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**The role of potassium as an osmoticum in barley leaf cells.**

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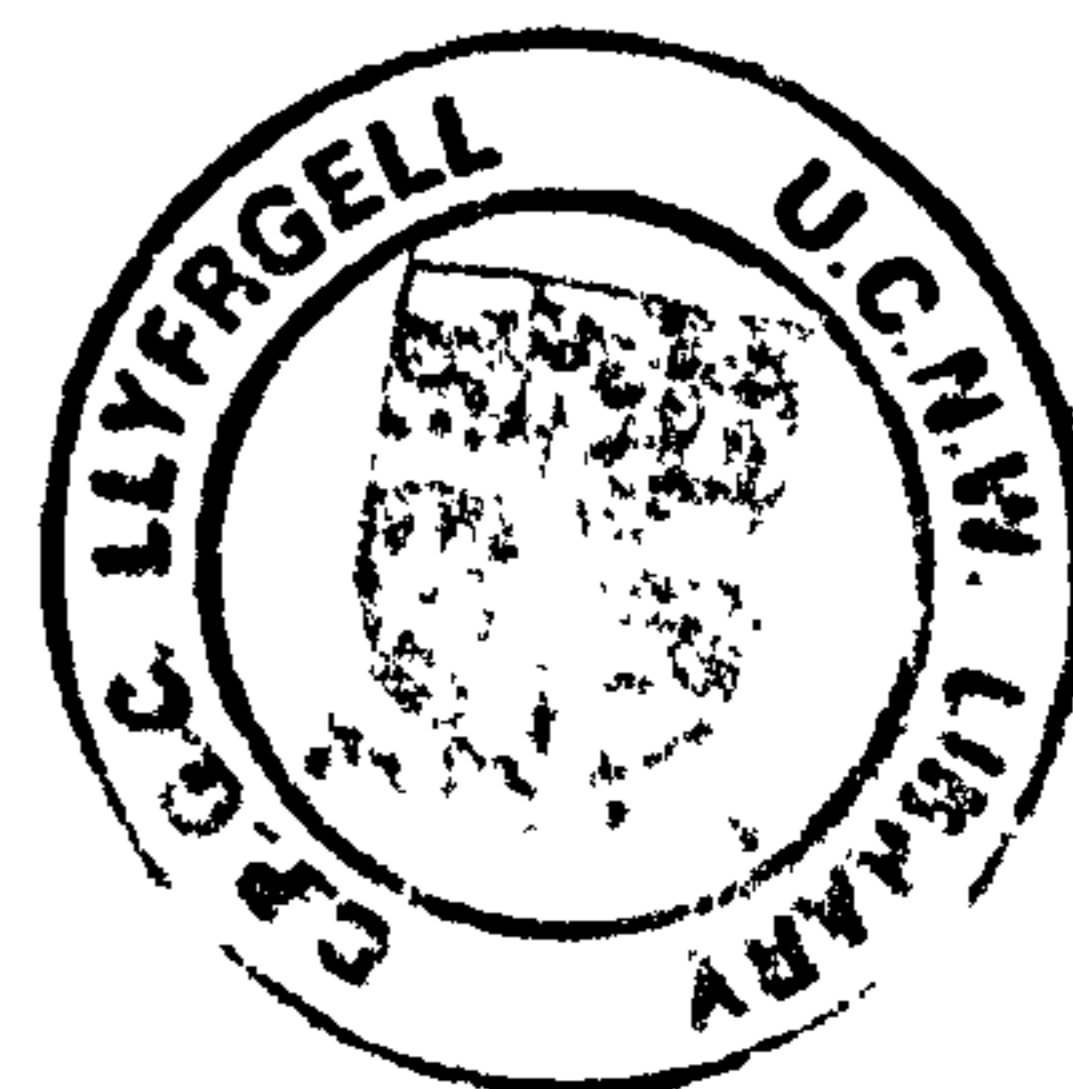
# THE ROLE OF POTASSIUM AS AN OSMOTICUM IN BARLEY LEAF CELLS

by

Peter Hinde

A thesis submitted to the University of Wales  
for the degree of Doctor of Philosophy

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## List of abbreviations

EDAX	Energy dispersive analysis of X-rays
NADP(H)	(Reduced) nicotinamide adenine dinucleotide phosphate
SD	Standard deviation
qoc	Quasi osmotic coefficient (see page 116)

## Summary

A recent X-ray analytical technique for the measurement of inorganic solutes in dried vacuolar sap has been improved and tested and was used to measure the content of barley leaf epidermal vacuoles. This method was compared with a technique which measured inorganic solutes in vacuoles of frozen leaf sections. The methods gave comparable results. They were used in conjunction with a range of other microanalytical methods to investigate the role of potassium as an osmoticum in individual leaf epidermal cells of barley grown in either low (0.2 mM) or control (4.0 mM) levels of potassium.

In the low potassium plants, both turgor and osmotic pressure were at first relatively low, but eventually rose to levels similar to the control plants. In both treatments, vacuolar potassium and its counterion accounted for all the osmotic pressure in young leaves. However, during leaf development, the potassium was replaced to varying extents by calcium. During this time gradients of ion concentrations developed between adjacent cells, depending on their proximity to vascular tissue.

In older leaves, the cellular concentrations of calcium and potassium were negatively correlated. The nature of this relationship was affected by potassium nutrition. In control plants, the ratio of calcium to potassium concentration was 2:3 (0.67). This value is consistent with the maintenance of osmotic pressure which was observed. In the low potassium plants, the Ca:K ratio was 0.76, which explained the increase in osmotic pressure over time in these plants.

It is proposed that the epidermis behaves as a storage tissue for potassium which is retranslocated to younger tissue as the leaf ages. The rate of export depends on the potassium nutrition of the plant. These events are also consistent with the hypothesis that turgor and osmotic pressure are regulated to compensate for the arrival of soluble calcium in the leaf.

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## **Chapter 1. Introduction**

Plants require both salts and water to grow. Specific ions are required in many biochemical systems as substrates ( $\text{NO}_3^-$ ,  $\text{SO}_4^{2-}$ ), activators ( $\text{Mg}^{2+}$ ) or intracellular regulators ( $\text{Ca}^{2+}$ ). Water is a primary reactant in photosynthesis, and is the solvent both for intracellular solutes and for long distance transport around the plant. In conjunction with the plasmalemma and cell wall, water and salts provide a mechanism by which pressure can be generated to provide both the driving force for cell expansion and the hydrostatic skeleton which constitutes the structure of all non-woody plants.

Since land-based plants in general live in a wide range of habitats that vary in their supply of salts and water, the ability to use these efficiently is beneficial. This thesis reports an investigation into the intercellular compartmentation of potassium. Potassium is the most abundant cation in the cytoplasm (Marschner 1985) and is the major cation accumulated in higher plants as an osmoticum (Leigh and Wyn Jones, 1984). Barley leaf epidermal cells were used as a model system to test a quantitative X-ray microanalytical technique to measure the composition of single-cell sap samples. The technique was used to investigate what degree of variation of cell composition exists within a single tissue and to examine the effects of such variation on the water relations of the cells.

### **1.1 Compartmentation and the heterogeneity of leaf cells**

In intact plants, exclusion or accumulation of solutes by different organs leads to differences in their solute contents (Martin, 1982; Bogemans *et al.* 1990; Wolf *et al.* 1990,1991). Such partitioning of solutes reflects the behaviour of the component cells of that tissue which may display compartmentation at two distinct levels.

1) At the subcellular level there are differences in solute concentrations between different membrane bound compartments within a cell (for reviews see

MacRobbie 1971; Cram 1974; Flowers and Läuchli 1983; Wyn Jones and Pollard 1983).

2) At the level of the single cell there may be differences in the proportion of vacuole and cytoplasm and differences in vacuolar composition. For instance, there is a clear distinction in the solute contents of mesophyll and epidermal cells. (Storey *et al.* 1983a; Outlaw *et al.* 1984; Leigh *et al.* 1986; Dietz *et al.* 1992a,b). There may be distinctions between adjacent cells of the same tissue type (Van Steveninck *et al.* 1982a).

### 1.1.1 Intracellular heterogeneity

#### 1.1.1.1 *The cytoplasm.*

The compartmentation of solutes reflects the functions of these compartments in the plant. For instance the composition of the cytoplasm is constrained by the requirements of the enzyme systems contained within it. Ions known to be controlled in this compartment include H<sup>+</sup> (e.g. Smith and Raven 1979); inorganic phosphate (e.g. Lee and Ratcliffe 1983); sodium, (e.g. Flowers and Läuchli 1983); potassium (e.g. Wildes and Pitman 1975; Wyn Jones and Pollard 1983; Gibson *et al.* 1984; Leigh *et al.* 1986) and calcium (Kauss 1987; Miller and Sanders 1987; Felle 1988; Evans *et al.* 1991). Ion homeostasis also occurs in compartments contained within the cytoplasm. Chloroplasts of spinach maintain constant concentrations of phosphate, sulphate and magnesium despite being subjected to treatments which increase the leaf concentration of these ions (Schröppel-Meier and Kaiser 1988a,b).

### 1.1.1.2 *The vacuole.*

The content of vacuoles also reflects their various roles. These roles have been summarised by Wagner (1982):

- 
- 1) Osmotic effects (in concert with the cytoplasm and cell wall)
    - a) Mechanical support
    - b) Tissue movement
    - c) Motive force for cell expansion
    - d) Stomatal function
  - 2) Ion balance and storage
  - 3) Metabolite storage
  - 4) Metabolite sequestration
  - 5) Lytic functions, senescence
  - 6) Minimisation of the volume of the cytoplasm and provision for efficient distribution of the photosynthetic apparatus
- 

### 1.1.1.3 *Cell solutes*

Any solutes dissolved in the vacuolar sap will contribute to osmotic pressure (Leigh and Wyn Jones 1984). As a result, the osmotic pressure within vacuoles can be generated by a wide range of solutes. Preferred solutes vary according to species but include inorganic compounds (eg sodium chloride in a wide range of marine algae) and organic compounds (e.g. sucrose in sugar beet) (Zimmermann 1978). The nature of the solute accumulated is also dependent on nutrient status and environmental conditions, and both ionic and organic solutes may occur together in single vacuoles.



In cereals (the topic of this investigation), whole tissue analyses suggest that the osmotic pressure is due largely to potassium (e.g. Leigh and Johnston 1983; Leigh and Wyn Jones 1984; Dietz *et al.* 1992b) although the nature of the solutes accumulated depends both upon their availability (e.g. Leigh and Wyn Jones 1984; Lynch and Läuchli 1985; Atkinson 1991; Richardson 1994), and other growth conditions (e.g. Veen and Kleinedorst 1986).

The importance of potassium as an osmoticum is probably related to its cheapness (in energy terms) relative to other solutes. When potassium is withheld, its vacuolar concentration decreases and other substances are accumulated to replace it. Potassium can be replaced to varying extents by  $\text{Na}^+$ ,  $\text{Ca}^{2+}$ ,  $\text{Mg}^{2+}$  or sugars depending upon the treatment (Pitman *et al.* 1971; Mott and Steward 1972; Mengel and Arneke 1982; Leigh *et al.* 1986).

There is a distinction between the homeostats necessary to control cytoplasmic and vacuolar solutes (Cram 1976; Glass and Siddiqi 1984; Memon *et al.* 1985; Leigh and Wyn Jones 1986). The cytoplasmic homeostat controls specific ions, hence osmotic adjustment of this compartment must be accomplished using a solute that can be tolerated at a wide range of concentrations. In contrast, osmotic adjustment of the vacuole may be effected by a wide range of potentially acceptable compounds. The ability of the vacuole to accumulate a wide variety of solutes might suggest that the level of no single solute need be regulated in the vacuole.

In fact, mechanisms exist in certain higher plants that restrict the accumulation of some ions to within maximum levels. In carrot, chloride is accumulated to steady levels which are insensitive to changes in turgor. In contrast in beet, chloride accumulation is stimulated by reductions in turgor (Cram 1976). It is not known whether or not specific regulation exists for other ions within the vacuole. For instance cereals accumulate a maximum of approximately 200 mM potassium in their shoots (Leigh and Johnston 1983; Leigh *et al.* 1986) although this apparent maximum could result from turgor- or osmoregulation rather than ion homeostasis.

The hypotheses of Leigh and Wyn Jones (1984, 1986) and Memon *et al.* (1985) suggest that the homeostats might interact. It was proposed that potassium was maintained constant within the cytoplasm despite changes in its concentration in the vacuole. Growth was predicted to be adversely affected only when the potassium

concentration in the cytoplasm had declined to a critical level which would occur only after the vacuolar potassium concentration had declined to a critical minimum. Thus the accumulation / retention of potassium by the cytoplasm would take precedence over the use of potassium as an osmoticum by the vacuole. Meanwhile, other solutes would accumulate in the vacuole to replace potassium in its osmotic role. (The relationship between cytoplasm and vacuole are discussed further in Wagner 1982, Glass and Siddiqi 1984, Memon *et al.* 1985, Leigh and Wyn Jones 1986. See also Cram 1988, Martinoia 1992.)

### 1.1.2 Intercellular heterogeneity

Solute partitioning also occurs at a higher level than the single cell and various studies utilising X-ray microanalysis have revealed differences between tissue types (e.g. in *Triticum aestivum* (Hodson and Sangster 1988), in *Sorghum bicolor* (Boursier and Läuchli 1989), in *Atriplex spongiosa* (Storey *et al.* 1983a,b) and in *Lupinus luteus* (Van Steveninck *et al.* 1982a,b,c) (see also review by Leigh and Tomos 1993). In leaves of *Hordeum vulgare*, Leigh *et al.* (1986) and Leigh and Storey (1993) demonstrated major differences in the composition of adjacent epidermal and mesophyll cells. Vacuolar calcium and chloride were restricted almost exclusively to the epidermis, whilst phosphorus was only detected in the vacuoles of the mesophyll. The compartmentation of calcium into the barley epidermis was proposed to be a sequestration mechanism preventing inhibition of calcium sensitive processes in other cell types.

The observed heterogeneity may result from a combination of whole organ structure and cell specific transport properties. Tomos and Wyn Jones (1988) drew an analogy between cells within the tissue and micropartition chromatography. This analogy suggested that properties of individual cells within the tissue would influence their composition, as would their relative positions to the source of nutrient supply.

Biochemical heterogeneity is generally accepted (for instance there are recognised differences between bundle-sheath and mesophyll cells of C4 plants and between stomatal guard cells and other epidermal cells), but such reasoning has not greatly influenced studies of solute compartmentation where analyses of whole tissues are often interpreted in terms of a 'generalised' cell. Martineau and Taylor (1986) demonstrated that the strict localisation of phosphoenolpyruvate carboxylase in maize leaf mesophyll cells is associated with exclusive restriction of its mRNA there. Similarly, Clark *et al.* (1992) found a number of mRNAs which were exclusive to the epidermis in *Pachyphytum*, although their functions were unknown. Recent characterisations by Dietz *et al.* (1992 a,b) of barley leaf protoplasts have revealed that epidermal and mesophyll cells are different in both their protein complement and their solute composition. These investigations suggest that heterogeneity between tissues might be explained solely by the behaviour of individual cells. Such a conclusion is also supported by the data of Outlaw *et al.* (1984) who demonstrated that intercellular compartmentation of phosphate into the epidermis of leaves of *Vicia faba* was the reverse of that found in barley (Leigh *et al.* 1986, Dietz *et al.* 1992b). Preferential accumulation of phosphate into the epidermis of *Lupinus* has also been reported (Treeby and Van Steveninck 1988). Collectively, the evidence shows that cells of different morphological types may possess quite different properties, and that these properties may vary according to the species.

The influence of nutrient stress on the compartmentation between cells has been largely neglected. Is there, for instance, a tissue which has priority for certain compounds within the plant, or which responds to nutrient stress by forfeiting its own nutrients? Leigh *et al.* (1986) showed that compartmentation of calcium into the epidermis of barley was strict, and that calcium was accumulated as a response to potassium deficit. It was suggested that calcium was acting as a replacement osmoticum in this species although interpretation of the data was complicated by the semi-quantitative nature of the data obtained by X-ray microanalysis. To date, parallel investigations of water and solute relations at single cell resolution have been hampered by the lack of reliable quantitative techniques capable of analysing the water and solute balance at the appropriate resolution.

## 1.2 Water and solute relations

The interdependence of water and solute relations (see 1.2.1) implies that water relations of the plants may change under conditions that perturb the solute balance (Mengel and Arneke 1982; Leigh *et al.* 1986). Osmotic pressure is particularly susceptible to changes in nutrition because this is absolutely dependent on solute concentrations that are known to change under nutrient stress (e.g. Leigh *et al.* 1986) and are predicted to change during senescence (see Bogemanns *et al.* 1990; Wolf *et al.* 1990,1991).

In the following considerations of water and solute relations, descriptions of water flow and predicted responses are based on the concept of an isolated idealised cell. There are three principal reasons for simplifying water relations to this level. Firstly, the important water relations parameter "turgor pressure" is manifested by individual cells. Secondly, this is the resolution at which it has been possible to measure the water relations properties of the plant. Thirdly, it is the level at which the cell itself may be able to exert some control.

The 'generalised' cell is regarded here as a large central vacuole bounded by a tonoplast surrounded by a thin layer of cytoplasm; itself surrounded by a semipermeable plasmalemma. The plasmalemma appresses tightly to a water- and ion-permeable cell wall that represents the apoplast. In fact, the epidermal cell of barley conforms well to this description since the vacuole occupies 99% of the symplastic volume of the cell (Winter *et al.* 1993). Solute concentrations here will therefore determine the maximum turgor pressure that a cell can exhibit.

