±107)	-	34 (±18)
C. communis leaf, open stomata (r_s)	13.6 (±15.1)	-	6.8 (±0.8)	-
C. communis leaf, closed stomata (r_c)	304 (±751)	198 (±125)	-	-

of the atmosphere was noted (Fig. 6.3). This appeared to be a logarithmic relationship (Fig. 6.4).

The effect of atmospheric humidity upon stomatal aperture has only recently been shown. Wilson (1948, quoted in Meidner and Mansfield, 1968) found little indication of any effect whilst Raschke and Kühl (1969) concluded that humidity could only modify stomatal aperture indirectly via leaf water potential. More recently however Raschke (1970 has found evidence for reduced stomatal opening at low atmospheric humidities in maize leaves whilst Lange et al. (1971) and Schulze et al. (1972) showed similar effects on the isolated epidermis of Polypodium vulgare and a variety of desert plants in the field respectively. Other reports using a variety of species have confirmed these findings (Jarvis and Slatyer, 1970; Drake, Raschke and Salisbury, 1970; Camacho-B, Hall and Kaufman, 1974; Schulze et al., 1974, 1975 a, b; Hall and Kaufmann, 1975; Meidner, 1976). Thus it would appear that atmospheric humidity, presumably acting directly on peristomatal transpiration can modify stomatal aperture. The findings of this investigation are in agreement with this though the apparently logarithmic relationship was unexpected, particularly in view of the linear relationship between evaporation from a free water surface and atmospheric humidity. It could function to lessen the increase in transpiration caused by increased leaf temperature which would result from a smaller closure.

Cuticular resistance to water vapour loss (Table 6.1) was clearly much greater than stomatal resistance and compared favourably with the values reported for maize by Dube et al. (1975). A similar response to different atmospheric humidites was noted, it was again logarithmic (Fig.6.4) and had a very similar slope. Moreshet (1970) has described an analogous effect in Helianthus annuus though he recorded a smaller proportional change, from 24 $cm^{-1}s$ at a WVPD of

Fig.6.3: Effect of water vapour pressure deficit (WVPD) upon the resistance to water vapour loss of excised maize leaves in the light. WVPD was modified as described in the text; temperature was 25°C (±2)

(WVPD) upon
excised maize
as described

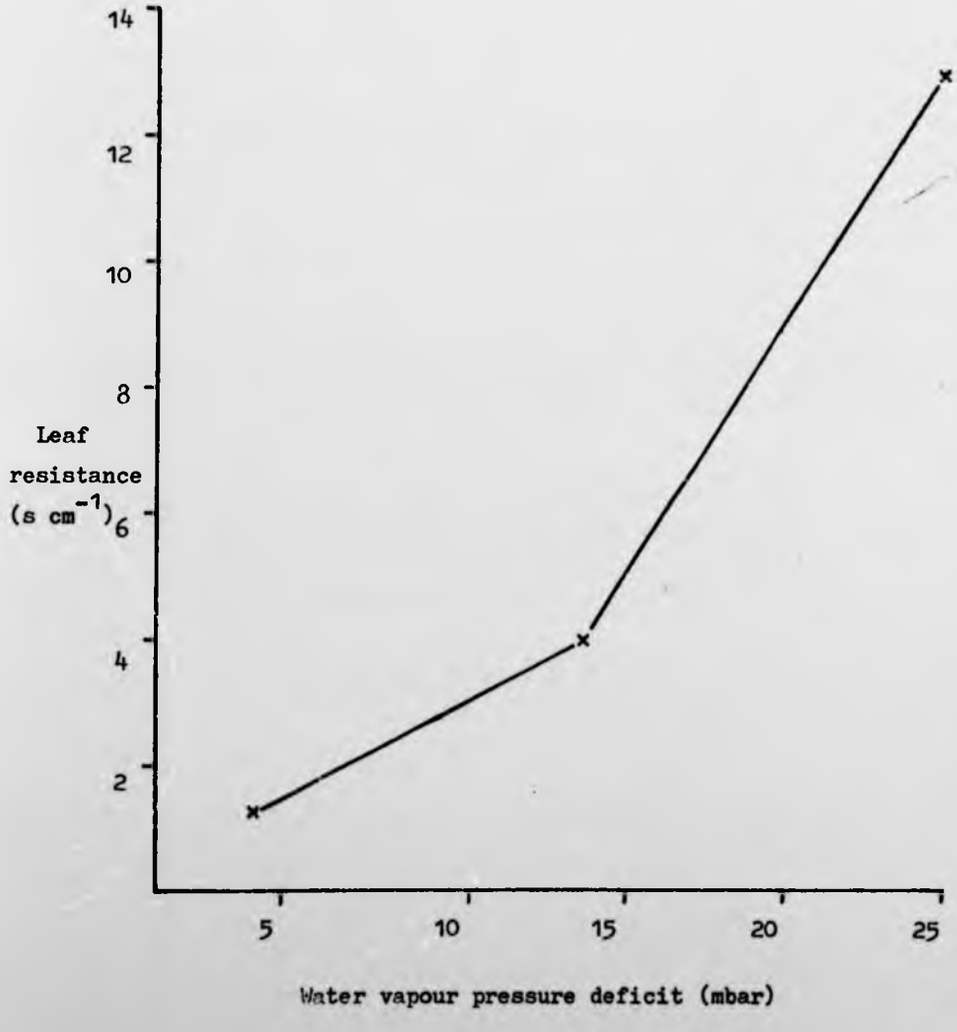


Fig 6.4: Effect of water vapour pressure deficit (WVPD) upon the resistance to water vapour loss of excised maize leaves. Resistance is plotted logarithmically. WVPD was modified as described in the text; temperature was 25°C (±2).