### 1.2.1 Water potential

The interaction between solutes, water and turgor pressure can conveniently be described using the convention of water potential (Nobel 1991). This describes the free energy associated with water and is defined as:

$$\Psi = P - \Pi + \rho_w g h \quad \text{Eqn 1.1}$$

where  $\Psi$  = Water potential  
 $P$  = Hydrostatic pressure  
 $\Pi$  = Osmotic pressure  
 $\rho_w g$  = a gravitational component  
(0.0098 MPa m<sup>-1</sup>)  
 $h$  = height (m).

For the purposes of calculating water potential in single cells, the gravitational component ( $\rho_w g h$ ) can be ignored since water movement in a vertical direction is negligible when the height of the cell is regarded as the reference height hence Eqn 1.1 simplifies to

$$\Psi = P - \Pi$$

For the purposes of convention, pure water is taken to have a water potential of zero at ambient temperature and pressure and is a useful tool to describe and predict water flows within plants and plant cells. Water within a system containing regions of differing water potential will move down water potential gradients until an equilibrium is established and the water potentials equalise. It follows therefore that solute accumulation by cells will disturb this equilibrium and lead to changes in the osmotic pressure of the cell. This change in osmotic pressure will lead to corresponding changes in the turgor pressure unless compensated for by corresponding decreases in the cell water potential.

### 1.2.2 The cell wall and the elastic modulus

The cell wall has to withstand the generated turgor pressure although its mechanical properties may vary. Its ability to expand or contract is a function of the volumetric elastic modulus ( $\epsilon$ ) of the cell (Philip 1958) which describes the relationship between the change in pressure and the change in volume of a cell (Eqn 1.2).

$$\epsilon = (\Delta P/\Delta V).V \quad \text{Eqn 1.2}$$

Under drought stress, highly elastic cells (low elastic modulus) can maintain higher turgors than relatively inelastic cells (Morgan 1984; Richter and Kikuta 1989). The marine alga *Halicystis parvula* maintains a fairly constant turgor following dilution of its osmotic environment by swelling (Zimmermann and Hüsken 1980). This "elastic adjustment" mechanism is essentially a hydration phenomenon that relates water content to turgor pressure. It has been observed in *Beta vulgaris* as a shrinking of the taproot during transpiration (Palta *et al.* 1987). However, turgor also decreased during this period.

### 1.2.3 Osmotic pressure and the van't Hoff relationship

The osmotic pressure component of water potential is clearly an important determinant of turgor pressure. Its relationship to solute concentration is described by the van't Hoff equation (Eqn 1.3).

$$\pi = miRT \quad \text{Eqn 1.3}$$

Where  $\pi$  = the osmotic pressure (MPa)

m = the molality of the solution (moles of solute/1000g H<sub>2</sub>O)

i = a constant allowing for ionisation of the solute and other deviations from ideality

R = the gas constant (0.831 litre MPa/mol deg).

T = temperature (K)

#### 1.2.4 The reflection coefficient

In order to accurately assess the degree of turgor pressure generated in a cell, another factor should be taken into account. This factor, the reflection coefficient<sup>1</sup> ( $\sigma$ ) describes how effectively the osmotic pressure of a solute can be exerted across a particular membrane or other barrier (Nobel 1991). When solutes are unable to penetrate the membrane, Eqn 1.1 holds, as the reflection coefficient is generally equal to 1. If the membrane is penetrated by solutes, this value declines and so does the maximum attainable turgor pressure.

In summary, turgor is the hydrostatic pressure generated when water enters a cell to restore a water potential disequilibrium caused by solute accumulation. In concert with the cell wall this pressure imparts a structural rigidity to the cell. (For further reviews of the roles of water in plant cells the reader is referred to Cram 1976; Zimmermann 1978; Zimmermann and Steudle 1978; Morgan 1984; Munns 1988a; Tomos 1988; Nobel 1991 and references therein.)

#### 1.2.5 Turgor regulation

The definition of turgor regulation used throughout this thesis is that advocated by Cram (1976) and Munns (1988a). Turgor regulation describes a process whereby water relations parameters of a cell are altered such that a target value of turgor is achieved, or tends to be re-achieved after perturbation. In short the term is applied wherever the plant cell adjusts its turgor pressure in order to reach a desired turgor pressure. The target turgor pressure may lie within a range of values depending upon cell type, physiological conditions, the availability of osmotica and the degree of

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<sup>1</sup> For the purposes of this thesis, the reflection coefficient has been assumed to be one. It follows that if the reflection coefficient is less than this for either internal or external solutes, then errors will be introduced if turgor is calculated on the basis of osmotic pressure and cell water potential. Direct measurement of osmotic pressure has allowed for the expression of cell solute concentrations as percentage components of osmotic pressure although variations between individual solutes in their reflection coefficients may affect their contributions to turgor pressure.

perturbation imposed. Osmotic adjustment, where used, describes a change in the osmotic pressure of cell sap resulting from more (or less) solute molecules per cell rather than from an altered cell volume.

### 1.3 Turgor in plant cells

Turgor is important to most plants for three reasons. Firstly, it drives growth (Lockhart 1965). Secondly, by inflating cells, it provides a hydrostatic structure (Dale 1988), and thirdly it is used to control the volume and movement of guard cells (Tallman 1992). To perform the 3<sup>rd</sup> function efficiently, the plant must be able to manipulate turgor, and, indeed it is generally recognised (Cram 1980) that some form of turgor homeostat must operate in all higher plants. In his review of the physics of turgor and osmoregulation, Zimmerman (1978) lists a range of marine algal cells that display various yet fairly constant turgors despite being subjected to a wide range of external osmolarity. (e.g. *Chaetomorpha linum* (1.0 MPa) and *Codium decorticans* (0.23 MPa)). Because of the constancy of the environment these cells adjust their turgor by control of their intracellular solutes.

The majority of higher plant cells are highly vacuolate, hence control of intracellular solutes is often synonymous with control of the vacuolar component. However, higher plants may adjust turgor through alteration of either their vacuolar solutes or wall water potential. The latter is not widely considered but has the advantage that the extracellular space is small relative to cell volume so small changes in the amount of solutes outside the cell can lead to large changes in the cell water potential and hence turgor. In the growing taproot of *Beta vulgaris*, sucrose accumulation leads to an increase in osmotic pressure of the storage cells yet no corresponding increase in turgor (Tomos *et al.* 1992a). Young tissue measured before appreciable accumulation of sucrose had a turgor pressure equal to the osmotic pressure and turgor pressure did not increase concomitant with the increase in osmotic pressure. This constancy of turgor suggests that wall solutes accumulate progressively as the root sucrose concentration increases in order to regulate turgor. Leigh and Tomos (1983) demonstrated that free potassium in the cell walls of red beet was



sufficient to account for the wall osmotic pressure in this tissue. Fresh excised discs of red beet tissue bathed in silicone oil to restrict the extracellular volume had an initially low turgor due to flooding of the apoplast by broken cell contents. Turgor recovered at a rate faster than that in which red beet tissue was bathed in a bulk osmoticum suggesting that control of the restricted apoplast allows rapid manipulation of turgor (Tomos 1988).

Although Clipson *et al.* (1985) demonstrated turgor maintenance in leaves of the halophyte *Suaeda maritima* subjected to a range of salinities, little evidence exists of turgor regulation in leaves of other higher plants (Munns 1988a). An investigation of *Phaseolus* by Mengel and Arneke (1982), however, concluded that potassium was indispensable for attaining an optimum turgor in young leaves. Recently, Tomos *et al.* (1992b) in an investigation of excised illuminated wheat leaves discovered that turgor in both epidermal and mesophyll cells remained constant despite the accumulation of sucrose, although the nature of the regulation was unclear. Certainly, several turgor-dependent processes exist within plants which may effect turgor regulation (Patrick 1984; Reinhold *et al.* 1984; Wolswinkel and Ammerlaan 1984; Wyse *et al.* 1986; Wolswinkel *et al.* 1992 see also Higgins *et al.* 1987,1988)).

#### 1.4 Retranslocation of solutes during ageing

At a constant water potential, turgor increases as vacuolar solutes are accumulated. A corollary of this is that turgor will decrease if solutes are exported. It is now well established that potassium is retranslocated from the leaves of plants as the leaf ages (Ward 1958; Greenway and Pitman 1965; Jeschke and Wolf 1985; Bogemans *et al.* 1990). Retranslocation of potassium is not restricted to plants of low potassium status (Greenway and Pitman 1965) nor to plants under salinity stress (Bogemans *et al.* 1990), and is sufficient to lead to net decreases in the potassium contents of leaves of both wheat and barley. This decrease shows that retranslocation of potassium is not solely a phenomenon restricted to transfer of potassium from the xylem to the phloem, and is consistent with leaf cells functioning as nutrient stores. ✓

Salinity may increase the quantity of potassium exported. Ward (1958) demonstrated that the rate of potassium retranslocation doubled if the barley was grown in a solution containing a high concentration of sodium chloride. Similarly, Wolf *et al.* (1990,1991) demonstrated increased losses of potassium from the oldest leaf and stem of barley grown with 100 mM sodium despite a nutrient potassium concentration of 6.5 mM. In old leaves, phloem export of potassium greatly exceeded xylem import whereas sodium export was restricted and sodium was retained within the leaf. It was found that the older leaves towards the base of the plant tended to export potassium through the phloem to the roots, where subsequent transfer to the xylem allowed transport to the shoot. In contrast, the upper leaves tended to transport potassium directly towards the apex. In the xylem sap of barley, potassium concentrations reaching individual leaves increased towards the apex, but concentrations of sodium, nitrate and chloride declined. The net effect of retranslocation and nutrient cycling was that a good supply of nutrients was available for the growth of expanding tissue, whilst the import of deleterious ions such as sodium were restricted. Because transport or accumulation of solutes may affect osmotic pressure, these movements of solutes may perturb the water relations in the plant. To date, however, our understanding of these processes has been restricted by lack of appropriate techniques to study them.

### 1.5 Analytical techniques

Whole organ measurement necessarily provides an average value of all cells, hence tissue and cell dependent characteristics are masked. The logical approach to overcome these difficulties is to match the resolution of measurement to the resolution of variation. For instance, investigations of turgor pressure are ideally made at the level of the single cell because this is the level at which turgor is manifested. Similarly, because turgor is dependent upon solutes contained within single cells, ideally, these too should be measured at single cell resolution. Indeed, Michelena and Boyer (1982) recognised that water relations of the cell elongating region could not be inferred from data obtained from fully expanded regions of the leaf blade,

suggesting that factors influencing growth should be studied at the scale at which it actually occurs.

Measurement of solutes in plants at the resolution of the single cell is a difficult process because of the invasive nature of most analytical techniques. Cram (1988) suggested that *in vitro* measurements of the parameters of isolated cells did not necessarily reflect the behaviour of these cells *in vivo* because isolation removes neighbouring cells and therefore cannot account for interactions between structures. Analytical techniques can therefore be categorised into two groups. There are those that measure the physiological parameters of excised isolated cells and those that measure non-destructively *in vivo*. Examples of the former include studies that utilise isolated protoplasts/ vacuoles (e.g. Leigh *et al.* 1979,1981; Dietz *et al.* 1992a,b), and isolated membranes (e.g. Sanders and Slayman 1989; Tester 1990). Examples of the latter that will be considered shortly (see Sections 1.5.2.1 to 1.5.2.3) are those studies that utilise the micropressure probe and microelectrodes.

The isolated cell approach for solute analysis is complicated by post-surgical changes. Firstly, the time delay of tissue preparation and analysis means that the tissue may leak solutes. Secondly, wounding itself may introduce changes in the physiology of the cell. Profound changes can be observed in tissue after excision. Calcium influx has been shown to increase on wounding (e.g. Zocchi and Hanson 1982). This influx may initiate a change in the cytosolic free calcium levels sufficient to elicit fundamental physiological changes. Ryan and An (1988) demonstrated changes in gene expression following localised wounding of shoots of some solanaceous plants. In sugar beet tap roots, excision of tissue initiates a series of events leading to increased respiration and the synthesis of a vacuolar invertase (van Steveninck 1976).

Aside from the biochemical responses to wounding, biophysical changes may also occur. Water fluxes from vacuoles to the isolation medium, or from it to the vacuoles may influence measured vacuolar concentrations. Furthermore, isolated membrane preparations must always be under conditions of zero turgor and cannot fully represent intact cells. Turgor sensitive transport mechanisms are known to exist for both ions and sucrose (e.g. Gutknecht 1968; Wyse *et al.* 1986; Perry *et al.* 1987). Even at the whole plant level the cells in plants are hydraulically connected to the xylem and thereby to other cells (Malone 1992). This association with other tissues

would suggest that excision and isolation of any tissue from a plant might subject the tissue to immediate change.

In contrast, the intact plant '*in vivo*' approach exemplified by techniques such as the pressure probe (Section 1.5.1), microelectrodes (Section 1.5.2.1) and confocal microscopy (Oparka and Hawes 1992) is less invasive. Cellular parameters are measured soon after wounding which is restricted to a few individual cells and therefore the degree of tissue response to wounding is minimised.

The microanalytical techniques that have been applied to plant tissue all have various benefits and drawbacks in addition to their varying effect on wound physiology. These techniques are briefly reviewed in Sections 1.5.1 to 1.6.1. Emphasis has been placed on the application of X-ray microanalysis because much of what is known about cell heterogeneity has been obtained using this technique after a variety of preparatory stages. Attention is also given to applications of the micropressure probe because this approach has been used in combination with X-ray microanalysis to quantify vacuolar solutes concomitant with vacuolar osmotic pressure and turgor pressure.

### 1.5.1 Measurement of turgor and osmotic pressure

Direct measurement of single cell pressures was first attempted more than 25 years ago (Green and Stanton 1967; Green 1968). Pressure in giant algal cells of *Nitella axillaris* was estimated by observing changes in the volume of an air bubble introduced into a capillary tube sealed at one end. This technique was limited in its application since the pressure readings obtained were of low precision. A significant advance in the area of turgor measurement in individual cells was made by Zimmermann *et al.* (1969) who introduced the pressure probe. This was used to obtain water relations values in giant algal cells (e.g. Steudle and Zimmermann 1974; Zimmermann and Steudle 1975) and was of higher precision than Green's method. It was also used to measure higher plant cells (Steudle *et al.* 1975, 1977) although its use was restricted to cells above a minimum size.

A breakthrough in turgor pressure measurements in smaller higher plant cells was achieved by Hüsken *et al.* (1978) who miniaturised the pressure probe. Turgor

pressure and water relation parameters have now been measured in many higher plant cells (e.g. Tomos *et al.* 1981; Cosgrove and Cleland 1983; Pritchard *et al.* 1988). Although use of the pressure probe is ideally suited to visible surface cells, Pritchard *et al.* (1989) were able to demonstrate radial turgor pressure gradients within the mature zone of wheat root cells by equipping the pressure probe with a linear variable displacement transducer (LVDT), thus allowing accurate determination of capillary tip depth within the tissue. Malone *et al.* (1989) further modified the miniaturised pressure probe by attaching an instant sampling valve. Operation of the valve facilitates rapid sample extraction of cell sap (for subsequent measurement of its osmotic pressure by freezing-point determinations using a modified nanolitre osmometer), and minimises the osmosis-driven dilution reported by other workers (Shackel and Brinckmann 1985, Shackel 1987). The value of the technique was further increased by applying a quantitative technique of X-ray microanalysis to the sap thereby making it possible to measure its inorganic composition (Malone *et al.* 1991, Hinde *et al.* 1994). A modified version of this technique was adopted for virtually all of the experiments described in this thesis and is considered in Section 1.6.1.4. ✓

## 1.5.2 Measurement of cell solutes

### *1.5.2.1 Microelectrodes*

Microelectrodes have been widely used in investigations of cellular ion concentrations (see review by Felle 1993). A major advantage of microelectrodes is that they provide measurements of the ion activity within cell compartments over a wide concentration range (Zhen *et al.* 1991). To date, they have not been applied to studies of heterogeneity in leaves. A major drawback in their use to quantify water and solute relations is their inability to provide biophysical data and to measure more than one parameter at a single point in time. They are also susceptible to "poisoning" and interferences from other ions (Miller and Zhen 1991). ✓

### *1.5.2.2 Isolated protoplasts and vacuoles*

The method of analysis adopted by e.g. Leigh and Tomos (1983) or Dietz *et al.* (1992a,b) of isolating and separating protoplasts and vacuoles from plant tissue has proved valuable in allowing semi-quantitative measurement of concentrations of both ions and organic solutes. The measurements represent the mean of a large number of cells, and thus do not account for cell heterogeneity. Dietz *et al.* (1992a,b), however, were successful in separating epidermal protoplasts from mesophyll protoplasts by density-gradient centrifugation, thereby accounting for the variation exhibited between these tissues, although not between cells within each tissue. A fundamental criticism of the technique as a quantitative method is its susceptibility to solute leakage during protoplast or vacuole isolation (Leigh and Tomos 1983) and to water movement during isolation. This susceptibility has led to the practice of expressing concentrations on the basis of cellular constituents presumed to be constant (e.g. relative to  $\alpha$ -mannosidase activity, Dietz *et al.* 1992a,b). This presumption, and the extended isolation procedure, may also introduce artefacts.

### *1.5.2.3 Organic solutes*

Full quantification of the osmotica within a cell depends on knowledge of organic solute concentrations. It is not possible to account for these solutes either with X-ray microanalysis or ion-specific microelectrodes. Quantitative analysis of very small quantities of sucrose was first achieved by Lowry and Passonneau (1972). The method was further modified to include malate and starch (Outlaw and Lowry 1977, 1979; Outlaw and Manchester 1979; Outlaw 1980) and was improved by Hampp *et al.* (1987) to enable the analysis of sucrose down to  $10^{-14}$  moles. Essentially all the techniques involve the dissection of cells from freeze-dried tissue. These are weighed and analysed by pyridine nucleotide coupled reactions by delivering the isolated cells into nanolitre volumes of enzyme/substrate cocktails submerged in liquid paraffin to minimise evaporation. Both enzymes and substrates can be assayed by monitoring the fluorescence of the pyridine nucleotides microfluorimetrically. The techniques require skilled handling and are imprecise because of averaging of cell contents and of lack

of data on the original cell volume. They may also be susceptible to solute redistribution during preparation. Limitations aside, modifications of these techniques have provided the basis by which the measurement of organic solutes has been achieved in extracted sap samples in this thesis. Use of extracted sap circumvents the problems of solute redistribution and enables other water relations parameters to be obtained from the same cell. In addition, further solute analyses can be performed on subsamples of extracted sap. ✓

### 1.6 X-ray microanalysis and quantitative measurement

X-ray microanalysis has been used by many workers to identify differences in the elemental composition between plant cell compartments. Essentially, the method identifies and counts characteristic X-ray photons emitted when atoms are struck by an electron beam. The spatial resolution of such an analytical system is restricted to the interaction volume dictated by the accelerating voltage and the variable presence of atoms of higher atomic number. In a thin specimen the spatial resolution depends, primarily, on the diameter of the incident beam (Goldstein *et al.* 1977). Because the electronic interactions of X-ray microanalysis occur within the inner electron shells of the atom, the technique is unable to provide information as to the chemical state of the measured elements.

The X-ray intensity from a specimen in which all the incident electrons are stopped (termed a bulk specimen) is dependent upon 3 interrelated factors:

*(1) The primary generated X-ray intensity (atomic number correction)* This is a factor that corrects for differences atoms have in their "stopping power". It describes the likelihood of X-ray generation from atoms before the energy of the excitation beam decays to zero, and also takes into account the quantity of "backscattered" electrons generated.

*(2) The absorption of primary X-rays* X-rays are absorbed exponentially with distance in a manner that can be estimated from Beer's law.

**(3) The secondary generation of additional X-rays (secondary fluorescence) Generated X-rays may interact with inner orbital electrons of other atoms if they are of certain energies. These interactions produce secondary X-rays of lower energy.**

Collectively, these 3 factors are by convention known as ZAF interactions (Morgan 1985) and need to be accounted for if meaningful quantification is to be achieved. Because accurate measurement requires prior knowledge of the specimen composition, complicated computing procedures must be used to "correct" the spectra. It is therefore advantageous to analyse thin specimens. The main criterion for quantitative analysis of thin specimens is that the incident electrons must be transmitted through the specimen with negligible average loss of energy and should emerge with negligible internal absorption. In general, thickness criteria are ignored in analyses of microsamples and thin sections, but may be important in analysis of whole tissue specimens.

#### **1.6.1 Specimen preparation for analysis**

Correct preparation of biological tissue is paramount if the data are to be meaningful. The plant cell is a dynamic hydraulic entity, surrounded by and containing discrete, yet interacting, aqueous domains, and susceptible to water and ion flows caused by combinations of both osmosis and diffusion. The object of tissue preparation then is threefold:

- (1) to preserve the ion distribution and compartmentation existing in the living cell, and instantly halt any physiological activity;**
- (2) to present this prepared tissue to the electron beam in a manner that fulfils the requirements of X-ray analysis and enables quantification.**
- (3) to provide adequate structural information.**

Full reviews of preparatory procedures are given by Coleman and Terepka 1974; Morgan *et al.* 1978; Morgan 1979,1980,1985; Echlin and Taylor 1986; van



Steveninck and van Steveninck 1991. In this section the major sources of error commonly encountered in a number of specimen preparatory techniques are discussed. The discussion will be restricted to those techniques that have been applied to plant tissue for the purpose of subsequent quantification. After acknowledgment of these possible sources of error, it will be possible to relate these both to the frozen-hydrated whole tissue method tested in one section of the thesis, and to the extracted-sample technique used for the majority of the work.

#### *1.6.1.1 Chemical fixation*

Many of the investigations that utilise the scanning electron microscope are of dehydrated specimens that have been fixed and stained. The process of fixing is designed to maintain structural integrity, whilst staining improves the definition and resolution of the resultant image. Significantly, these processes are performed by submerging tissue samples in a series of aqueous solutions. Water will also come into contact with tissue if sections are floated on water after cutting. Clearly any contact between the tissue and water may lead to leaching and retranslocation making subsequent quantification meaningless. Indeed, several early attempts at quantification adopted these wet chemical approaches that led to loss of electrolytes (Harvey *et al.* 1976ab, 1979; Coetzee and Van der Merwe 1984).

Harvey *et al.* (1981) recognised the deficiencies of these approaches as quantitative methods and stated that the information obtained was only valid when "ion redistribution is prevented, and ion identification is unequivocal". Difficulties of accounting for artefactual errors in these instances led Morgan (1985) to conclude that wet techniques are generally unsuitable for most microanalytical purposes.

#### *1.6.1.2 Frozen tissue*

Freezing offers an alternative to chemical fixation. To prevent movement of diffusible solutes, tissue must be frozen at a rate sufficient to prevent the formation of ice crystals to produce ice in a vitrified form. The cryoprotocols that are used vary (Morgan 1985), but include plunging specimens into liquid or melting cryogen

(e.g. isopentane or liquid nitrogen slush) or pressing the tissue against cold metal surfaces.

Willison *et al.* (1984) and Pearce and Beckett (1985) reported the existence of water droplets lining intercellular spaces in leaves of *Triticum* and *Hordeum*. These droplets were further investigated by Jeffree *et al.* (1987). By freezing *Phaseolus* tissue at varying rates it was demonstrated that the droplets were artefacts of preparation strongly dependent upon the rate of cooling. Indeed, the fastest cooling rates they used ( $>965^{\circ}\text{C}\cdot\text{s}^{-1}$ ) were approximately three times faster than those normally achieved and still resulted in deposits. Sublimation under vacuum at  $-70^{\circ}\text{C}$  suggested that the deposits were largely water, yet in certain instances non volatile residues were observed. These residues indicate that solute movement may occur occasionally. It may therefore be concluded that no freezing mechanism yet used is sufficiently fast to completely avoid these freezing artefacts. The lack of information on the extent of water and ion redistribution leads to some doubt about the veracity of all the results obtained using freezing techniques.

All the freezing methods used to date yield specimens where only the surface layer (10-15  $\mu\text{m}$  deep) is vitrified, deeper vitrification being inhibited by the relatively low thermal conductivity of the frozen surface layer (Morgan 1985). Strictly, this means that analyses should be restricted to this vitrified layer. Unfortunately, when cut for freezing, the excised tissue must be small to reduce its heat capacity and to afford a large surface to volume ratio. This method of preparation may lead to a proportionally large degree of flooding of the apoplast by solutes released from cut cells. Freezing is unlikely to occur at a very rapid rate beneath this damaged surface layer, and may therefore lead to ice crystal formation beneath it. Since the tissue surface is planed to reveal a flat surface for X-ray microanalysis, this may present for analysis tissue that is not vitreous in character which may also be influenced by solutes released into the apoplast at the time of excision. It is clear therefore that the freezing techniques may limit the resolution of measurement in a number of ways.

Frozen tissue has been used for quantitative (Harvey *et al.* 1981; Pitman *et al.* 1981; Marshall 1982; Echlin and Taylor 1986; Treeby *et al.* 1987; Koyro and Stelzer 1988; Drew *et al.* 1990) and qualitative, (Leigh *et al.* 1986; Hodson and Sangster 1988; Huang and van Steveninck 1989; Leigh and Storey 1993) studies of plant

material and is an initial step in the procedure of freeze-substitution (see below). In general, X-ray microanalysis of plant tissue is performed after the tissue has been excised, frozen, cryoplaned and etched (and then coated with an electron conductive element). Much of the value of this technique lies in the area of distinguishing qualitative and semiquantitative differences between the vacuolar contents of distinguishable cell types which due to their relatively large volume are less susceptible to problems originating from solute redistribution and in defining the origin of the emitted X-rays.

Application of X-ray microanalysis to measurement of subcellular compartments smaller than the vacuole is more susceptible to gross quantitative change of electrolytes, and also necessitates greater beam current densities to achieve similar count rates. High beam current densities can lead to thermal damage resulting from volatilisation of elements ("mass loss"; Morgan 1985). In addition, it is difficult to ascertain the precise volume of tissue excited by the electron beam because of the proximity of the cell wall and cytoplasmic organelles. Drew *et al.* (1990) argue that although the depth of penetration of the electron beam in their study was calculated as 5.7  $\mu\text{m}$  for potassium, most of the signal would be derived from the superficial layers (90% within the upper 1.8-2.0  $\mu\text{m}$ ), and that its lateral resolution would be similar. It was recognised that the etched layers would have a resolution poorer than the underlying ice although by using digitised maps they showed that resolution was acceptable overall. Since they were not able to quantify the effects of surface etching on the resolution of potassium, it was suggested that the element concentration differences between vacuole and cytoplasm be regarded with reservation. No mention was made of the likelihood of mass loss in their system. Mass loss is retarded at low specimen temperatures (Morgan 1985) although the author writes "Where thermal damage by the electron beam caused gross distortion during mapping, the analysis was abandoned". However, thermal damage not leading to visible changes was not accounted for and may therefore have been underestimated.

The quantitative approach to analysis of frozen-hydrated tissue relies upon the analogous behaviour of specimen and standards during the freezing and etching stages. Storey and Walker (1987), in an attempt to prepare meaningful calibration curves using this method, equilibrated dead tissue of red beet with a range of potassium

chloride standards and treated these the same as the specimen. Similarly, Lazof and Lauchli (1991) calibrated their system by equilibrating lettuce leaf tissue with large volumes of standard solutions. These approaches may be able to account for some of the problems of whole tissue analysis, but still ignore the problem of electrolyte redistribution. Their use may also be restricted to tissue of limited air spaces, since flooding of these otherwise vacuum filled voids could conceivably affect the etching rate of the tissue. (The problems associated with this approach when applied to leaves have been further addressed in Chapter 10 where two methods of analysing solute concentrations in *Hordeum* primary leaves are compared.) Because quantitative X-ray analysis of frozen tissue relies on the identical thermal conductance of samples and standards, it is possible that the technique is better suited to those tissues where insulating air spaces are absent.

### *1.6.1.3 Freeze substitution and dry sectioning*

Freeze substitution describes the process whereby the ice in a tissue is replaced first by anhydrous solvents and then by resin. The necessity of freezing the tissue incurs all of the problems mentioned above, but has the advantage that ultrastructural preservation is good (Morgan 1985). The influence of the resin substitution on solute redistribution is unknown, but would be in addition to redistributions caused by freezing. Harvey *et al.* (1981) advocated the use of a freeze substitution technique followed by dry sectioning to overcome the problem of electrolyte leakage, and were able to demonstrate over 95% retention of the total Na<sup>+</sup>, K<sup>+</sup> and Cl<sup>-</sup> in 1 mm long *Suaeda* leaf segments (intra-leaf redistribution was not quantified). Freeze substitution was certainly better than an earlier method where tissue was resin substituted after being fixed in an aqueous medium (Harvey *et al.* 1976a,b).

In their analysis of the subcellular compartments of *Suaeda* mesophyll cells, Harvey *et al.* (1981) identified three populations of cells with vacuolar sodium concentrations ranging from 286 to 565 mM, chloroplastic concentrations ranged from 93 to 257 mM, cytoplasmic/ cell wall concentrations ranged from 71 to 146 mM and cell wall/ intracellular space concentrations ranged from 0 to 132 mM. Analyses of whole leaves gave a mean vacuolar sodium concentration of 543 mM, close to the

mean value obtained from averaging the results obtained by X-ray microanalysis (494 mM). This similarity is consistent with their proposal that the technique is accurate, however it was also suggested that "Since the distribution of ions differs in individual cells, it is unlikely that the measured concentrations are a consequence of the procedure but reflect the real *in vivo* ion distributions". This statement is possibly too simplistic a view on ion redistribution. Given that leaf tissue is heterogenous, the observation of compartmentation after the freeze-substitution process merely shows that complete re-equilibration of the cellular contents has not occurred. For instance, it is possible that due to the large surface to volume ratio of the cytoplasm that relatively small transmembrane fluxes in and out of the cytoplasm could drastically alter ion concentrations there. Such redistribution may easily be effected in the initial freezing phase of the preparation (see 1.6.1.2), and may account for the enormous range of ion concentrations found by Harvey and Thorpe (1986). In their analysis of salt stressed wheat, cytoplasmic sodium concentrations ranged from 9 to 3657 mM and potassium from 32 to 3502 mM. Such large ranges are not consistent with either the water relations of these plants nor with current views on cytoplasmic homeostasis.

It is too easy to accept the data obtained using this approach as quantitative, relying on the apparent similarity between the average vacuolar concentration estimated from whole tissue analyses and averaged measured vacuolar solute concentrations. There is danger of misinterpretation of such data. Firstly, calculations based on whole tissue analyses, although providing a crude benchmark of likely concentrations, ignore the proven heterogeneity between different cell types. Secondly, any imperfections and artefacts introduced by the measurement system adopted may be advanced as evidence of heterogeneity. Harvey *et al.* (1981) identify three distinct sources of inaccuracy: minor losses occurring during specimen preparation (possibly due to loss from cut tissue edges), inherent inaccuracies in analysis and instrument calibration, and variations arising within the replicate analysed samples. It was stated that actual values for the first two mentioned sources of inaccuracy were impossible to attribute for individual analyses, and that it would be wise to estimate a general standard error of the mean of not less than 20% for their data. This statistical treatment of the results is somewhat arbitrary and not suited for proper quantitative investigations. Some idea of the measurement errors might be

obtained simply by performing replicate measurements on multiple standards. This, however, could not account for the effect of the preparatory procedure on ion redistribution. Coleman and Terepka (1974) suggested use of gelatine 'model' plant tissue loaded with elements to test the extent of ion translocation during a preparatory sequence, but this would only have been applicable (if at all) to larger cell compartments such as the vacuole for reasons outlined above.

Consistent demonstration of three mesophyll populations may be suggestive of heterogeneity, despite plant variation. Until, however, the errors of the measurement system have been investigated it is difficult to place firm belief in the quantitative values obtained. To conclude, it is difficult to recommend any technique as quantitative if it is not possible to gain any insight as to the precision and accuracy of the measurement system itself.

#### *1.6.1.4 Analysis of dried constant volume microdroplets*

The uncertainties and difficulties arising from the application of X-ray microanalytical methods to analyses of whole tissue are surmounted if samples of the compartment of interest can be isolated from the tissue. The need for analysis of small volumes of renal fluid and of plasma led Ingram and Hogben (1967) to develop a system whereby nanolitre droplets of sample and standard were pipetted in constant volumes onto the surface of a quartz slide and allowed to air dry before X-ray microanalysis. The drying technique adopted is designed to yield samples free from crystals and of identical size to allow direct comparison of samples with standards. Both freeze-drying (e.g. Bonventre *et al.* 1981; Malone *et al.* 1991) and air-drying (Ingram and Hogben 1967; Hyatt and Marshall 1985) have been used successfully.

Quantitative X-ray microanalysis of the resultant standard droplets has been achieved by direct comparison of sample and standard droplets (e.g. Bonventre *et al.* 1981; Malone *et al.* 1991), and by the incorporation of an internal standard which is later used to normalise the data (Ingram and Hogben 1967; Hyatt and Marshall 1985). This latter approach makes the quantification process independent of droplet size, beam current and counting time. Since its inception, the technique of microdroplet analysis has been considerably improved. Lechene (1974) introduced the use of the

constriction micropipettes and proved their accuracy as better than 1% standard error of the mean, whilst the introduction of thin film supports eliminated the toxicity problem previously encountered with the use of beryllium, and increased the signal/background ratio (Quinton 1979; Van Eekelen *et al.* 1980; Bonventre *et al.* 1981).

Quantitative X-ray microanalysis of vacuolar sap extracted from plant cells was first achieved by Malone *et al.* (1991) who analysed microdroplets from wheat leaf epidermal cells after measurement of their osmotic pressure. In this thesis, this technique has been further modified to incorporate a normalising standard of rubidium. As an analytical approach the dried-microdroplet technique offers several advantages over the whole-tissue approach. Firstly, it is relatively easy to conform to the essential thin film criterion, hence calibration curves are linear. Secondly, since the sample is wholly encompassed by the electron raster, the excitation volume is absolutely defined and the question of ion redistribution is irrelevant. Further advantages for analysis of plant cell composition include its capacity to be integrated with other techniques (e.g. microfluorimetric enzyme assays (Zhen *et al.* 1991; Fricke *et al.* 1994a,b; Richardson 1994), turgor and osmotic pressure measurements (Malone *et al.* 1989,1991,1992)) on the same sample. Furthermore, volumetric addition of organic compounds may stabilise the samples against mass loss. These compounds provide a matrix in which the elements of interest may be dispersed (Roinel and de Rouffignac 1982; LeRoy and Roinel 1980,1983). The inclusion of mannitol (Malone *et al.* 1991) performs this function and it can be added to all samples concomitant with the addition of rubidium as the internal standard.