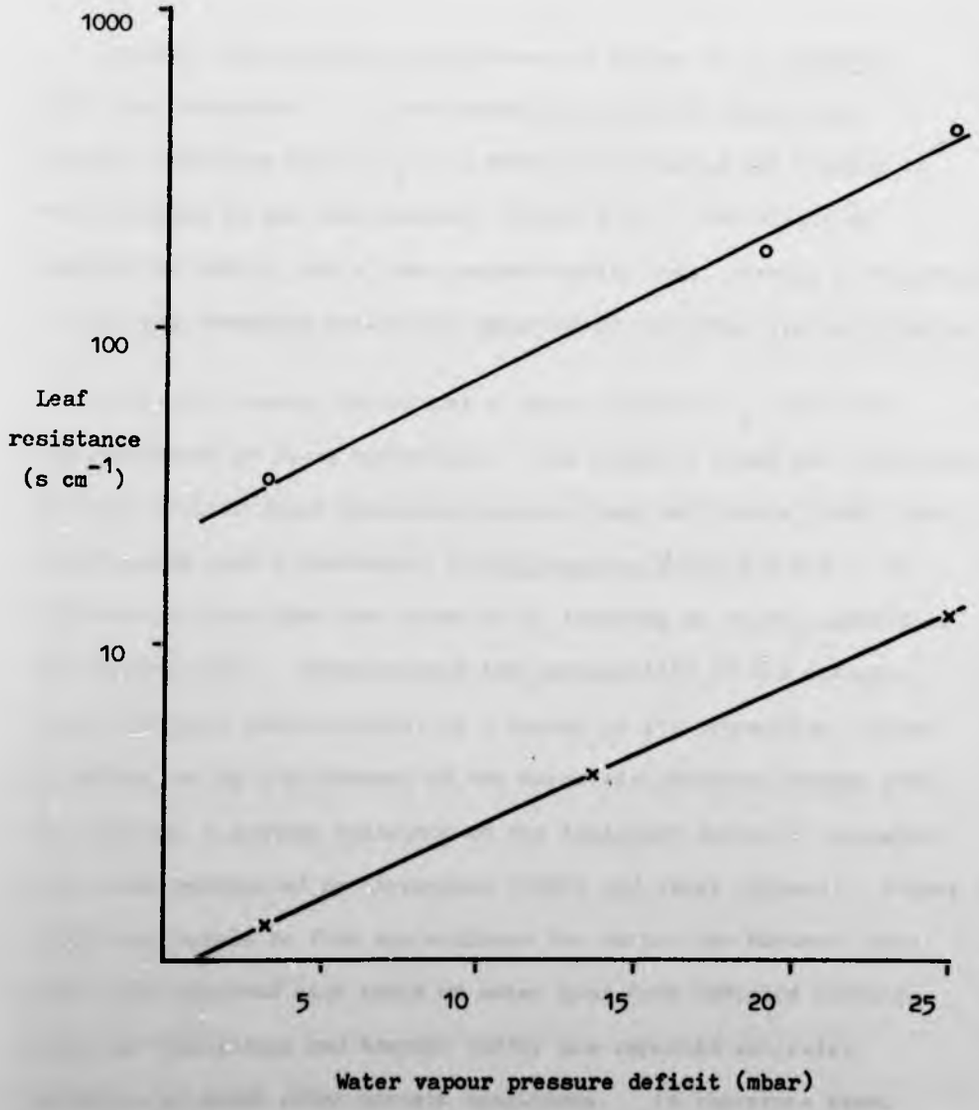
x : Leaves with open stomata

o : Leaves with closed stomata

it (WVPD)
loss of
plotted
as described
2).

ata

omata



9.6 mbar to 120 at one of 22.1 mbar. Similar responses have also been reported in locust cuticle (Loveridge, 1968), human skin (Buettner, 1965) and the epidermis of Tradescantia virginiana (Meidner, 1976). However Kuiper (1969) has produced conflicting data showing no effect of humidity upon r_c; the resistances recorded were low (< 20 $cm^{-1}s$) however and perhaps were caused by the leaf having slightly open stomata.

Stomatal and cuticular resistances of leaves of C. communis were also determined. r_s was higher than that of maize under similar conditions whilst r_c at a WVPD of 19.0 mbars was similar to that of maize at the same humidity (Table 6.1). The effect of humidity on both r_s and r_c was proportionally less, perhaps a reflection of the less demanding conditions under which the plant typically grows.

There are several mechanisms by which apparent r_c could have been increased at lower humidities. One probable cause was increasing stomatal closure under increasing stress, Begg and Jarvis (1968) have demonstrated such a phenomenon in Stylosanthes humilis H.B.K. If this was the case then the values of r_c recorded at higher humidities were falsely low. Alternatively the permeability of the cuticle could have been reduced either by a change in its properties, caused by drying, or by a withdrawal of the water-air interface deeper into the cuticle, a process analogous to the incipient drying of mesophyll cell walls postulated by Livingston (1906) and later authors. Kuiper (1969) was unable to find any evidence for either but Meidner (pers. comm.) has observed high rates of water loss from hydrated cuticles under low humidities and Raschke (1975) has reported cuticular guttation in maize under certain conditions. It therefore seems probable that at least one of these mechanisms was operating. A recent paper (Schönherr, 1976) has suggested that the pH of the cuticle may be of importance in controlling its water permeability and

so it is likely that a change in cuticular properties rather than incipient drying is the more important mechanism.

(ii) Porometry:

The mean value of r_s recorded by diffusion porometry was less than that measured by weighing (Table 6.2). The value of r_s was considerably greater than the values reported by Dube et al. (1975), possibly a reflection of the low humidity in the growth rooms in which the experiments were carried out.

The abaxial leaf surface of C. communis had a slightly higher r_s than did those of maize, a situation similar to that recorded in the weighing experiments.

It proved impossible to detect water loss from either surface of maize or C. communis leaves in the dark or from their adaxial surfaces in the light, even using the shortest path length available (0.25 cm). The porometer chamber was sealed on the leaf surface for periods as long as twenty minutes without any significant deflection of the meter needle.

This lack of response was found to be caused by leakage from the sensor chamber, principally between the clamp and leaf surface but also via the entry ports into the chamber for the thermistor and air circulator. Long transit periods also increased the likelihood of small temperature fluctuations modifying sensor sensitivity.

The significance of leakage could be demonstrated by sealing the porometer chamber against a piece of moist filter paper backed by a maize leaf. When full scale deflection on the meter was attained the paper could be quickly withdrawn and the chamber resealed on the leaf without the loss of deflection on the meter. The transit time for the meter readings to pass from 60 to 20 could then be recorded and

Table 6.2: Diffusive resistances (s cm^{-1}) to water vapour loss from leaves of maize and C. communis measured by diffusion porometry. Water vapour pressure was 25mbar; temperature was 25°C. The results shown are the means of at least twenty reading in each case.

Tissue	Leaf resistance (s cm^{-1})
Maize leaf, open stomata	5.44 ($\pm$0.85)
Maize leaf, closed stomata	Not recordable
C. communis, open stomata	5.81 ($\pm$1.68)
C. communis, closed stomata	Not recordable

this value used as an estimate of leakage. The procedure was not totally suitable because of the hysteretic response of the sensor (Monteith and Bull, 1970) but it was thought satisfactory as it was impossible to obtain true values of the resistance to leakage because of the unknown dimensions of its pathway. Very variable results were obtained ranging from transit times as short as 5 seconds (equivalent to a leaf resistance of 4.4 $cm^{-1}s$) up to 265 seconds (r - 52 $cm^{-1}s$) a mean time of 6.1 sec was recorded.

Though the leakage problem was certainly compounded by the low ambient humidity in the growth room which meant that a steep water vapour concentration gradient existed between the chamber and external atmosphere it is clear that leakage is a potential major source of error in the use of this design of porometer, particularly if the experimental material has an irregular surface architecture and a low rate of water loss. If leakage is found to be a problem in an investigation then experiments would best be conducted in an atmosphere having a humidity corresponding to the mid-point of the meter range, leakage into and out of the sensor chamber would be then balanced.

At the lower end of the meter range, the small concentration gradient between the porometer chamber and the outside atmosphere meant that leakage was slow. Therefore the absence of any movement in the meter needle in all attempts to measure cuticular resistance indicate that r_c is considerably larger than the apparent resistance of the leakage. Thus it would appear that the values recorded in the weighing experiments are reliable estimates of cuticular resistance.

It is interesting to note the apparent inactivity of the stomata of the abaxial leaf surface of maize leaves; repeated attempts with a number of plants failed to reveal any water loss from the upper epidermis even though the stomata of the lower were clearly open.