The major disadvantage of the technique is its restriction of application to samples large enough to be pipetted accurately (in turn dependent upon cell size, cell turgor and the cell elastic modulus (Section 1.2.2)). Other criticisms do not apply strictly to the accuracy of the technique *per se*, but on problems associated with identifying the source of the sample. Depending upon the cell sampled there is some susceptibility to cytoplasmic contamination and dilution at the instant of extraction (these problems are discussed in Chapter 10). Extraction techniques have been improved to minimise dilution (Malone *et al.* 1989) and the maximal extent of such dilution may, at any rate, be determined by measurement of the half times for water influx using the pressure-probe. Because the osmotic pressure is measured after

extraction, data can, at least, be related to this parameter. Lastly, the technique so far has only been attempted on the large easily accessible surface epidermal cells. Analysis of deeper smaller and less vacuolate cells may prove to be more difficult, and the identity of the cell type analysed becomes less certain.

### 1.7 The model system for experimentation

In order to investigate the relationship between turgor and potassium nutrition, the barley primary leaf has been chosen as a model system. As an experimental plant material, barley has several advantages aside from those of being a well-researched crop species with ready availability of well-defined genotypes. Possibly that of most relevance is its simple leaf structure (Plate 2a). Because 70-80% of the leaf volume is composed of just two tissue types (see below) then if one tissue type is investigated, inferences can be made of the other by comparison with whole tissue values. Also, single-cell analysis is facilitated by the large epidermal cell size and high turgor pressure. These allow the extraction of samples sufficiently large for subsampling, thereby enabling measurement error to be distinguished from cell to cell variation. Large samples also make it easier to observe the melting of ice crystals during osmotic pressure measurements. Cross sections of both frozen and resin embedded tissue show that the epidermis at least has a very high vacuole:cytoplasm ratio (99% in barley epidermal cells (Winter *et al.* 1993)). In addition, since the barley leaf is uniformly and predictably constructed it is possible to choose an area of interest and repeat measurements in a similar position in other plants. The cultivar 'Klaxon' had a very rapid (overnight) and even germination, close to 100%. Therefore experimental plants were broadly comparable at a given age within any one batch of plants. Developmental changes within the first leaf of barley were fairly complete after about 13 days which aided experimental planning.

The appearance of the typical leaf reflects its function as an interceptor of radiation, with a large surface to volume ratio to maximise gas exchange. In the measurements of leaf compartments performed by Dietz *et al.* (1992a) and Winter *et al.* (1993), the epidermis amounted to 27% of the leaf volume. (The mesophyll cells accounted for 42-59%, and the apoplasmic space and the vascular tissue 4-8% and 6-



8% respectively). On its atmospheric side, the epidermis is covered with a thin cuticle that may or may not extend to cover inner surfaces of the epidermis and the mesophyll to varying extents (Boyer 1985). The innermost side is loosely connected to the irregularly shaped mesophyll cells that loosely pack the sections of leaf about the vascular tissue. The vascular tissue itself is encased within a layer of small cells termed the mesophyll sheath, that is further enclosed by a layer of larger cells, the bundle sheath.

### 1.7.1 The role of the epidermis in cereals

Because the epidermis constitutes some 30% of the leaf, the function of this tissue may be of considerable importance for normal operation. Dietz *et al.* (1992a) suggest that the obvious function of the epidermal cell layer is to form the boundary with the surrounding environment, recognising that its ability to synthesise and deposit cuticular substances confers upon it the property of reducing water loss from the leaf. It is unlikely that this represents the main function of the epidermis in barley since the epidermal volume is considerably greater than that necessary to synthesise such a layer. The large vacuole of this tissue, however, suggests that it may play an important role.

The epidermis is a structural tissue (Dale 1988). The proposed structural properties of the epidermis stem both from its observed turgor and from its architecture. Since the cells are intimately connected to each other, they may be able to withstand forces that would not be tolerated by mesophyll cells alone. The epidermis presumably assists in the development of tissue tension in conjunction with the reinforcing strands of sclerenchyma that run down the length of each leaf. This tension would hold the leaf in an efficient light intercepting position whilst the transparency of the epidermal layers to light would allow for photosynthesis of any cells contained between them. It also provides the structure within which the stomata work (Meidner 1990). It is clear therefore that some relationship exists between vacuolar solutes and structure in the epidermis.

A second role suggested for the epidermis is as a sink for toxic ions (Outlaw *et al.* 1984; Boursier and Läuchli 1989; Huang and van Steveninck 1989; Leigh and

Storey 1993). This role is consistent with the proposal of vacuoles acting as nutrient stores. Surrounding the mesophyll cells, such an arrangement might afford some degree of protection against pathogens.

### 1.8 The problem addressed

To date, no study has simultaneously addressed the concepts of tissue heterogeneity, turgor maintenance and solute movement. Potassium and barley have been chosen as the subject of study because potassium's important role as an osmoticum in barley can be replaced when its supply is restricted (Section 1.1.1.2), and because it is mobilised as the leaf ages (Section 1.4). Hydroponically grown barley can thus be used as a model system where nutrition can be controlled to test specific hypotheses. Barley is particularly suitable for measurement because it has large epidermal cells that constitute a significant proportion (about 30%) of the volume of the leaf and is highly vacuolate (Section 1.7). This means that is easy to test simple hypotheses that may or may not perturb the water and solute relations of these cells.

A simple analysis suggests that there may be a subtle distinction between responses to lack of potassium incurred by potassium deprivation in contrast to potassium retranslocation. Plants cannot foresee nutrient deficits in their growth medium, hence the response of the plant must be geared towards the rapid accumulation of alternative osmotica to enable leaf expansion and subsequent photosynthesis. In contrast, the retranslocation of potassium already present as an osmoticum is presumably under some kind of control. As a result, the need to accumulate alternative osmotica may be less urgent because this accumulation may occur at a rate dictated by either the arrival of a preferred alternative solute, or by the demand of the retranslocated potassium. The stimulation of potassium retranslocation by salinity (Section 1.4) suggests that the former may be true.

To date, our understanding of solute partitioning within the leaf has been hampered by limitations in traditional analytical techniques. Of all those available only

the extracted sap technique (Section 1.5) offers the potential to measure both the water relations parameters and also the complete solute complement, and it is therefore the method of choice for experiments in this thesis. For purposes of comparison, the method of X-ray analyses of frozen-hydrated whole tissue has also been investigated to assess its suitability as a quantitative method and to provide more information on the distribution of ions within the leaf, especially in sub-surface cells of the mesophyll which are difficult to measure using the pressure-probe approach.

In conclusion, it is hoped to be able to answer, at least in part, the question:

**"What is the role of potassium as an osmoticum in individual leaf epidermal cells of barley developing under potassium sufficient and deficient growth conditions?"**

In answering this question an important role of the epidermis in terms of its ion partitioning capability is postulated.

## Chapter 2. Materials and methods

### 2.1 Plant material

All measurements were performed on the primary leaf of barley (*Hordeum vulgare* L.), at different stages of development from day 3 (the day before full leaf expansion as signified by the appearance of the leaf ligules) until day 13 (the start of early senescence, as signified by drying of the extreme leaf tip, and the presence of brown necrotic patches with dead epidermal cells).

### 2.2 Chemicals

Unless specifically stated, all chemicals were from BDH (Poole, Dorset, England) and were of standard laboratory grade. Silicone oil was supplied by Wacker (siliconöl AS4 Kontr. Nr. 1541 IG, Wacker-Chemie GmbH, München). Liquid paraffin was supplied both by Boots and BDH and was water saturated by shaking the oil vigorously with water for 5 minutes. Pure water-saturated liquid paraffin was collected from the surface layer after centrifugation at 100 g for 10 minutes. This was stored over water at 4 °C until required. The water used for the solutions was purified by reverse osmosis and ion exchange and had a resultant resistivity of not less than 20 Megohm cm<sup>-1</sup>.

### 2.3 Plant cultivation

Seeds of *Hordeum vulgare* L. cv Klaxon were rinsed for 10 min in several changes of tap water to remove excess dressing, and were imbibed overnight in aerated deionised water. The germinating seeds were transferred onto a thick polythene mesh suspended over 6 l of aerated 0.5 mM CaSO<sub>4</sub>, so that the seeds were kept moist but not saturated. Seedlings were grown in the dark until the primary leaf tip had just emerged from the coleoptile (approximately 4 d), whereupon the seedlings were transferred to the light for 2 d to allow the shoot to grow to approximately 4 cm. At this stage (designated in all experiments as day 0) 40 plants were suspended

over 6 l of nutrient solution in 8 groups of 5 plants held in foam set into expanded polystyrene supports (see Plate 2.1).

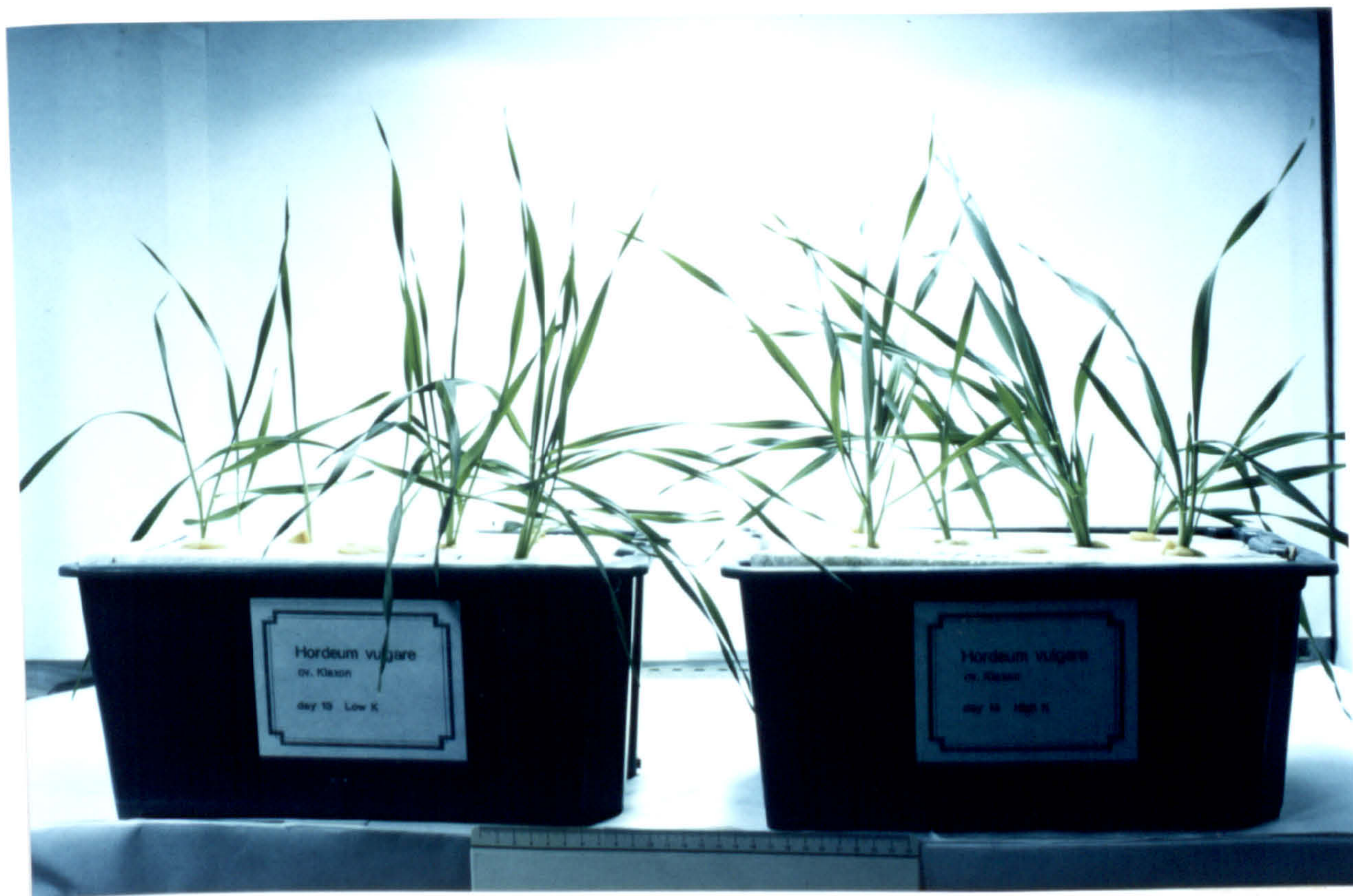


Plate 2.1. Hydroponic system used for the growth of the barley plants in all experiments. The nutrient deficiency imposed led to smaller, less sturdy plants. This difference was less obvious in plants grown under higher light intensities (as shown above). The control (4.0 mM K) plants are on the right whilst the potassium deficient (0.2 mM K) plants are on the left. The plants at this stage have been grown in nutrient solution for 13 days.

#### 2.4 Growth conditions in Bangor

The plants were aerated continuously and grown under 250 W metal halide lamps (Wotan) giving a light intensity of approximately  $140 \mu\text{molm}^{-2}\text{s}^{-1}$  at plant height level on a 16 h day/ 8 h night. Neither temperature nor humidity was controlled but would rarely have exceeded the range of 20-30°C at a relative humidity of 60-90%. Nutrient solutions were changed every 7 days.

## 2.5 Growth conditions in Hannover

The plants were aerated continuously and grown under 400 W metal halide lamps (Wotan) giving a light intensity of approximately  $10^4$  lux at plant height level on a 14 h d/ 10 h night. The day temperature was 22-25 °C decreasing to 12-16 °C at night. Humidity remained at a constant 70-80% both day and night. Nutrient solutions were changed every 7 d.

Throughout all experiments plants were removed from the growth system without replacement. The techniques used in Hannover required that more plants be sampled at each sampling date. Effectively this meant that the rate of nutrient depletion in the potassium-deficient treatment may have been slower than for plants grown in Bangor. This may have been one of the factors leading to the different nutritional characteristics of these plants (see results).

## 2.6 Nutrient solutions

The nutrient solutions used for growth were based on those of Epstein (1972) (Table 2.1). They were formulated to contain either 0.2 mM (Low K<sup>+</sup>) or 4.0 mM (High K<sup>+</sup>) potassium, achieved by varying the potassium nitrate concentration. The variation in nitrate or total cations was not compensated for. Calcium chloride (0.5 mM) was added to the medium as a chloride supplement to swamp the possible effects of airborne chloride contamination (H-W. Koyro, pers com).

Table 2.1 Nutrient Solution Composition.

COMPOUND	[NUTRIENT SOLUTION] (mM)
$\text{KNO}_3$	0.2 or 4.0
$\text{Ca}(\text{NO}_3)_2$	4.0
$\text{NH}_4\text{H}_2\text{PO}_4$	1.0
$(\text{NH}_4)_2\text{HPO}_4$	1.0
$\text{MgSO}_4$	1.0
NaFeEDTA	0.015
KCl	0.05
$\text{H}_3\text{BO}_3$	0.25
$\text{MnSO}_4$	0.002
$\text{CuSO}_4$	0.002
$\text{H}_2\text{MoO}_4$	0.00025
$\text{CaCl}_2$	0.5

## 2.7 Nomenclature used to describe the cells

The adaxial (upper) epidermis of barley consists of at least 2 cell types identifiable both by their appearance and their position relative to each other and to the stomata (see Plate 2.2 (a,b)). All measurements were made on cells from the upper epidermis. The term ridge cell is used to describe those elongated large epidermal cells that overlie the vascular tissue and lie above the stomata of the upper epidermis. The term trough cell is reserved for those swollen shorter bulliform epidermal cells that lie between the ridges overlying the vascular tissue and below the rows of stomata (looking from above; see Plate 2.2 b). These ridges can be discerned with the naked eye, and are numbered according to the convention of Kuo *et al.* (1972). In their analysis they describe large lateral veins that are numbered outwards from the midrib, and separated by up to 3 smaller intermediate veins. Generally, experiments were performed on sap extracted from cells at the tops of the ridges overlying the second lateral vein and the bottoms of the troughs adjacent to this lateral and towards the midrib of the leaf. This sampling approach ensured that representative cells of each type were measured. Those cells near the stomata that were smaller and often displayed characteristics of both cell types were generally avoided.



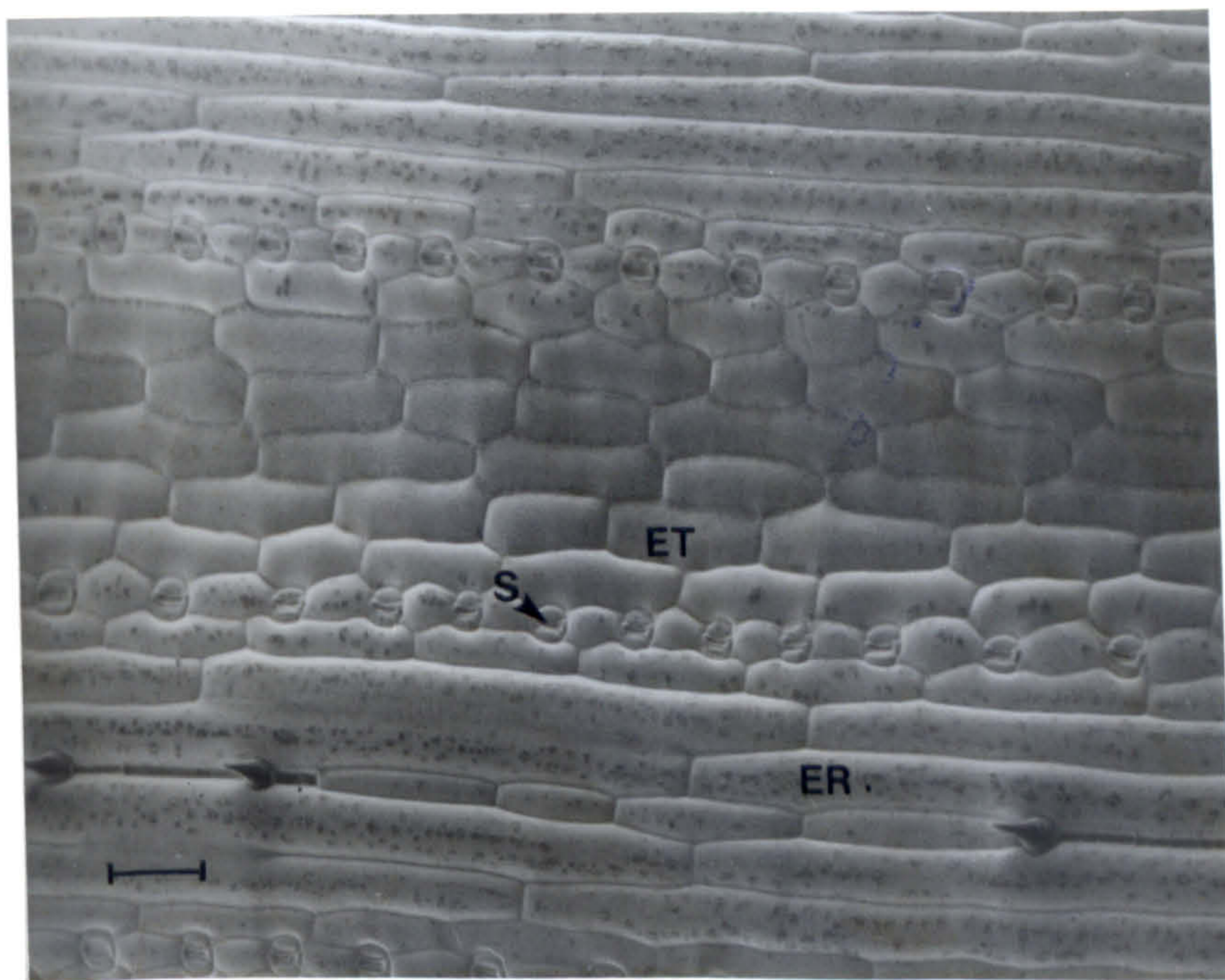
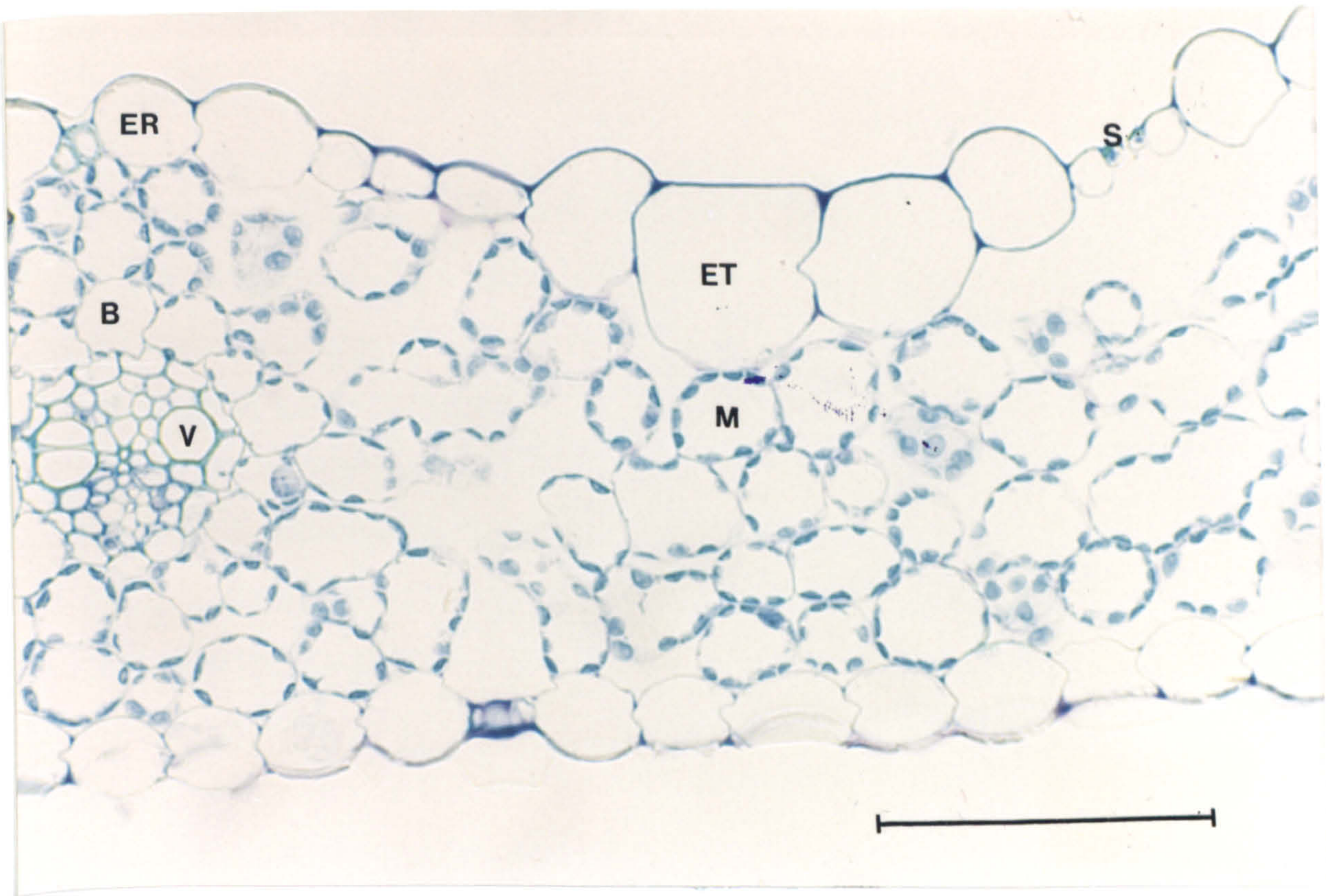


Plate 2.2. Anatomy of the barley primary leaf illustrating the cell types analysed, and the general proportions of different tissue types. (a) is a light micrograph of a transverse section stained with toluidine blue to show the proportions of cytoplasm. (b) is a scanning electron micrograph of frozen leaf tissue, illustrating the different morphology of the ridge and trough cells. ET = epidermal trough cell; ER = epidermal ridge cell; M = mesophyll; B = bundle sheath; V = vascular tissue; S = stomata. Scale bars are approximately  $100\mu\text{m}$ .

## 2.8 The selection of cells for measurement

An area of plant tissue 4 cm from the leaf tip was chosen to standardise measurements since longitudinal variation was evident in the leaf (see results). Furthermore, since it was evident that senescence progressed from the edge of the leaf towards the centre, a repeatable area was defined in which all sampling took place. This involved sampling cells from around the second large lateral vein. Cells in this region had started to senesce by day 13. Starting sampling at day 3 ensured that the solute concentrations in the epidermal cells were measured over a developmental period including both a phase prior to full leaf expansion and senescence.

Usually three cells of each cell type were sampled from at least one plant during measurement. Apart from instances where measurements of adjacent cells were required, cells were sampled that were separated from punctured or damaged areas by at least one cell longitudinally, and two cells laterally. This approach minimised complications introduced by osmotically driven water flow. Although attempts were made to sample randomly, samples were taken from cells that were angled such that they could easily admit the tip of the capillary.

## 2.9 Determination of osmotic pressure of whole tissue sap

Sap was extracted from whole tissue by freeze-thawing according to the method of Tomos *et al.* (1984). Leaf sections were sealed into 1.5 ml Eppendorf tubes (obtained from BDH, Poole, England) and plunged into liquid nitrogen to disrupt the cells. Sap was extracted by centrifuging the leaf material for 2 minutes at full speed (13000 g) in a microfuge. A small hole in the base of the Eppendorf allowed collection of the sap in another tube. Care was taken to avoid condensation and samples were measured for osmotic pressure within 30 min to minimise the effects of evaporation and enzymatic/bacterial hydrolysis. Osmotic pressure was measured using a Wescor 5100 B vapour pressure osmometer (Wescor, Logan, Utah 84321) calibrated against commercially available standards.

## 2.10 Determination of inorganic anion content of whole tissue sap

To measure anions, the above sap samples were first diluted 1 in 10 with 20% (v/v) propan-2-ol and then further diluted 1 in 17 with HPLC eluant (2 mM Na<sub>2</sub>CO<sub>3</sub>, 0.7 mM NaHCO<sub>3</sub> in 2% propan-2-ol). Inorganic anions were measured using a Dionex 2010i ion chromatograph (Dionex UK, Camberley, England) equipped with an HPLC-AS4A column and conductivity detector. These were calibrated against known concentrations of nitrate, chloride and phosphate (prepared as potassium salts).

## 2.11 Determination of the potassium and calcium content of whole tissue

Potassium and calcium were measured in acidic extracts of dried leaf material. Approximately 200 mg of fresh leaf tissue was weighed, oven dried at 80°C for 3 d and weighed again to obtain the leaf water and dry matter content. One ml of 0.1 M HNO<sub>3</sub> was added, and the samples were extracted by rotation for at least 4 d at room temperature. Subsamples of this extract were taken and diluted appropriately for measurement using an SP2900 atomic absorption spectrophotometer (Pye, Cambridge, England) in absorption mode, using an acetylene/air flame.

## 2.12 Preparation of tissue transverse sections for light microscopy

Leaf material (approximately 5 mm) was fixed in 3% (v/v) glutaraldehyde in 0.05 M sodium cacodylate buffer for approximately 3 d in an evacuated desiccator. The samples were dehydrated in a graded series of ethanol/water (25,50,75 and 100% v/v) over 2 d. Subsequently, the material was embedded in LKB historesin (Reichert-Jung, Heidelberger Str 17-19, D6907) according to the manufacturers instructions. (Plant material was rotated for 2 d in a 1:1 mixture of ethanol and LKB infiltration solution (50 ml basic resin plus 0.5 g activator). The solution was then exchanged for pure infiltration solution and rotated for a further 4 d after which the specimens were embedded in moulds (15 ml infiltration solution plus 1 ml of hardener)). Specimens were left for 1 week to fully polymerise and then 5 $\mu$ m sections were cut on a microtome, equipped with disposable blades. Sections were stained in toluidine blue to aid visibility of the cytoplasm.

## 2.13 Single cell analyses

The advantages of measuring solute and water relation parameters at single cell resolution have been emphasised in the introduction. For such measurements to be of potential use in describing the characteristics of single cells at single cell resolution, the errors inherent in the measurement system have to be quantified and either demonstrated to be of no importance to the conclusions made or be corrected for at the appropriate time. The experimental system used in collection of the data evolved over a period of three years and an outline set of instructions has recently been published (Tomos *et al.* 1994).

### 2.13.1 Design and organisation of the micro sampling system

The entire procedure of turgor and osmotic pressure measurement and subsequent micropipetting procedures were performed under a Wild M8 (Leica UK Ltd, Milton Keynes MK5 8LB) stereomicroscope equipped with 20X eyepieces and a 1.6X extender, and illuminated with a 150W cold light fibre optic source (Intralux

5000, Leica) running at the low power setting. The primary leaf of barley was held securely in a Perspex clamp with the minimum of pressure to avoid leaf damage. The clamp itself was mounted on a micromanipulator (Prior Scientific Instruments, Unit 4, Bishops Stortford, Herts) enabling precise three dimensional movement. The roots of the plants were bathed in fresh nutrient solution appropriate to the treatment. The container for the plant was mounted on the Perspex clamp such that movement of the plant on the micromanipulator caused a parallel movement of the plant container. This arrangement avoided strain on the roots and ensured that they remained covered by nutrient solution.

The picolitre osmometer (constructed at UCNW, Bangor) was a custom made device consisting essentially of a very accurate temperature controlled stage. It was set up on the same micromanipulator assembly (Prior) as the leaf holder, such that movement of the micromanipulator along one axis resulted in a change in field of view from the leaf to the osmometer stage. The capillary tip and microscope remained stationary during this period. The pressure probe was mounted on the top plate of a micromanipulator (Leica) enabling three dimensional movement with a tilt capability. A brass arm fastened to the top plate allowed for the clamping of glass microcapillaries for turgorless sampling and micropipetting. The capillary tip of the pressure probe was inserted into the target cell by appropriate control of the micromanipulator. Set up in this way it was possible to take a sample from a leaf cell and transfer it to beneath the surface of the liquid paraffin set upon the osmometer stage within 3.5 s.

### 2.13.2 Measurement of turgor pressure and collection of vacuolar sap

The turgor pressure of individual epidermal cells of barley was measured directly by means of a pressure probe (Hüsken *et al.* 1978) adapted with an instant sampling valve (Malone *et al.* 1989). This valve allowed for the rapid removal of vacuolar sap with the minimum of dilution. Penetration of the cell wall results in cell sap being forced into the capillary tip. The silicone oil/vacuolar sap boundary was visible as a clear meniscus. Using the motor-driven piston, the meniscus was driven back towards the tip of the capillary so that it came to rest as close as possible to the

cell wall and was only just visible. The pressure of the system at this point was recorded by the pressure transducer and taken to be the cell turgor pressure. During the measurement of this pressure, the motor driven piston was used intermittently to move the meniscus slightly about a fixed point. Observed movement of the meniscus under these circumstances proved that the tip was not blocked. Cells that showed a decline in measured pressure were considered to be leaking and were discarded. In general, cells that maintained a constant pressure for approximately 20 s were deemed non-leaky and suitable for sampling. Good pressure probe practice would normally entail holding a constant turgor pressure for somewhat longer (Hüsken *et al.* 1978) whilst undergoing pressure compressions and relaxations. The time saved enables the measurement of a greater number of cells, thus taking into account cellular and plant variation, and allowed for more time to be spent on subsequent osmotic pressure measurement and micropipetting. Furthermore, a long measurement period was not attempted for the majority of the pressure probe readings since the vibrations caused by the pressure probe used occasionally induced leakiness.

Following the measurement of turgor pressure a sample of sap was taken. To reduce the osmotically driven water flow during sampling the instant sampling valve was opened and the tip removed a fraction of a second later. This approach often yielded samples large enough for osmotic pressure measurement and for the subsequent elemental analysis of several subsamples.

A second method of extracting vacuolar sap was used when turgor pressure measurements were not required. A capillary tip mounted on a brass arm was backfilled with silicone oil and was rapidly inserted into a cell and immediately withdrawn in a 'stabbing' action. Generally larger samples could be taken by this method with the tip geometry optimised for this purpose only.

### 2.13.3 Measurement of vacuolar osmotic pressure

The osmotic pressure of extracted vacuolar sap was measured by observation of its melting point essentially as described by Malone *et al.* (1989) and Malone and Tomos (1992). Samples contained within the microcapillary tip were expelled onto the surface of a blackened and scratched coverslip set onto the osmometer stage. Good

thermal contact between the coverslip and the stage was maintained by the use of a zinc oxide paste (RS components, Birchington Road, Corby, Northants, U.K.). Evaporation of the samples was minimised by performing all depositions and measurements underneath a droplet of water-saturated liquid paraffin. The picolitre osmometer used was a purpose built device (UCNW Bangor) using a thermistor as the temperature sensor which offered a larger working platform on a smaller heatsink than the commercially available version (Clifton Technical Physics, Box 181, Hartford, New York). Condensation was prevented by minimal use of a stream of dry air provided by passing air from an aquarium pump over silica gel contained within a Dreschel bottle, and was only used when adding and removing samples. A guard coverslip overlying the top plate of the picolitre osmometer excluded water vapour whilst the samples were being frozen and their melting point assessed. The picolitre stage was calibrated on every measurement day and each time the glass stage was replaced by preparing a straight line calibration curve from a set of standards (0-921 mOsmolal NaCl) (see results). If the samples were to be further analysed, they were drawn into a "storage constriction pipette" between larger volumes of water-saturated liquid paraffin and stored at 4 °C in a sealed container at 100% humidity (provided by layers of wet tissue paper within the container). Storage was for a maximum of 5 h. Water loss during the various stages of sample manipulations was calculated from the increase in osmotic pressure over time.

#### 2.13.4 Capillary tip production

Microcapillaries were prepared on a commercial capillary puller (Harvard Apparatus, Sheerness, Kent ME12 1RZ) from 1 mm glass capillary tubing (Clark Electromedical Instruments). Exact dimensions were varied according to the heater and solenoid-puller settings and chosen by trial and error to give a glass tip of approximately 0.1  $\mu\text{m}$ . The extreme tip was removed by mounting the capillary in a De Fonbrune microforge (Alcatel, 98 Avenue de Brogny, 74009, Annecy Cedex, France) and brushing the tip against the platinum element.