This finding is in apparent disagreement with the work of Stigter (1974) and Turner and Begg (1973) who reported similar diffusion resistances for both leaf surfaces of field grown maize. This difference could again be caused by the low humidity of the growth room, under conditions of strong atmospheric drying it would be most effective to close the stomata of the adaxial surface which are certainly exposed to a greater evaporative demand from both solar radiation and atmospheric turbulence than the abaxial.

(iii) Cuticular Resistance to CO_2 transfer:

It was clear that in both maize and C. Communis r_cH_2O was many times greater than r_sH_2O under similar conditions. Since rH_2O and rCO_2 are related by a constant factor (see Introduction) the same must apply for resistances to CO_2 diffusion.

The values recorded for maize leaf were similar to that recorded in C. communis and so the absence of CO_2 efflux from illuminated maize leaves cannot be explained upon the basis of cuticular resistance alone. The conversion of r_cH_2O to r_cCO_2 could only be approximate for the reasons discussed in the Introduction, but because the conversion probably resulted in a falsely low value of r_cCO_2 (as a result of the additional liquid phase resistance) it was thought valid to make it. At a WVPD of 19.0 mbar r_cH_2O was 182 scm^{-1} in the case of maize and 198 in C. communis; this was equivalent to values of r_cCO_2 of 291 and 317 scm^{-1} respectively. Clearly the cuticles of both species were relatively impermeable to CO_2.

In maize if CO_2 was being produced by respiration immediately adjacent to the outer cell wall of an epidermal cell than it could either diffuse outward across the cuticle or inwards toward the mesophyll. It was impossible to measure the latter resistance directly but it was possible to estimate it. As the pH of the epidermis

as a whole was low (see Chapter 4) the only molecular species of carbon dioxide present in any quantity was CO_2 itself which has a diffusion coefficient of 0.16×10^{-4} cm^2 s^{-1} at 15°C (Jarvis, 1971). The mean epidermal cell thickness was 22 µm, so if the cell contained pure water the resistance to CO_2 transfer would be about 1.38 s cm^{-1}. This estimate is probably below the true value because of the presence of solutes in the cell and the additional resistance involved in diffusion across the cell wall to the mesophyll cells. However it must also be remembered that the cytoplasm was probably at a higher pH than the tissue as a whole permitting diffusion of more than one ionic species of carbon dioxide through it and consequent decrease in resistance. It therefore seems certain that the resistance to CO_2 transfer across the epidermal cell would be insignificant compared to that across the cuticle and therefore the CO_2 loss from an illuminated maize leaf via the cuticle would be minimal.

CHAPTER 7

DISCUSSION

Experimental Work

As may be judged from the data presented in the previous chapters the major problem faced in this study was the almost uniformly poor quality of the results which has made the drawing of any firm conclusions impossible. The great variation obtained was caused primarily by the problem of isolating sufficient tissue of suitable quality.

The inefficiency of the epidermal isolation techniques meant that a steady supply of maize plants of similar age and quality were required and this proved very difficult to ensure. Winter grown plants were particularly liable to abnormal development despite supplemented lighting and heating, a common problem being premature flower initiation. Unfortunately the large number of plants required meant that they could not be grown in a growth room having a comparatively constant environment. The inefficiency of the isolation procedure also meant that a number of leaves, from several plants were required to obtain sufficient tissue and that tissue had to be removed from the whole length of the leaves, which of course vary in age along their length. Thus the different samples of epidermal tissue probably varied considerably in their growth history and this probably resulted in some of the differences in the results that were encountered.

It is likely that the greatest source of error, however, originated in the actual preparation of the tissue. Whichever technique was used considerable damage, either physical or chemical was inflicted to varying extents (depending upon the quality of the leaf)on the

tissue and this, coupled with changing levels of contamination, resulted in extracts of very variable quality.

The problems faced in the actual isolation of the epidermis were considerable (see Chapter 3) and while most were unavoidable, one of the most critical - the detrimental effect of enzyme digestion upon the metabolism of the tissue, could perhaps have been lessened if more satisfactory controls had been implemented.

If further work is to be carried out on this subject then considerably more sophisticated techniques must be used involving not only an improved technique for the bulk isolation of tissue but also the use of much smaller scale methods and investigations of epidermal activity in situ on the leaf using perhaps autoradiography (Appendix 2), histochemistry and pulse-chase techniques (Outlaw, 1975).

Epidermal Activity

The first question to be answered was whether the epidermis of maize was metabolically active or whether it was either largely dead, as is perhaps the case in many grasses (Brown and Pratt, 1965) or had dead epidermal cells as is the case in Commelina cyanea (Pearson and Milthorpe, 1974). The evidence for stomatal activity was overwhelming, stomatal cells took up neutral red; had large and apparently functional mitochondria and chloroplasts; showed a consistently detectable respiratory activity (using either Warburg manometry or Cartesian diver microrespirometry) and displayed a typical response to light-dark regimes (Chapter 6). This conclusion is reinforced by numerous reports of stomatal control of transpiration in maize (e.g. Raschke and Kühl, 1969; Stigter, 1974).

Evidence for metabolic activity in the long epidermal cells was more circumstantial, but still, I feel, conclusive. Though it proved

impossible to clearly demonstrate respiratory activity in the intact epidermis the long epidermal cells could take up neutral red; they had a cytoplasm having mitochondria and it has been reported that their nuclei move in response to fungal attack (Contreras and Boothroyd, 1974). However their small cytoplasmic content and the apparent absence of any additional respiratory oxygen uptake in enzyme isolated strips probably indicates that their activity was low.

Epidermal tissue of Commelina communis displayed a much greater respiratory activity than that of maize, perhaps surprising in view of its lower stomatal density. Though this could reflect a significant reduction in respiratory activity in maize epidermis, possibly as a result of a more efficient stomatal mechanism, there are several other potential causes such as increased respiration due to mechanical stimulation during isolation of C. communis epidermis or reduced activity in maize tissue because of permanent damage caused by peeling.

Light had no apparent effect on respiratory activity in either species. However C. communis stomata certainly fix carbon dioxide as they have appreciable carboxylase activity (Willmer, 1973 a, b; 1974) which is clearly functional (Appendix 2; Willmer et al. 1973b). The stomata of maize also probably do (again having carboxylase activity and chlorophyll containing chloroplasts, though fixation could not be demonstrated - Appendix 2). Thus in both species it seems probable that photosynthesis either occurs but does not have any apparent effect upon respiration because of a reduced Hill reaction activity or fixation occurs in both the light and dark utilising energy derived from substrates supplied by the mesophyll. The absence of any light effect also indicates that, unless a respiratory inhibitor produced in the mesophyll enters the epidermis, respiration probably occurs in both the light and dark to an appreciable extent. Indeed it is possible that respiration is promoted in the light as a result

of increased substrate availability from photosynthate being translocated through the epidermis (Outlaw, 1975).

Using values derived from Warburg manometry it was possible to estimate the overall respiratory balance of the maize leaf (Table 7.1). Using the value of epidermal respiration measured in normal epidermal peels by Warburg manometry, which is probably conservative because of the large number of damaged long epidermal cells and damage to the stomatal cells it is apparent that the retention of respiratory CO_2 in the epidermis in the light could reduce CO_2 loss by the whole leaf in a 24 hour period by 7%. Epidermal respiration in C. communis may be more significant but the asymmetric distribution of the stomata makes quantification difficult.