The final tip and shank dimensions varied according to the function. For example, for turgor measurement the tips had an aperture of approximately 1  $\mu\text{m}$

diameter. These tips minimised leakage when turgor pressure was being measured. For turgorless sampling, tips were of 2-3  $\mu\text{m}$  diameter to reduce the impedance against rapid sap ingress and to allow the collection of relatively larger sap samples.

#### 2.13.5 Constriction pipette production

Constriction pipettes were produced from "turgorless sampling tips" by increasing their tip diameter to approximately 5 or 6  $\mu\text{m}$  by abrasion against the microforge heating element. A constriction was then made in the glass wall of the capillary by advancing the capillary wall towards the incandescent heating element of the microforge. An attempt was made to make the constriction equal to the tip diameter as these tended to be easier to use. Pipette sizes varied according to their function. Pipetting for subsampling for X-ray microanalysis was performed using pipettes of approximately 10-20  $\mu\text{l}$ , whilst for the osmotic pressure standard curve pipettes of approximately 40-50  $\mu\text{l}$  were manufactured. Short term storage pipettes were manufactured with a double constriction to minimise evaporation and were > 50  $\mu\text{l}$  in size between the constrictions.

Pipette tips were silanised to prevent spontaneous filling of pipettes by capillary action and to reduce the chances of protein adherence and tip blockage. After forging and prior to silanisation, pipette tips were baked for at least 3 h at 200°C in an oven. The pipettes were held upright in a drilled aluminium holder in a 600 ml beaker covered with aluminium foil. Silanisation was achieved by pipetting 50  $\mu\text{l}$  of dichlorodimethyl silane (Sigma) into the hot beaker via a hole cut into the foil. Following this addition, the samples were baked for a further 2 h to allow the silane to react with the glass surface. Pipettes were stored tip uppermost in this container until needed.

#### 2.13.6 Use of constriction pipettes

All pipettes were controlled via a pneumatic system. A 50 ml syringe was connected via a T piece to the constriction pipette using plastic tubing. The open T piece in the pressure line was connected to a length of soft silicone rubber tubing that



was open to the atmosphere yet could be closed by the action of a footswitch-controlled solenoid. The pipette was first backfilled with liquid paraffin and samples of identical volume were obtained by drawing samples up to a constant point in the narrow constriction, using the syringe with footswitch valve closed. (The accuracy of this procedure was often enhanced if visible marks were present on the constriction). Droplets were expelled whenever necessary by applying pressure via the syringe with the footswitch valve closed.

For X-ray microanalysis, constriction pipettes were used to allow the collection and deposition of vacuolar subsamples of identical volume. Although it was possible to calibrate pipettes absolutely the appropriate use of calibration-standards pipetted in the same manner and with the same pipette eliminated the need for precise knowledge of volume. In general, pipettes were chosen that allowed at least 3 subsamples of sap to be obtained although, if possible, six replicates of any one sample were taken. This replication allowed for the expression of results as the mean  $\pm$  standard deviation and gave some indication of the errors of measurement involved with pipetting and subsequent X-ray microanalysis. Within any one batch of pipettes used for any particular experiment it was attempted to keep pipette sizes as similar to each other as possible. This approach allowed for the use of replacement pipettes when blockages or breakages of the original occurred. Up to 12 samples could generally be drawn into the constriction pipette, separated from each other by equal volumes of water-saturated liquid paraffin and from the atmosphere by larger volumes ( $\sim 100$  x the volume).

Larger (and easier to use) constriction pipettes were used to deposit the standards used for calibration of the osmometer. Volumes were chosen to be approximately equal to the volumes of extracted vacuolar sap. Small deviations did not affect osmometer readings. (Results not shown).

The osmometer stage was used to cool the aluminium holder used for subsequent micropipetting. It was therefore necessary to store extracted vacuolar sap for short time periods (up to 3 h). For storage, samples were removed from the osmometer stage immediately after osmotic pressure measurement. Double constriction pipettes were used for storage, and after the backfill of liquid paraffin to isolate droplets from the atmosphere, a water "guard-droplet" was incorporated to further minimise water loss. The pipettes had a second constriction to minimise the cross

sectional area across which water might diffuse. Once samples were within the pipette they were labelled and transferred tip uppermost to a sealed container containing moist tissue paper and kept refrigerated at 4°C. Samples were generally stored for a maximum of approximately 3 h without subsequent osmotic pressure measurement.

#### 2.14 X-ray microanalysis of dried sap microdroplets

The method used was a modification of that used by Malone *et al.* (1991) in their analysis of the elemental content of epidermal vacuoles of wheat. The original method relied upon the comparison of dried equal volumes of samples vs standards. Its accuracy was dependent upon the similarity of circular lyophilised samples and standards in terms of their densities and cross sections. Since the freeze-drying facilities available in Bangor could not guarantee such reproducibility the method was modified to make geometric similarity of dried microdroplets less important. The method used was similar in concept to that of Hyatt and Marshall (1985) who incorporated cobalt as a normalising element into samples and standards. The actual method adopted entailed drying droplets of fixed volume onto a thin film support alongside standards. Incorporation of rubidium fluoride as an internal standard facilitated quantification utilising the integral of the rubidium  $L_{\alpha}/L_{\beta}$  peak to normalise the data.

##### 2.14.1 Production of the pioloform coated support grids

Pioloform coated grids were used for all X-ray microanalyses to afford the advantages offered by thin films (see Quinton 1979; Van Eekelen *et al.* 1980). In general the preparation relies on all equipment and reagents being scrupulously clean and dry. The pioloform solution used is twice as concentrated as that normally used for electron microscopy (Stockem, 1970). This increased concentration was found to significantly improve both the strength and ease of preparation of the grids without any obvious adverse effects upon the background radiation.

Folding copper electron microscope grids (100/ 200 mesh from Agar scientific Ltd, (cat No G234)) were washed by sonication in acetone (AR) and allowed to dry in an oven at 50° C. Meanwhile, glass microscope slides with ground edges (Agar Scientific Ltd) were precleaned in ethanol and covered in a layer of wet soap (Imperial Leather, Cussons, UK). The soap was rubbed into the surface of the slide with the fingers until completely dry. All traces of soap were removed with a dry chamois leather, and the slides stored in a drying cabinet at 50° C.

A 1% solution of pioloform (Agar Scientific Ltd) was prepared by allowing 1 g of pioloform to dissolve for 24 h in 1,2-dichloroethane. The solution was decanted into a glass separating funnel with parallel sides of sufficiently small diameter to hold the glass slide upright. The warm microscope slide was placed in the funnel and the pioloform solution was run out via the tap in approximately 3 s. The coated slide was allowed to dry *in situ* for 30 s and then removed and allowed to dry beneath an inverted beaker for 10 min.

Meanwhile, a crystallising dish (set upon black paper to aid visibility) was filled to a positive meniscus with distilled water. Surface dust was removed by sweeping with a glass rod. Using a razor blade, the coated slide was scored to release the pioloform coating from the slide. To aid release, the coating was breathed on, and the slide was submerged into the water at an angle of approximately 25°. The coating was visible as an iridescent film under oblique lighting and against the dark background. The slide was gently removed, leaving the film floating on the water.

The washed grids were coated by placing them dull side down onto the film (avoiding dirty and torn areas). In one movement, a piece of vellum slightly larger than the film area was used to scoop out the grids, the paper contacting the uppermost surface of the film first. The grids together with the paper were allowed to dry for several days at 60° C. They were dissected individually from the enclosing membrane using forceps, and stored individually in gelatin capsules until required.

#### 2.14.2 Pipetting of the samples and standards

Subsamples of sap of equal volume (approximately 10 pl) were pipetted onto a pioloform coated double copper electron microscope grid alongside a series of

standards of known concentration. Identical volumes of 200 mM rubidium fluoride (99.9% Sigma, Poole, England) prepared in 150 mM mannitol (Sigma) were immediately added to each droplet within 3 min of its deposition, using the same constriction pipette. Cross contamination was not accounted for but was unlikely to have been great because distilled water standards pipetted after the calibration standards had been pipetted were never observed to contain contaminants.

Deposition and pipetting of the samples was carried out under a 5 mm layer of water-saturated liquid paraffin held in a purpose-built aluminium container with transparent plastic sides. The container plus its contents were kept at a low temperature (approximately 4°C) by means of the osmometer stage. Excessive condensation was controlled either by switching off the osmometer or by minimal use of a stream of dry air. After all the samples and standards had been pipetted with their internal standard, the droplets were ready for drying.

#### 2.14.3 Drying of the samples and standards

The drying procedure was designed to yield a homogenous lyophilised non-crystalline sample of constant area and cross section. This approach obviates the need for subsequent corrections in quantification. Liquid paraffin was removed from the grid in two successive rinses of water saturated hexane at 4°C (10 ml for approx 10 s each), followed by a further rinse in liquid isopentane at 4°C (10 ml for approx 5 s).

Two approaches were used to dry the droplets. Initially, samples were freeze-dried. Following the isopentane rinse, the grid, held using the unused half was plunged rapidly into melting isopentane (mp -160°C). The grid was lowered into a precooled aluminium holder within the isopentane, which was transferred immediately to a Quickfit boiling tube containing liquid nitrogen. A vacuum was applied by means of a rotary pump, and the tube was lagged with tissue paper and allowed to rise to room temperature overnight. Shock freezing using isopentane was found to yield more consistent freeze dried samples than did liquid nitrogen alone.

The second method was to flash evaporate the samples. Following the isopentane rinse, the grid was flicked to remove excess isopentane and then held in

the airstream of a hair-dryer for 5 s (air temperature of 55 °C). This air-drying method was as successful as the freeze-drying method yet was far faster and required less skilled manipulation of the sample. It is the recommended method.

#### 2.14.4 X-ray microanalysis

After drying, the grid was secured to the carbon stub using double sided sticky tape such that the grid overlaid the carbon at a height of approximately 3 mm at an angle of 45° to the beam and detector. Electrical conductivity was ensured by earthing the grid to the stub with colloidal graphite (Aquadag, Agar Scientific Ltd). The samples were analysed in a Hitachi S520 scanning electron microscope in conjunction with a LINK QX2000 energy dispersive X-ray analyser equipped with a beryllium window. The magnification chosen was approximately 1200X, the acceleration voltage was 14 kV, and the condensor lens was set to 4. The dried sample was scanned with an electron raster just large enough to encompass the entire volume. Under normal conditions count rates of approximately 1000 counts per second were achieved, and the spectrum was collected for 100 'live' seconds (10% deadtime).

#### 2.14.5 Interpretation of the results

Peak integrals were obtained for the peaks of interest (Rb.  $L_{\alpha}/L_{\beta}$ , K.  $K_{\alpha}$ , Cl.  $K_{\alpha}$  and Ca  $K_{\alpha}/K_{\beta}$ ), and the count rates were normalised with respect to the peak integral of the rubidium. The interpretation of the results for calcium are complicated by the overlap of the potassium  $K_{\alpha}$  peak. Since the ratio of the peaks in an element are a constant, 10% of the potassium  $K_{\alpha}$  peak integral was subtracted from the calcium  $K_{\alpha}$  peak reading to take this into account. (This value was obtained from the literature and confirmed empirically by confirming the ratio on a pure potassium salt). The window for calcium was expanded slightly in its energy range from 3.47-3.83 keV to ensure that all potassium  $K_{\alpha}$  signals were collected. The success of the calibration method for all elements was confirmed in each experiment by the linearity of the calibration curves obtained, and by the incorporation of additional internal standards (see results).

#### 2.14.6 Choice of standard solutions and their concentrations

Calibration solutions were prepared to reflect the range of concentrations expected in the plant vacuole. The potassium and chloride concentrations ranged from 0 to 300 mM whilst the calcium and nitrate concentrations ranged from 0 to 200 mM. The composition of the standards was such that when the potassium concentration was high, the calcium concentration was low and *vice versa*. This had two functions. Firstly, it mirrored the concentration trends that plant vacuolar sap was found to display, and was hence more representative of the sap being measured. Secondly, it provided a test of the efficacy of the method used for accounting for the overlap of the calcium  $K_{\alpha}$  and potassium  $K_{\alpha}$  peaks.

The rubidium concentration was chosen to be comparable in size with the major peak of interest. For analyses of the high potassium treatment, the concentration was 200-250 mM  $Rb^{+}$  whilst 100-150 mM  $Rb^{+}$  was used for analysis of the low potassium treatment.

The rubidium solution was prepared in 150 mM mannitol. The mannitol was incorporated to swamp the effect of organic solutes that may have been present in the

sap sample, and was a precaution against mass loss (see Section 1.6). It also ensured good mixing of the dried sample because the mannitol boiled during beam scanning. Straight line calibration curves were prepared from the potassium, chloride and calcium standards (Fig 3.4) from which the concentrations of elements in sap samples were calculated.

## 2.15 Preparation of leaf tissue for X-ray microanalysis of frozen-hydrated specimens

Other quantitative approaches have been made to analyse elements at single cell resolution (see Section 1.6). Clearly, all valid measurements should yield similar quantitative results after analysis of plants grown under similar conditions. The method used for comparison purposes was one which had been used previously for the quantitative analysis of elements in root tissue of 3 grass species where the technique of X-ray microanalysis had been applied to frozen etched tissue, prepared alongside frozen etched standards (Koyro and Stelzer 1988). *This work was performed at the Botanisches Institut, Tierärztliche Hochschule Hannover, D3000 Hannover 71, FDR. in collaboration with Dr H-W. Koyro.*

### 2.15.1 Tissue preparation

Thin strips of leaf tissue of approximately 2-3 mm were cut transversely from the primary leaf of barley approximately 4 cm from the leaf tip, using a razor blade. (This position was chosen in order to closely coincide with the region used for single-cell sampling). The resultant section was then cut longitudinally to yield a section of tissue containing the region of interest approximately 1-2 mm wide.

Immediately after cutting, the tissue was transferred to a 0.75 mm hole drilled into a threaded precleaned<sup>2</sup> copper block. Good thermal contact between the leaf

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<sup>2</sup> Cleaning was performed by polishing the copper face of the block with a commercial metal cleaning polish. This was removed initially with a dry cloth followed by subsequent sonication in 3 rinses of distilled water and one in ethanol. The blocks were then oven dried at 50°C and sputter coated with gold.

tissue and the copper was ensured by allowing a drop of weak detergent solution to flow into the hole and about the tissue by capillary action.

Following the mounting of the leaf a range of (usually) four multiple standards of approximately 1  $\mu$ l each were pipetted onto the copper block such that they encircled the leaf. Using forceps, the block was plunged sample side down into liquid nitrogen where it was stored until required.

### 2.15.2 Tissue planing and etching

Analyses were performed on the freezing stage of an E.T.E.C. Corporation autoscan scanning electron microscope equipped with a Tracor Northern TN2000 X-ray microanalytical facility based on a Kevex detector with beryllium window. The leaf tissue was rapidly (within 10 s) transferred to the electron microscope stage with a clamping tool. Both were precooled in liquid nitrogen, good thermal contact being maintained between the copper block and the holder by screwing the block firmly onto the stage.

Transverse fracturing of the leaf tissue was achieved manually using a razor blade precooled in liquid nitrogen, mounted within the vacuum chamber of the microscope. Sectioning was performed until approximately 0.5-0.75 mm of leaf tissue of flat surface was left protruding from the copper block surface (Plate 2.3). Etching improves the intensity of the signal peaks and visibility of the cells in the tissue. Etching of both standards and samples was performed within the chamber of the electron microscope by means of a substage radiant heater constructed from an electron microscope filament. Heating was applied whilst monitoring the process and removed when cell detail became apparent and a slight feathering appeared over the surface of the fractured cell (Plate 10.1). Etching started at a temperature of approximately  $-110^{\circ}\text{C}$ , and the whole process generally took 220-260 s under a vacuum of  $1-2 \times 10^{-6}$  torr (1 torr = 133.3 Pa = 1 mm Hg).

X-ray microanalysis was performed under an accelerating voltage of 10 kV, with an emission current of 175  $\mu\text{A}$  (condensor set to 278) for 15 s. The channels monitored were from 0-5 keV. In common with the single cell approach, the electron



raster was operated in reduced area mode, such that an area was scanned within the region of interest.