While much of the respiration in maize epidermis is probably occurring in the guard cells where the released CO_2 could be refixed photosynthetically some must also occur in non-chlorophyllous cells, in particular the subsidiary cells which have an appreciable cytoplasmic volume and numerous mitochondria. Since these cells appear to lack any cytoplasmic connections with the guard cells, they and the long epidermal cells must rely upon non-autotrophic means to prevent CO_2 escape. It is unlikely that the escape is prevented by impermeable inner cells walls in view of the recent reports suggesting that these walls are a principal site of evaporation in many species.

The distribution of carboxylase enzymes in the epidermis of maize was investigated and evidence for the presence of two with appreciable activities, RuBP carboxylase and PEP carboxylase obtained. The evidence for the occurrence of PEP carboxylase was the most convincing; however the overall distribution was similar to that reported in the epidermis of C_3 species (Willmer, Pallas and Black, 1973), furthermore its activity was much less and it appeared to be associated with malic

Table 7.1: Overall respiratory balance of the maize leaf. Respiration rates were derived by Warburg manometry, leaf respiration was assumed to remain constant throughout a 12 hour dark period and epidermal respiration constant during the entire 24 hour light-dark cycle.

Oxygen uptake by 1g DW equivalent of whole leaf in 12 hour dark period	: $21.780 \times 10^3 \mu l$
Oxygen uptake by the epidermis of 1g DW equivalent of whole leaf in 12 hour light period	: $1.751 \times 10^3 \mu l$
Total oxygen uptake by 1g DW equivalent of whole leaf tissue in 24 hour period	: $23.531 \times 10^3 \mu l$
Proportion of total oxygen (and hence CO_2) respired by epidermis in 24 hours	: 7.4%

enzyme, a decarboxylase. When converted to an area basis its activity was adequate to account for the respiration of normal epidermal strips measured by Warburg manometry; 5938 µl CO_2 g DW h^{-1} compared to a respiratory oxygen uptake of 384 µl O_2 g DWh^{-1}.

RuBP carboxylase was also recorded in appreciable quantities, apparently mainly in long epidermal cells, its activity was also sufficient to account for all respiratory CO_2 released by normal epidermal peels. This finding was most unexpected, all previous reports of this enzyme have indicated that it is purely chloroplastic in its distribution and this together with the fact that much of the data was derived from extracts prepared by enzyme digestion means, I feel, that it cannot be taken as meaningful, clearly the work needs repeating with extracts prepared by a non-inhibitory enzyme digestion technique and needs to be confirmed if possible using autoradiography.

The evolution of CO_2 reassimilation

Photosynthesis in many C_3 species is saturated at ¼ of full sunlight (Zelitch, 1971), any further increase in light intensity has no effect upon the rate of photosynthesis. Saturation is limited by CO_2 since an increase in external carbon dioxide concentration in bright light results in an increase in photosynthetic rate. Since the rate of photosynthesis can be increased so easily it is apparent that the capacity for photophosphorylation is not saturated and indeed excess ATP and reductant must be being produced or is only being prevented by end product inhibition. Under normal conditions C_3 plants may lose up to 50% of their fixed CO_2 by photorespiration and normal respiration in the light (Zelitch, 1975), though some of this may be refixed it is apparent that a large proportion of it escapes to the outside. Thus in a reasonable light intensity it is apparent that many C_3 species have both the need and the means of refixing carbon dioxide. There

are two reasons, I believe, why they do not:

(i) The leaf of a typical C_3 plant has one type of photosynthetic cell, the location of both the PCR cycle and photorespiration. Since it is thought that RuBP oxygenase activity is a source of glycollate for photorespiration any increased refixation would lower the CO_2 concentration in the cell, thus favouring the oxygenase reaction, and so tend to increase photorespiration whilst at the same time decreasing PCR cycle activity.

(ii) C_3 species typically grow under conditions of low water stress, hence water use efficiency is rarely a selective pressure and plants can usually maintain wide stomatal apertures. Therefore though the CO_2 concentration in the leaf may be comparatively high resulting in a small CO_2 concentration gradient from the outside a satisfactory flux of CO_2 may still be attained because of the low stomatal resistance.

The absence of significant refixation in C_3 species can no longer be explained on the absence of C_4 type metabolism in view of increasing numbers of workers reporting its occurrence (e.g. Willmer and Johnston, 1976). The only enzyme apparently virtually absent is pyruvate phosphate dikinase but CAM species demonstrate that other means of synthetising PEP are available.

C_4 species have a modified photosynthetic pathway which does not saturate even in full sunlight i.e. light is always limiting the rate of photosynthesis. Therefore the use of energy to refix carbon dioxide seems unnecessary as CO_2 is always available. Yet clearly C_4 species do reassimilate all CO_2 being released in the light in the leaf - why do they do this? There are I believe two reasons:

(i) The site of reassimilation is spatially separated from the site of PCR cycle and photorespiratory activity. Therefore low concentrations

of CO_2 can be tolerated at the site of refixation and indeed its concentration can be increased at the site of PCR cycle activity.

(ii) C_4 plants typically grow in environments where periods of severe moisture stress are frequent. Under such conditions efficient water utilisation becomes critical and plants that can maintain small stomatal apertures may have a selective advantage. Under such conditions large fluxes of CO_2 into the leaf can only be maintained by maximising the concentration gradient across the stomata and this can be achieved by preventing respiratory CO_2 escape from the cells into the air space system - hence reassimilation and the resultant CO_2 compensation point of zero.

The latter reason is, I feel, of particular significance and as a result I feel that C_4 photosynthesis and its associated characteristic leaf structure may be regarded as primarily an evolutionary response to water stress. The leaf anatomy shows several adaptations to reducing water loss (Laetsch, 1974), though the large volume of water storing epidermis is unusual, and I feel that it was the development of this anatomy that put a selective pressure on the plants to modify their photosynthetic pathways, selection favouring both a more efficient assimilation mechanism and the reassimilation of CO_2. It is probable that the large selective pressure in arid climates (Stebbins, 1952; Axelrod, 1972) gave the best conditions for a rapid evolution of a new photosynthetic process.

The theory that C4 photosynthesis has arisen as a response to photorespiratory CO_2 loss must, I feel be discounted for two reasons:

(i) Apparently all C_4 species have evolved in conditions of intermittent water stress.

(ii) It is becoming increasingly apparent that C_3 species, which also

have photorespiration, have the enzymes capable of providing C_4 photosynthetis yet it is only used in certain tissues. The reason for this is probably that there is no selective pressure to compartmentalise carboxylase activity and hence even in leaves having radially arranged mesophyll tissues such as certain C_3 grasses no cellular differentiation has occurred.

Thus it would appear that C_4 plants in evolving in response to water stress have also overcome the problems presented by photorespiration and this latter fact has permitted them to compete successfully in regions of lower water stress with C_3 species. Indeed it appears that they have a potential advantage only limited by their somewhat higher light and temperature requirements. CAM plants on the other hand have evolved a more complex system of CO_2 fixation under more extreme conditions and this system is too specialised to permit them to complete successfully in less demanding environments.

Evolutionary parallels to this scheme of C_4 evolution may be found in the animal kingdom (Young, 1962 p.775) perhaps the most well known being the suggested evolution of marine fishes. It appears that though life first evolved in the sea the marine fishes appeared from species that had migrated to the highly selective fresh water environment and had therefore quickly evolved. Species that then returned to the sea had a much greater efficiency and could displace species having the same ancestors that had evolved more slowly in the less demanding environment.

This view of C_4 photosynthesis leads to considerable doubts of the potential value of introducing C_4 characteristics into C_3 species. Clearly C_4 photosynthesis depends upon a synthesis of several characters, the introduction of one into a C_3 species is liable to be of limited value - either reducing water loss but also photosynthesis, or increasing

reassimilation but decreasing PCR cycle activity. I feel that the whole C_4 syndrome should be regarded as an indivisible entity - a view that is supported by the apparent absence of plants having characteristics intermediate between the two types.

Overall Conclusion

If one regards C_4 photosynthe is as an adaptation to decreasing stomatal aperture then it is apparent that the high cuticular resistance recorded in maize will further increase the importance of retention of CO_2 in the epidermis. A high cuticular resistance will mean that little CO_2 escapes from the epidermis to the outside and so it will tend to escape into the leaf air space system, so reducing the CO_2 concentration gradient, and thus decreasing water use efficiency.

Thus there are two clear reasons for CO_2 retention in the epidermis:

(a) Prevention of photosynthate loss. This appears to be of some significance when respiration of the leaf as a whole is considered, though doubts about the reliability of the data because of the damage to the tissue make precise quantification impossible.

(b) Maintenance of low internal CO_2 concentrations. This I feel is probably the more important, particularly to C_4 plants growing under water stress when the maintenance of a steep CO_2 concentration gradient is important.

In this study however I feel that I have not found any clear evidence for the existence of a clearly defined mechanism to prevent CO_2 escape. There do appear, however, to be several factors that could each assist in preventing it to a certain extent:

(i) Respiratory activity could well be depressed relative to that found in the epidermis of C_3 species. This applies particularly to stomatal respiration because on the basis of the greater stomatal

density recorded in maize a higher respiration rate could be expected but was not found.

(ii) PEP carboxylase is present in the stomatal complex and could prevent CO_2 escape from the subsidiary cells in the light if it is in those cells. Transfer of malate could then occur to the guard cell where it could be decarboxylated and refixed autotrophically. Substrate for refixation could be derived either from the mesophyll or from the guard cell. The occurrence of a greater activity of PEP carboxylase in a C_3 species does not disprove this idea as it is possible that low CO_2 concentrations are needed in the vicinity of the guard cell for stomatal operation and they too may require organic acids to maintain ionic balance.

(iii) Experiments showing that a principal site of water evaporation in some leaves are the subsidiary and guard cell walls leads to the possibility that CO_2 movement from respiratory sites in long epidermal cells is facilitated towards the stomatal complex (containing PEP carboxylase and active chloroplasts) by the 'mass flow' of water.

(iv) The tendency of mesophyll chloroplasts to cluster adjacent to the long epidermal cells would increase the probability of CO_2 moving from the long epidermal cells into the mesophyll being refixed, particularly if carbonic anhydrase was available to increase the solubility of the gas.

I feel that some, or all, of these factors acting together could reduce the amount of CO_2 escaping from maize leaf epidermis to a level undetectable by the techniques used in studies carried out up to the present time. It is clear however that firm conclusions about the validity of this statement must await considerably improved techniques of epidermal investigation.

APPENDIX 1

PROTEIN ASSAYS IN THE PRESENCE OF DITHIOTHREITOL

Introduction

The results of all enzyme assays carried out were presented on both a protein and chlorophyll basis. Since the protein concentration in most epidermal extracts was very low and the volume of extract available small the most sensitive technique available, that using Folin-Ciocalteu phenol reagent (Lowry et al., 1951), was used.

It was also necessary to use the thiol-group protecting agent dithiothreitol (Cleland, 1964) in the extracts prepared in order to prevent enzyme inactivation. However dithiothreitol, in common with similar agents such as mercaptoethanol, interacts strongly with the Folin phenol reagent to produce anomalous results. A variety of techniques to overcome this interference were investigated.

Methods

Protein determinations were initially carried out on solutions of Bovine Serum Albumin (BSA) dissolved either in the grinding medium used in the preparation of tissue extracts, which contained 5 mM dithiothreitol (DTT) or in water. All protein determinations were carried out by the method of Lowry et al. (1951) with slight modifications:-

Reagents:

Solution A : 2% $Na_2 CO_3$ in 0.1M NaOH

Solution B : 0.5% $CuSO_4.5H_2O$ in 1% sodium citrate

Solution C : 50 ml of Solution A and 1 ml Solution B. Prepared fresh each day.

Solution D : Folin-Ciocalteu phenol reagent diluted with an equal volume of water.

Method:

To 0.5 ml of protein solution, 5 ml of solution C was added with mixing and then left to stand for 10 minutes at room temperature. 0.5 ml of solution D was rapidly added with mixing and colour allowed to develop for 30 minutes at room temperature. Absorption was measured at 600 nm against a water blank.

It was necessary to modify this basic procedure in some of the three procedures investigated to remove interference by DTT:

(i) Protein (0.5 ml) was precipitated with 1 ml 20% TCA, left at room temperature for 30 minutes and centrifuged (3000g, 10 minutes). The supernatant was discarded, the precipitate gently washed with absolute alcohol to remove chlorophyll, recentrifuged and the supernatant again discarded. The protein was redissolved in 1M NaOH and its concentration determined as described above.

(ii) Excess DTT was driven off with hydrogen peroxide (Geiger and Bessman, 1972). 0.5 ml of protein, 5 ml of solution C and 0.2 ml of 0.3% H_2O_2 were carefully mixed and left for 30 minutes at room temperature before 0.5 ml of solution D was added and absorption at 600 nm measured after a further 30 minutes.

(iii) Excess DTT was removed by carboxymethylation with iodoacetate (Ross and Schatz, 1973). Reagents used were:

Solution A : 30g Na_2CO_3 (anhydrous), 4g NaOH per litre.

Solution B : 100 volumes of A, 1 volume 2% $CuSO_4.5H_2O$ and 1 volume 4% sodium potassium tartrate

Solution C : Folin phenol reagent diluted 1 : 1 with water.

Solution D : 0.375M Na_2CO_3, 5% deoxycholic acid and 0.01N NaOH.

Solution E : Sodium iodoacetate 2N pH 6.0 prepared by dissolving 3.72g of free acid in 10 ml 2N NaOH.

0.2 ml of solution D was added to 0.2 or 0.05 ml of protein, mixed thoroughly and 0.1 ml sodium iodoacetate added. 2.5 ml solution B and 0.25 ml solution C were added after a further 30 and 10 minutes respectively and absorption at 600 nm recorded after a further 30 minutes.

Leaf extracts were prepared by taking equal weights of maize leaf tissue from either side of the midrib and grinding one in HEPES buffer pH 8.3 and the other in complete grinding medium (with DTT). Both extracts were filtered through a 30 µm nylon mesh and protein concentration in the former determined by a standard Lowry technique, in the latter by the method of Ross and Schatz.