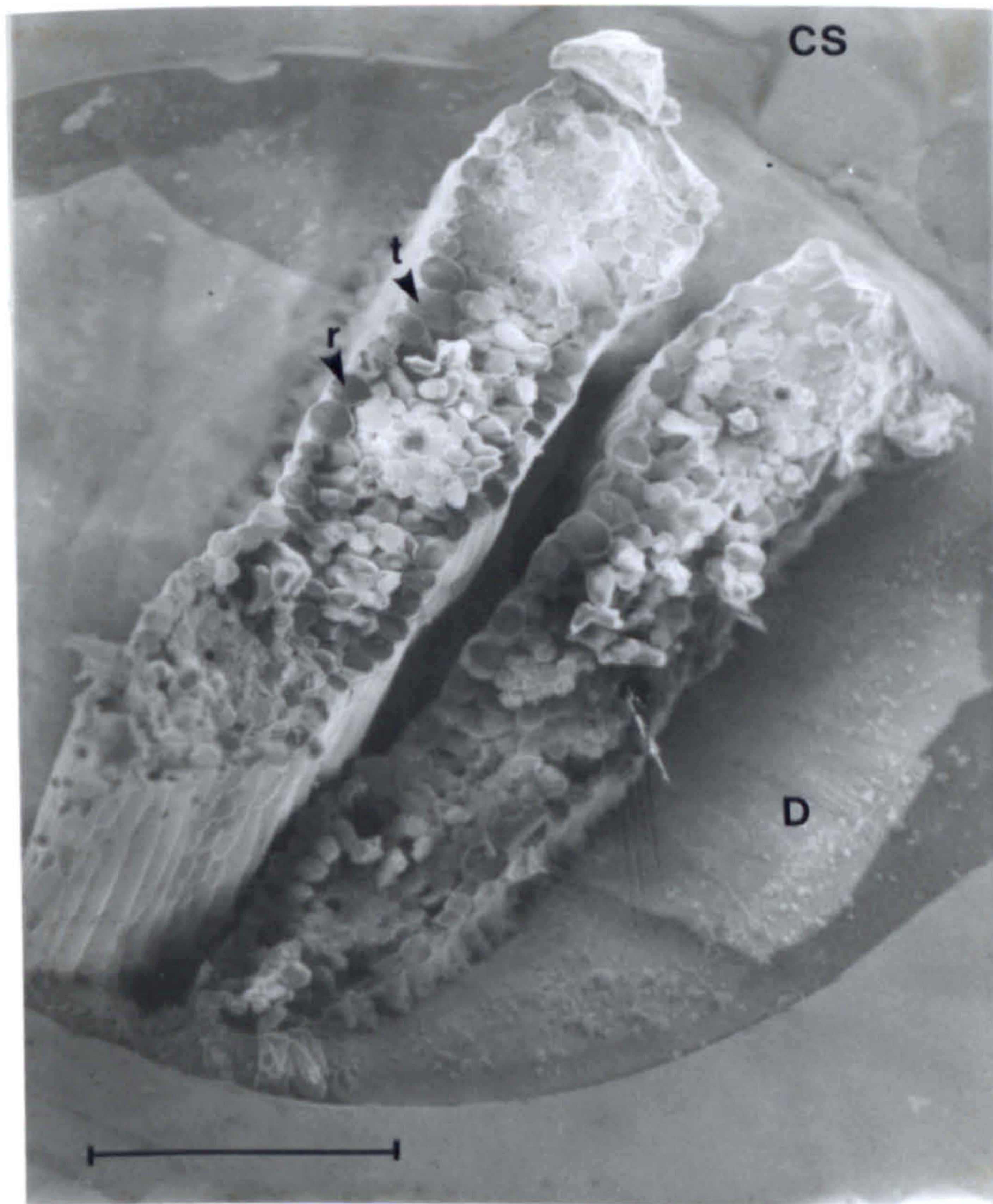


Plate 2.3 Fractured leaf tissue mounted within copper stub for etching and subsequent X-ray microanalysis. CS shows the copper stub, D is the frozen detergent solution used for mounting the tissue. Calibration standards were laid upon the copper surface surrounding the tissue and are not visible in this micrograph. Arrows indicate the epidermal cells (t = trough cells, r = ridge cells). Scale bar is approximately 0.25 mm.

### 2.15.3 Calculation and interpretation of data

Uncertainties of the geometry of the tissue within the system caused by unpredictability of the sectioning process meant that in certain instances, generated X-rays could be intercepted before reaching the detector (e.g. by projecting cut cell wall

material). Such uncertainties generate data that is impossible to correct for hence spectra having atypical<sup>3</sup> brehmsstrahlung signatures were discarded.

All data were expressed in terms of peak/background ratios, and concentrations were obtained from calibration curves prepared for each individual sample by fitting a straight line to a peak/ background ratios vs concentration plot. The interference of the potassium signal to the calcium peak was accounted for by subtracting 10% of the potassium peak integral as described previously.

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<sup>3</sup> The bremsstrahlung radiation originating from a specimen has a typical spectrum (Morgan 1985 p. 11). In general, the spectra obtained in this study reflected this typical spectrum. Any spectra that appeared to deviate from this were regarded as 'atypical'.

## Chapter 3. Characteristics of the microanalytical methods.

### 3.1 The validity of the microanalytical methods

In order to establish that the methods used for microanalysis were valid, a number of control experiments were performed. Essentially, it was necessary to account for the likely errors so that experimental variation could be distinguished from physiological variation. The following section addresses the problems associated with single cell analyses.

#### 3.1.1 The $t^{1/2}$ of water influx: Is it possible that significant dilution of the vacuolar sap occurs at the instant of sample extraction?

Penetration of a turgid cell by a capillary open to atmospheric pressure results in a pressure-driven flow of cell sap into the capillary. Loss of cell volume at this instant leads to an immediate reduction in turgor pressure and thereby lowers the water potential of the cell. The water potential imbalance that occurs re-equilibrates rapidly as water flows into the cell through the plasmalemma. The rate of this re-equilibration can be determined experimentally using the pressure probe. Turgor pressure is artificially reduced by increasing the volume of the pressure probe using the motor driven piston. Assuming instantaneous response of the pressure probe, the time it takes for the turgor to reach a new steady state is a measure of the rate of water influx for that cell under the prevailing conditions (Steudle and Zimmermann 1974). Water influx is commonly expressed as a half time ( $t^{1/2}$ ) (Dainty 1976), and can lead to sample dilution if the extraction procedure is slow. The  $t^{1/2}$  depends upon a number of variables including the cell volume (and its surface/ volume ratio), the cell elastic modulus and hydraulic conductivity. The actual degree of dilution may be further influenced by other factors including cell turgor (which will influence the rate of sample extraction) and the water capacity of the surroundings.

Typical values of the  $t^{1/2}$  for water influx in epidermal cells are shown in Fig 3.1. Turgor equilibration was generally complete within the response time of the

pressure probe, and was possibly faster than 0.5 s. Such rapid water influx is an explanation of those instances where turgor pressure was measured to be higher than osmotic pressure (cf Malone *et al.* 1989). Although no attempts were made to measure the rapidity of sample extraction it was estimated to be possibly faster than 0.25 s. Dilution under these circumstances shows that the  $t_{1/2}$  for water influx into barley epidermal cells is very rapid. Uncertainties regarding the extent of this dilution will reduce the value of the data and underestimate the real osmotic pressure, but these errors can be estimated. Assuming a vacuolar volume of 1000 pl, and an extracted sap volume of 100 pl, the maximum degree of dilution would be 10%. This worst case estimation is likely to be an overestimate, since the  $t_{1/2}$  values obtained using the pressure-probe are determined from movements of only small volumes of vacuolar sap when the water capacity of the surroundings may not be rate limiting. The observation that no great variation was obtained between samples obtained by stabbing, and those obtained using the instant sampler valve would support this hypothesis since the latter method was judged to be slower<sup>4</sup>. The similarity of osmotic pressure in these cases suggest that the initially rapid water movement may not be sustained.

It is concluded that although barley leaf epidermal cells are susceptible to sample dilution during extraction, that the errors are likely to be small. It was estimated visually that the volumes of sap extracted from the cell were less than 5% of the total cell volume in any case, hence errors due to dilution would be at maximum 5%. Nevertheless, extraction of all samples were performed as rapidly as possible to minimise osmosis-derived dilution.

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<sup>4</sup> Use of the instant sampling device of Malone *et al.* (1989) involves removing the capillary tip directly after switching off the sampling solenoid. In practice, since the valve was occasionally 'sticky', the tip was removed only after the sample was observed moving rapidly into the capillary. The 'stabbing' approach does not rely on visible confirmation of sample movement and occasionally yielded smaller samples consistent with a greater speed of tip removal.

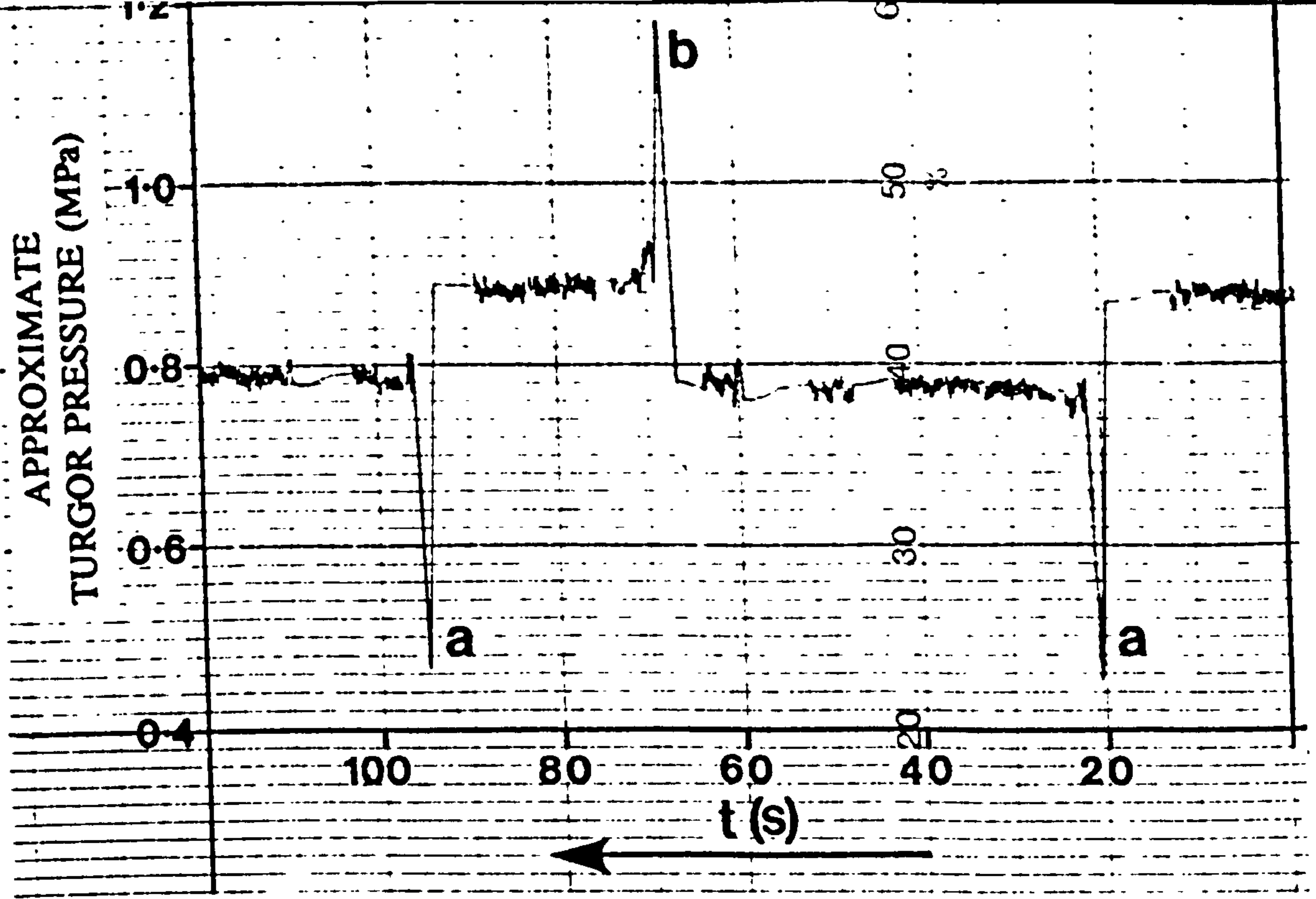


Fig 3.1 Typical response of epidermal cell of barley to an imposed sudden decrease (a) and increase (b) of stationary turgor pressure. The time taken to reestablish a constant turgor is a function of the rate of water flow into the cell. Here halftime values demonstrate that in barley, this reequilibration of water potential is fast and possibly faster than the response time of the pressure probe (see text).

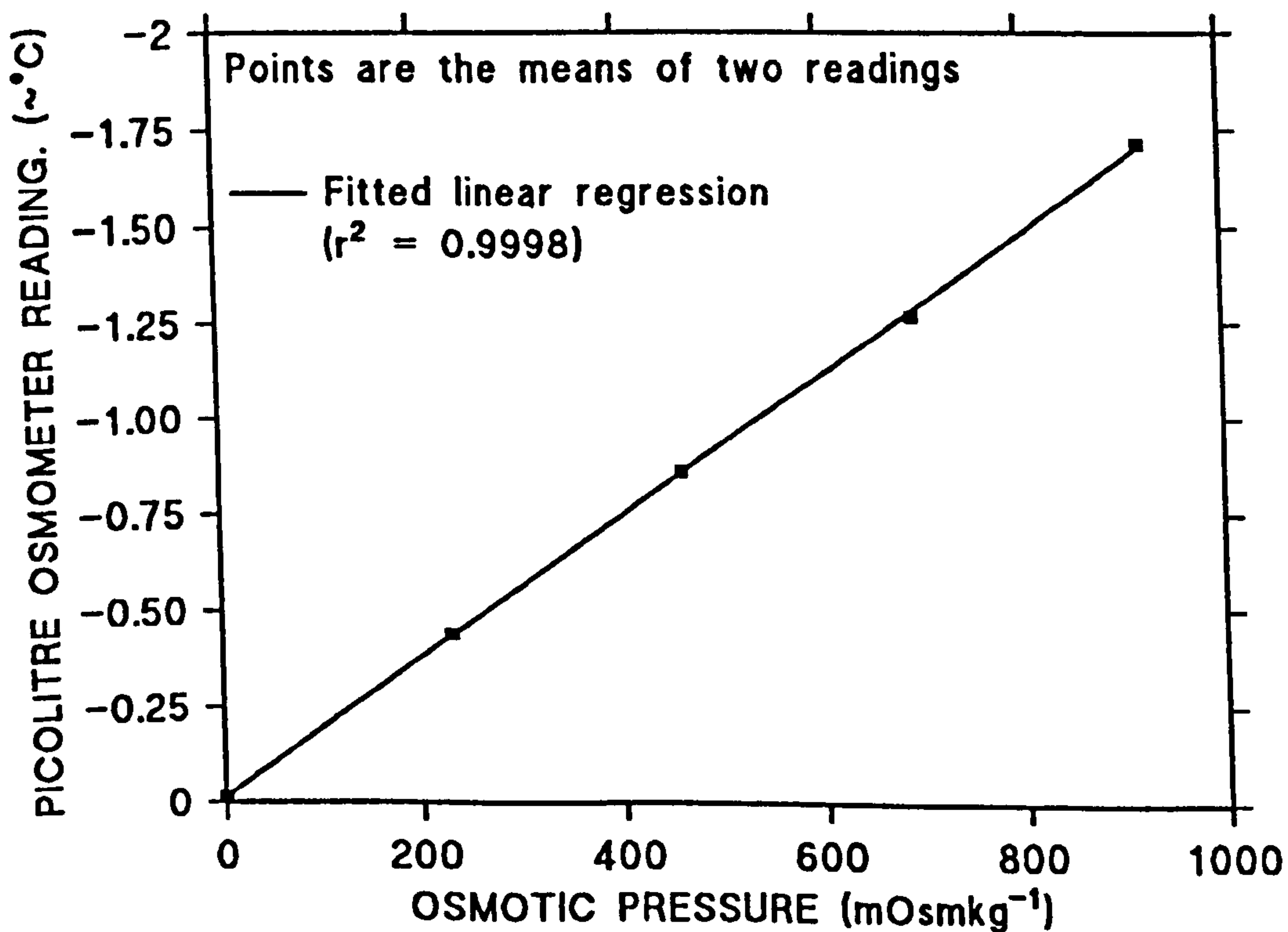


Fig 3.2 Typical calibration curve derived from the purpose built osmometer (see materials and methods)

### 3.1.2 Calibration and linearity of the osmometer

All of the osmotic pressure measurements performed in Bangor were made on a purpose-built osmometer (see materials and methods). The osmometer routinely gave straight line calibration curves with linear regression values of 0.999 (see Fig 3.2). In instances when the calibration curve gave a linear regression of less than 0.99 the stage was re-cleaned and new standards prepared. The resolution of the device was sufficient to give osmotic pressure readings accurate to within 10 mOsmoles/Kg.

### 3.1.3 Water loss from the osmometer stage

To establish the degree of water loss from the samples during handling, osmotic pressure was remeasured in the calibration standards at various intervals after deposition (Fig 3.3). During the first 45 minutes, the stage was subjected to a continuous stream of dry air. Thereafter, dry air was passed over the samples at the times of osmotic pressure measurements as a precaution against condensation whilst the droplets were being frozen. The increase in osmotic pressure after 250 minutes of storage on the osmometer stage and using extreme drying conditions was at maximum 3% (cf Malone and Tomos 1992). Thus evaporation during short term storage in the order of 10 minutes is negligible.