Results and Discussion

Protein concentrations in solution of BSA in either water or complete grinding medium were determined (Table A1) by the standard Lowry technique. Clearly interference by dithiothreitol was considerable and very variable, thus it was impossible to overcome the interference by either preparing a calibration curve of BSA dissolved in grinding medium or by doing protein determinations normally and reading the absorbance against a similarly treated grinding medium blank.

Precipitation was potentially the most suitable method since in addition to removing DTT it also removed any interference by phenols (Potty, 1969). However it was found to suffer from two disadvantages:

(i) A very large amount of variability was encountered despite all reasonable precautions being carried out. This effect, possible due to loss of protein (Ross and Schatz, 1973), was most significant at low protein.

Table A1: Absorbance of solutions of BSA in water or grinding medium after protein determinations by the method of Lowry et al.

Concentration of BSA ($mg\ ml^{-1}$)	Absorbance of solution at 600nm	
	BSA in water	BSA in grinding medium
0.4	0.52	1.70
0.2	0.27	1.90
0.1	0.16	1.78
0	0.02	1.85

Table A2: Absorbance of solutions of BSA in water or grinding medium treatment by the method of Geiger and Bessman.

Concentration of BSA ($mg\ ml^{-1}$)	Absorbance of solution at 600nm	
	BSA in water	BSA in grinding medium
0.4	0.62	0.46
0.2	-	0.46
0.1	0.39	0.43
0	0.25	0.31

(ii) When a satisfactory calibration curve was obtained a reduction in the sensitivity of the technique became apparent, the absorbance range for a normal curve was approximately 20% more than that of a curve prepared following precipitation (neither solution contained DTT).

Thus the method was felt unsuitable for low protein content epidermal extracts.

The method of Geiger and Bessman yielded more reproducible results but unfortunately the calibration curves had a greatly reduced range, only 0.15 absorbance units compared to 0.52 abosrbance units for the same range of concentration of BSA dissolved in water and assayed normally (Table A2). This reduction in range was a result of the high absorbance of the blanks, their absorbance was not reduced by increasing the amount of H_2O_2 added.

Carboxymethylation proved the best technique available and initial experiments showed that 0.1 ml of iodoacetate, the volume used in the original report, was also most suitable for this work. Initial calibration curves were prepared using 0.2 ml of protein solution and absorbance was read at 600 nm. The results obtained (Table A3) very reproducible and their only disadvantage was a slight reduction in range. The spectrum of iodoacetate treated samples compared favourably with that of normally treated samples as well (Fig. A1) It was therefore decided to use this method in further experiments.

When protein concentrations in whole leaf extracts were determined by the sodium iodoacetate technique considerable interference by chlorophyll became apparent (Fig.A2). Attempts to remove the chlorophyll by differential solubility in organic solvents failed and so it was necessary to measure the absorbance at a wave length where chlorophyll interference was minimal, 790 nm was chosen. This approach had the advantage that any protein complexed with chlorophyll was also

Table A3: Typical calibration curves prepared using solutions of BSA in grinding medium or water. Protein determinations were carried out normally or after treatment with sodium iodoacetate. Replicates are shown to demonstrate the reproducibilty of the technique.

Concentration of BSA (mg ml^{-1})	Absorbance at 600nm				
	BSA in grinding medium-treated		BSA in water treated		BSA in water untreated
0.5	0.410	0.410	0.415	0.450	0.50
0.35	0.340	0.325	0.320	0.310	0.38
0.25	0.204	0.250	0.245	0.235	0.30
0.10	0.142	0.131	0.129	0.124	0.164
0.05	0.081	0.092	0.079	0.071	0.102
0	0.041	0.052	0.029	0.03	0.046

Fig A1 : Spectra of solutions of BSA following treatment with Folins reagent. The upper curve is that of a solution of BSA in water treated normally, the lower that of a solution of BSA in grinding medium pretreated with sodium iodo-acetate.

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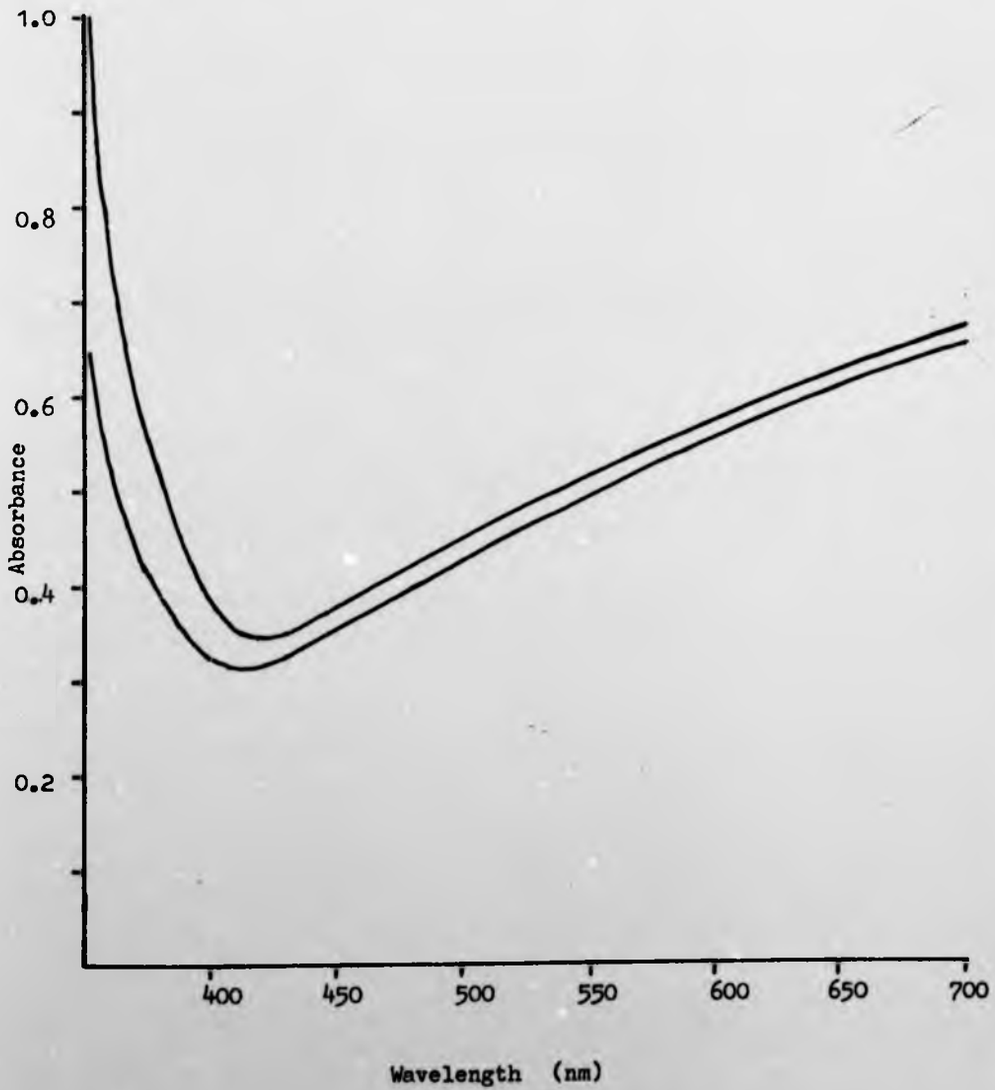

Fig. A2 : Spectra demonstrating the interference in protein determinations by chlorophyll present in leaf extracts.

a : Absorbtion spectrum of leaf extract after carboxymethylation and treatment with Folins reagent.

b : Absorption spectrum of BSA in grinding medium (0.7 mg ml^{-1}) after carboxymetylation and treatment with Folins reagent.

c : Absorbtion spectrum of solution containing 0.05 ml of extract and 3.0ml 100% acetone.

As can be seen the differences between (a) and (b) coincide with the peaks of (c).

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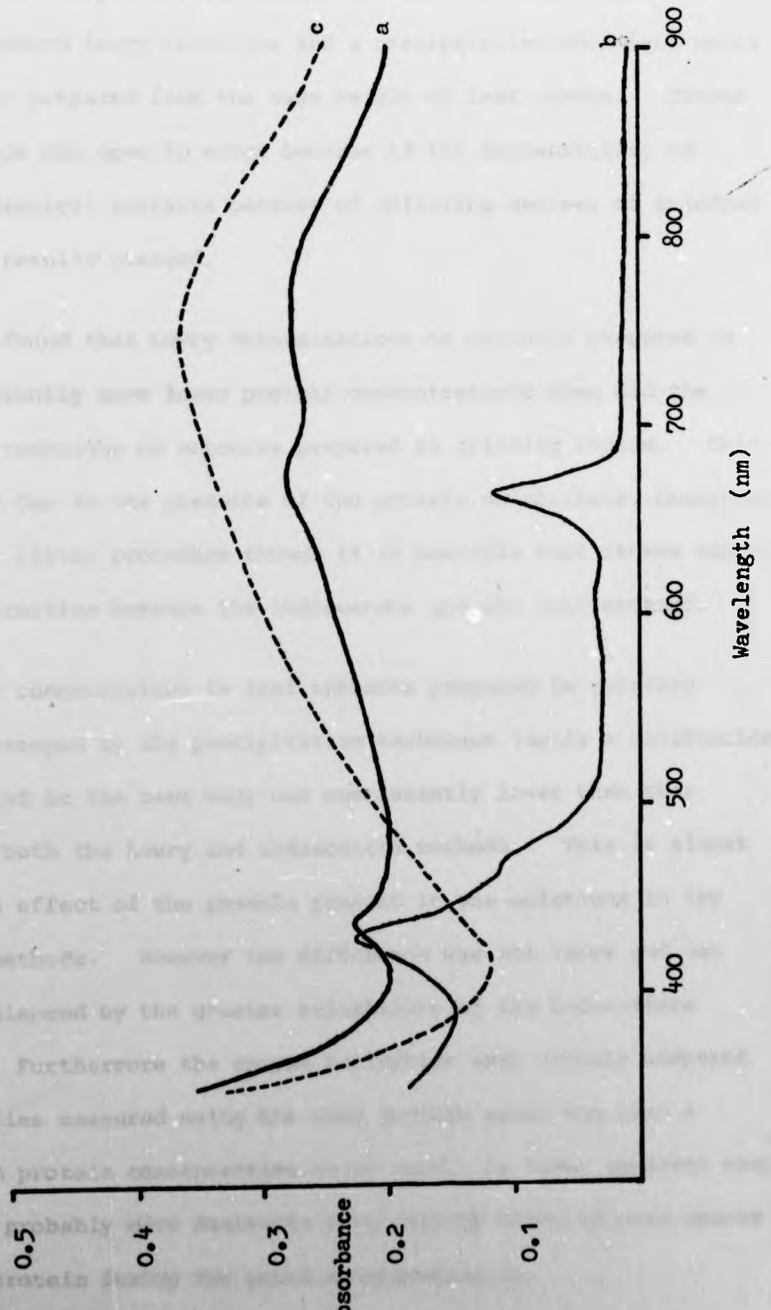


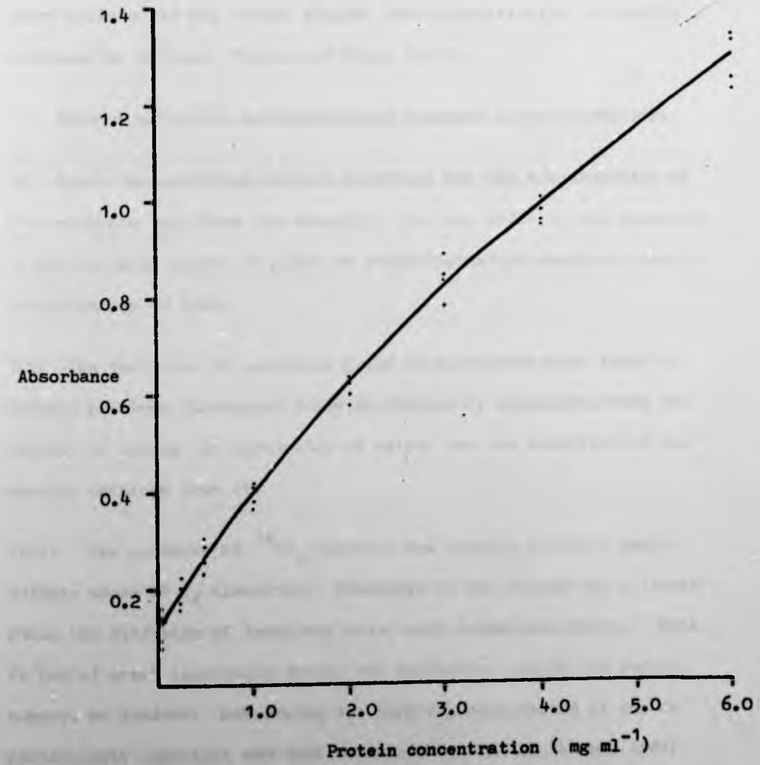
assayed. Chlorophyll and turbidity effects were further reduced by only using 0.05 ml of extract. Final calibration curves were therefore prepared using 0.05 ml of BSA solution and absorbance measured at 790 nm (Fig.A3). Calibration curves were checked during each protein determination.

As a final comparison the method of Ross and Schatz was compared with the standard Lowry technique and a precipitation technique using leaf extracts prepared from the same weight of leaf tissue. Though this technique was open to error because of the impossibility of preparing identical extracts because of differing degrees of grinding some useful results emerged.

It was found that Lowry determinations on extracts prepared in water consistently gave lower protein concentrations than did the iodoacetate technique on extracts prepared in grinding medium. This was probably due to the presence of the protein solubiliser, deoxycholic acid, in the latter procedure though it is possible that it was due to sort of interaction between the iodoacetate and the leaf extract.

Protein concentration in leaf extracts prepared in grinding medium and assayed by the precipitation technique (using a calibration curve prepared in the same way) was consistently lower than that recorded by both the Lowry and iodoacetate methods. This is almost certainly an effect of the phenols present in the solutions in the latter two methods. However the difference was not large and was certainly balanced by the greater reliability of the iodoacetate technique. Furthermore the enzyme activities were largely compared with activities measured using the same protein assay and also a falsely high protein concentration would result in lower apparent enzyme activities, probably more desirable than falsely elevated ones caused by loss of protein during the protein determination.

Fig. A3: Typical calibration curve prepared following carboxymethylation of solutions of BSA dissolved in grinding medium containing DTT.



APPENDIX 2

AUTORADIOGRAPHY OF EPIDERMAL TISSUES

Introduction

Autoradiography was potentially capable of showing the distribution of carboyxlases in maize epidermal tissue. It was first used for epidermal investigation by Shaw and MacLachlan (1954) to show that guard cells could fix carbon dioxide photosynthetically, a finding confirmed by Willmer, Pallas and Black (1973).

However epidermal autoradiography presents several problems:

(i) Since the epidermis acts as a pathway for the translocation of photosynthate away from the mesophyll (Outlaw, 1975) it was necessary to use isolated tissue in order to avoid misleading results caused by translocation of label.

(ii) The isolation of epidermis prior to experimentation leads to several problems (discussed fully in Chapter 4) concerning both the removal of tissue (in particular of maize) and the viability of any results obtained from it.

(iii) The products of $^{14}CO_2$ fixation are largely soluble, particularly those of C_4 fixation. Therefore if the tissue has a liquid phase the diffusion of label may occur with consequent error. This is not of great importance during the incubation period and cannot, anyway, be avoided; but during the long exposure period it can be particularly important and must be avoided (Roth and Stumpf, 1969).

(iv) The tissue must be in close contact with the photographic emulsion used because of the low energy of the β-particles emitted

by carbon-14 and the probably small quantity of isotope fixed in the tissue. This however may be difficult in epidermal tissue because of the irregular structure of the tissue and whilst firm pressure may overcome it, it may also result in artefacts caused by uneven distribution of pressure.

The last two of these are particularly difficult since they are largely incompatible - the best ways to get opposition of emulsion and tissue, liquid emulsion and stripping film, both involve the immersion of the tissue in liquids, impossible if diffusion of labelled products is to be prevented. A variety of techniques have been evolved which overcome this problem with some loss of sensitivity and reliability. The most widely used is that of Appleton (1972) which involves the placement of the frozen tissue on slides coated with dried stripping film followed by exposure at sub-zero temperatures. Perhaps the simplest is that of Willmer, Pallas and Black (1973) who freeze dried the tissue, mounted it on 35 mm film with sellotape and exposed it in a dry atmosphere. Other techniques have involved the covering of the tissue with a PVC film (Sawicki and Darzynkiewica 1964) and non-aqueous fixation techniques (Fritz & Eschrich, 1970).

A further problem that had to be considered was the possibility of chemography, either positive in which chemicals from the tissue cause false image production or negative where the tissue prevents image formation despite the presence of isotope.

Methods

(i) Preparation and incubation of tissue:

Epidermal strips were isolated from maize and C. communis leaf by the same techniques that were used in respirometry (Chapter 4). Pieces of whole leaf tissue were also used. The tissue was placed in a small (approximately 500 cm^3) glass chamber, either flat on pieces

of moist filter paper or suspended free. After sealing 1 mi C $NaH^{14}CO_3$ was introduced into a small vessel in the chamber containing excess lactic acid. The air in the chamber was circulated by means of small fan. After a period of illumination (30-60 minutes) the chamber was opened and the tissue quickly transferred to liquid nitrogen. Epidermal strips were removed from whole leaf tissue pieces before transfer.

(ii) Exposure:

Two techniques were used for exposure:

(a) Tissue was transferred from the liquid nitrogen to a cryostat cabinet and placed on slides covered with Kodak AR10 (previously dried on) under safe lighting (Wratton No.1 Filter). The slides were transferred to light proof boxes and exposed below 0°C.

(b) Tissue was freeze dried then placed against either Pan X or AR10 film for exposure at 4°C. It was either sellotaped in place or just placed on the film. All work with Pan X was done in the dark, with AR10 under a Wratton No.1 Filter.

Controls were carried out with all exposure techniques to check for positive or negative chemography.

(iii) Development:

Pan F film was removed from the tissue, loaded into a spiral, developed in Ilfosol for 8½ minutes and fixed in Metafix for 10 minutes. AR10 films were dipped in 1% gelatin and dried (to fix the tissue to the film), developed in Kodak D-19 for five minutes and fixed in Metafix for 10 minutes.

Results and Discussion

If a piece of labelled maize epidermal tissue was dissolved in Soluene (Packard Ltd.) and its activity determined by liquid scintillation counting it was apparent that it accumulated less than one fifth of the

activity of a similar sized segment of C. communis epidermis. This low activity was probably the reason why labelled maize epidermis showed little or no tendency to give meaningful autoradiograms. C. communis demonstrated clear stomatal uptake of $^{14}CO_2$ (Plate B1) similar to those published by Willmer, Pallas and Black (1973) with exposure against either AR10 or Pan X. There was some evidence of negative chemography between maize epidermis and AR10 film - film away from the tissue was often more background blackened than film adjacent to it. There was no evidence of this with Pan X and certain images could easily be correlated with features on the strip e.g. lengths of vascular tissue or patches of clinging mesophyll cells (in the case of enzyme treated strips) however there was no clear darkening in the rest of the autoradiograph even after exposure periods as long as three months.

This data confirms the low metabolic activity of isolated strips of maize epidermis. This is possibly because of damage inflicted during peeling, though the absence of any image formation in strips removed after peeling does not completely agree with this.

Plate B1 : An autoradiograph obtained by feeding epidermal strips of C. communis with $^{14}CO_2$ in the light followed by exposure on Pan X film for 6 weeks.

A : Labelled piece of tissue.

B : Autoradiograph obtained from it.

The correlation between stomata and accumulation of label is apparent.

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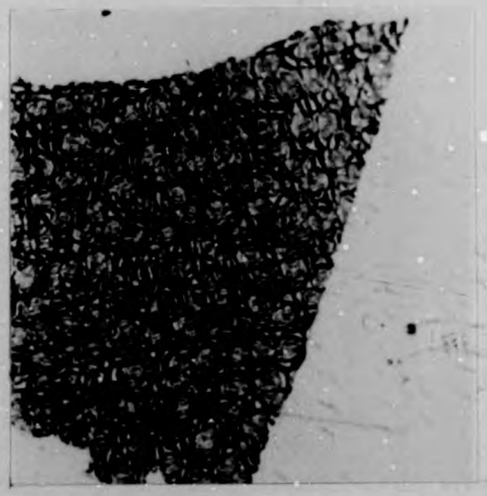

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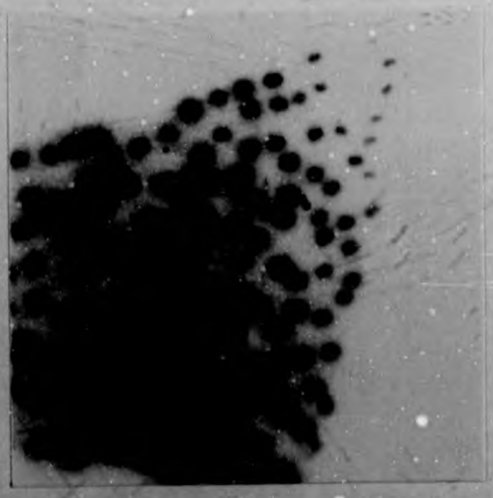

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