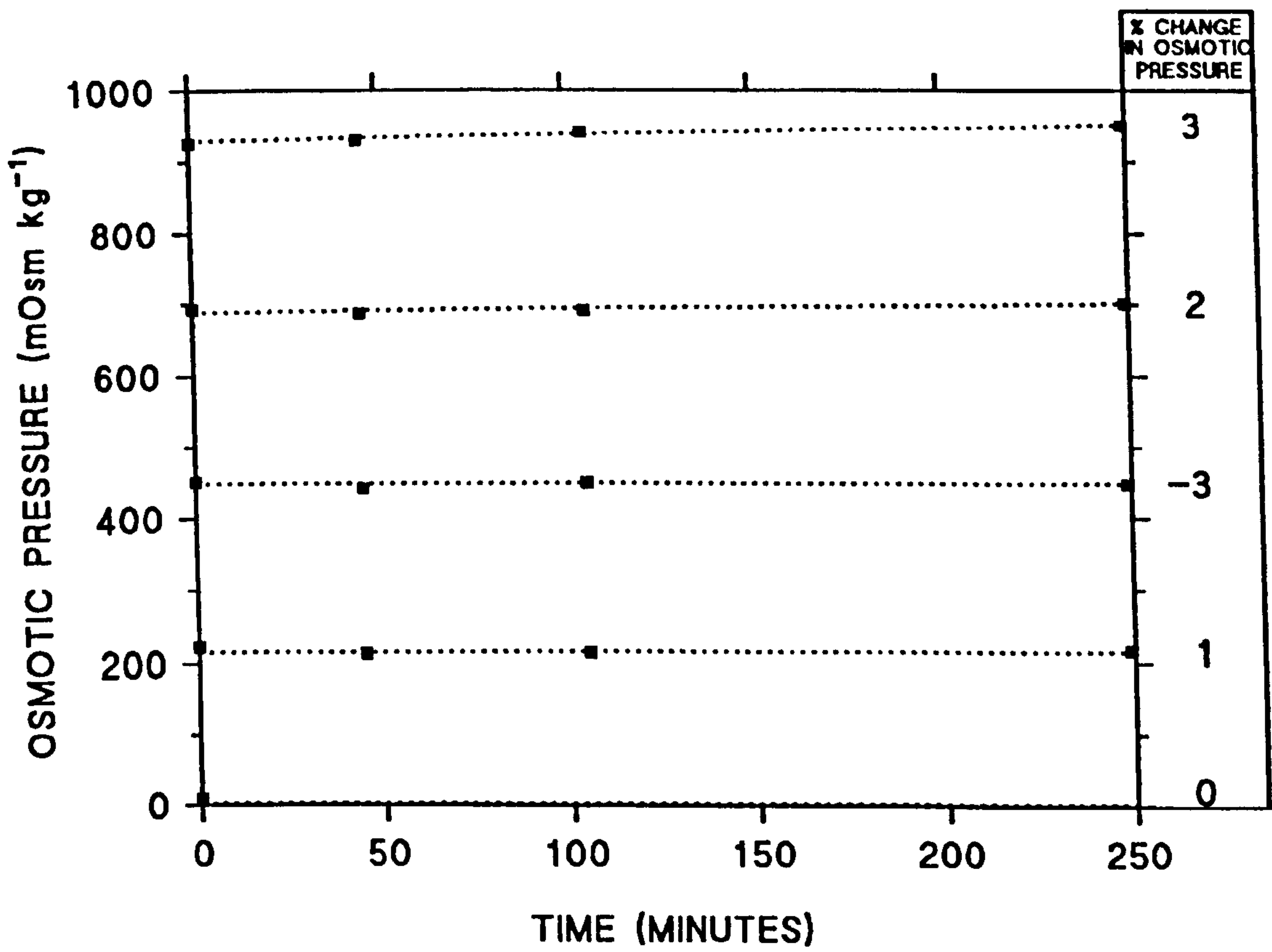


Fig 3.3 Assessment of the extent of water loss from typical samples during normal operation of the osmometer stage. Results shown are the mean of two samples. In general samples were only stored on the osmometer stage for a maximum of 15 minutes.

### 3.1.4 Water loss from "storage" constriction pipettes

Since the osmometer stage was required for the cooling of the aluminium holder during the latter stages of sample preparation, it was necessary to store the vacuolar samples for periods of up to 5 h. The change in osmotic pressure after storage of samples under various conditions is shown in Table 3.1.

ORIGINAL OSMOTIC PRESSURE (mOsmkg <sup>-1</sup> )	OSMOTIC PRESSURE AFTER STORAGE FOR 14 DAYS IN REFRIGERATOR	OSMOTIC PRESSURE AFTER STORAGE ON SHELF OVERNIGHT	OSMOTIC PRESSURE AFTER STORAGE ON SHELF FOR 12 DAYS
0	**	-12,-16	**
230.25	**	218,220	266
460.5	455,442/ 437,424.	437,441	437,394
690.75	**	668,663	628
921	**	924,920	937,888

Table 3.1 Assessment of the degree of evaporation of samples stored under various storage conditions (see text) as monitored by changes in their osmotic pressure.

The osmotic pressure of some samples changed significantly over periods of several hours although most remained fairly constant. The decrease in osmotic pressure observed in certain instances was thought to be possibly due to the presence of condensation. Since the variation of osmotic pressure after storage was not 100% predictable, samples were stored for as short a period as possible before being pipetted. (Generally, long term storage was avoided, yet storage of samples over a one week period was necessary for nitrate measurement. In these instances, the change in osmotic pressure was accounted for by remeasuring the osmotic pressure of the samples after storage, and by back correcting the concentrations obtained).



### 3.1.5 The validity of X-ray microanalysis for quantification

#### 3.1.5.1 Linearity of the X-ray calibration curves

The repeatable linearity of a calibration curve derived from mixed standards (Fig 3.4) suggests that the internal rubidium fluoride standard is a reliable aid to quantification. It would also suggest that some of the problems associated with X-ray microanalysis (ZAF) of thick specimens are not applicable to the thin samples analysed here (see Section 1.6).

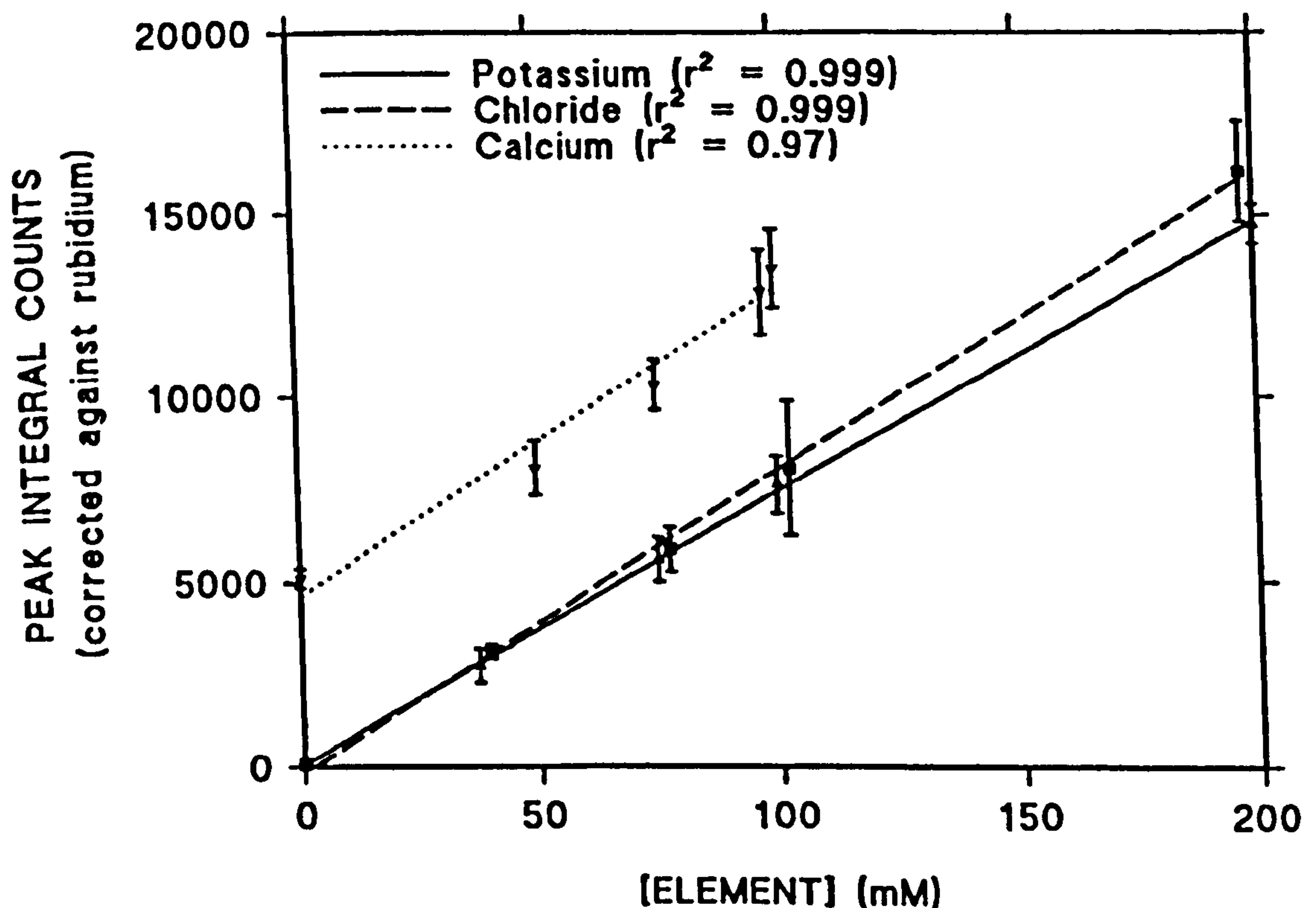


Fig 3.4 Typical calibration curve for quantitative analysis of potassium, calcium and chloride in dried microdroplets (approximately 10-20 pl). Data show the mean and SD of (usually) 6 subsamples. The curve for calcium has been offset from zero for clarity.

The linearity was maintained even in the calibration curve for calcium. This curve was constructed from standards prepared so as to represent concentration trends developed over time in real extracted sap and so had to quantitatively account for the presence of a variable interfering potassium  $K_{\alpha}$  peak. The larger error bars associated

with the calcium are a result of the additive errors incurred whenever potassium has to be accounted for. The value of the potassium  $K_a$  peak as a percentage of the potassium  $K_w$  peak was determined empirically by measurement of a pure potassium standard and found to be  $11.7 \pm 1.4\%$ . (data not shown). This value is close to other published values (e.g. 10%, Koyro and Stelzer 1988).

### *3.1.5.2 Are the standards representative of the samples?*

Further confirmation that the technique was reliable was provided by exploiting the technique of micropipetting to add standards ('spikes') of known concentration to sap samples to demonstrate that the increase obtained was that predicted. Pooled epidermal sap from a high potassium treatment plant was pipetted onto the grid, and 50 mM KCl/Ca(NO<sub>3</sub>)<sub>2</sub> or water added using the same micropipette. This experiment acted both as a test for internal interfering components and as a test of pipetting. The experiment was repeated on two separate occasions (Table 3.2).

SAMPLER (n)	[Cl](mM) ±SD	[K](mM) ±SD	[Ca](mM) ±SD
Epidermis (5)	0 ± 1	288 ± 32	35 ± 16.4
Epidermis (5) + water	1 ± 0.2	308 ± 24	32 ± 6.9
Epidermis (4) +50 mM Standard	55 ± 8.4	363 ± 45	82 ± 16.3

SAMPLE (n)	[Cl](mM) ±SD	[K](mM) ±SD	[Ca](mM) ±SD
Epidermis (8)	91 ± 13	252 ± 21	58 ± 7
Epidermis (8) + water	83 ± 2	254 ± 13	60 ± 11
Epidermis (8) +50 mM Standard	136 ± 6	300 ± 24	111 ± 13

**Table 3.2 a,b.** Demonstration of the predicted quantitative increases upon adding standard solutions to vacuolar sap on two separate occasions.

All samples increased to the extent predicted, upon addition of the "spike". These results in tandem with the linearity of the calibration curves throughout the collected experiments (Fig 3.4.) were accepted as proof that the system was quantitative, repeatable and reliable.

### ***3.1.5.3 Does irradiation under the electron beam lead to selective loss of elements?***

Bombardment of samples by an electron beam in the high vacuum of the scanning electron microscope can lead to the loss of light organic matrix components and a proportion of the heavier detectable elements. Such mass loss is a function of the delivered electron dose and manifests itself immediately in uncooled specimens (see Morgan (1985) and references therein). It was important to exclude the possibility of mass loss affecting the system in those instances where a low count rate led to longer counting times. If mass loss was proportional for all elements, then the system would be immune from change, as concentration predictions are made from relative peak sizes. Chloride is susceptible to sublimation (LeRoy and Roinel 1983) and since the susceptibility of rubidium fluoride to mass loss was unknown the extent of mass loss in the measurement system used was assessed. Samples were exposed to the electron beam for 600 seconds under standard measurement conditions. Peak integrals were taken after every 100 seconds livetime and the system reset between subsequent measurements. The results shown are the means of 5 separate samples in which elemental change was expressed as a percentage difference from the initial reading obtained after a 100 second scan (Fig 3.5).

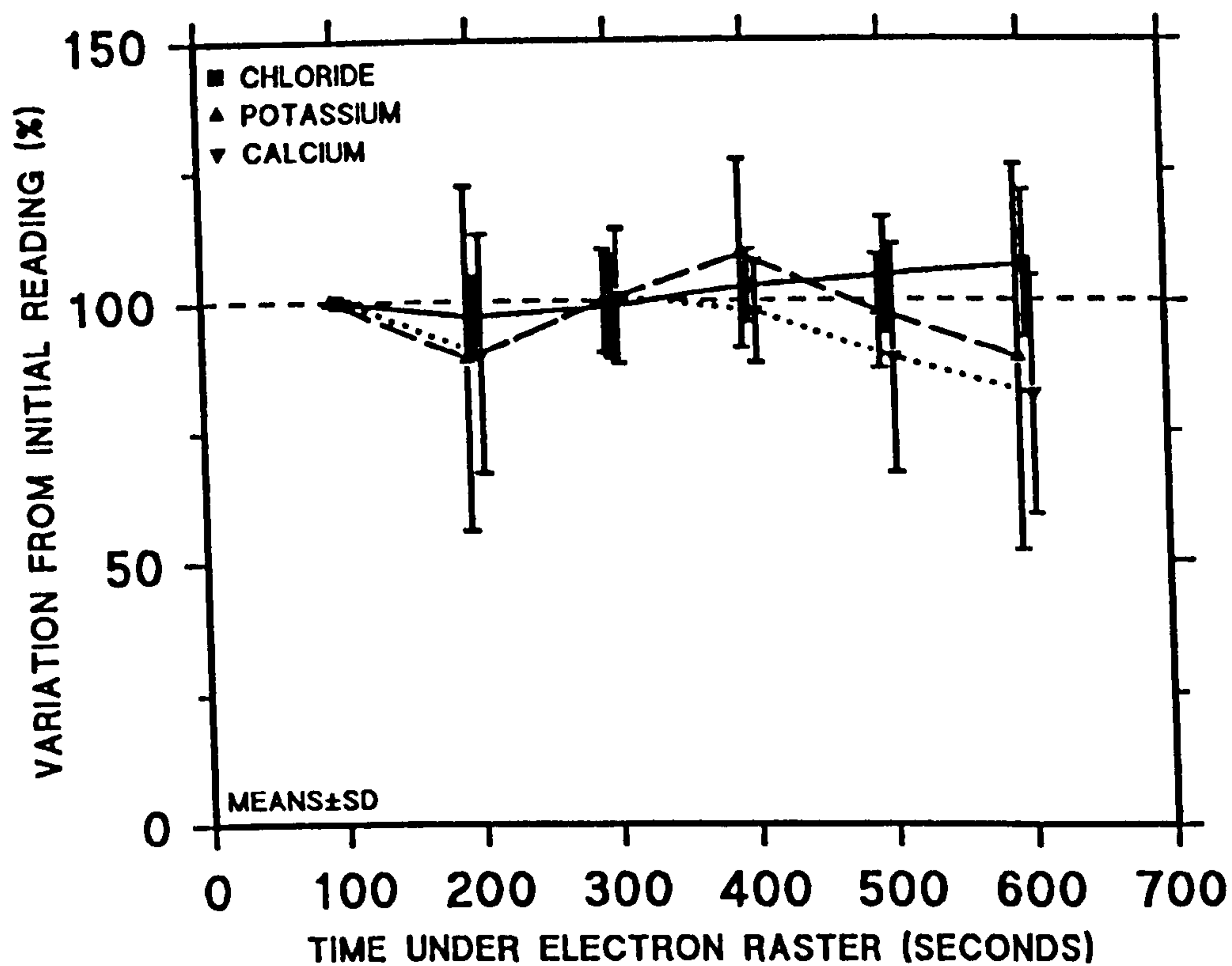


Fig 3.5 Stability of samples to irradiation by the electron beam. Samples were irradiated for 6 successive periods of 100 seconds under typical analysis conditions (12000X magnification at 14kV). The points represent the means and standard deviations of 3 samples differing widely in composition.

There is no trend indicative of mass loss of any of the elements (including rubidium) over the first 400 seconds. Since the samples were only read for 100 seconds, the problem of mass loss was deemed unimportant in the measurement system used. These results are in accordance with those of Lechene (1974) who using the dried microdroplet technique on a beryllium support demonstrated that such losses were within the statistical fluctuation of counting.

### 3.2 How well do the osmotic pressure and X-ray techniques integrate?

In combination, quantitative measurements of osmotic pressure and its elemental composition allows for accurate estimation of the water relation properties of extracted vacuolar sap. Accuracy is assisted by the straight line relationship of both techniques, which makes curve fitting more reliable. It is difficult to give precise estimations of the range of errors encountered since they are dependent upon sample

size, sample composition, and concentration. For instance, large samples aid ice crystal visibility for osmotic pressure measurement and allow for the multiple sub-sampling for X-ray microanalysis. Accurate sub-sampling itself is dependent upon both individual micropipette properties and sample volume. For X-ray microanalysis in particular, accuracy may be improved by increasing the counting duration, and by choosing a rubidium concentration comparable with the elemental concentration expected.

Although pipetting errors have not been strictly quantified, the general accuracy of the technique is indicated by the linearity and error bars of the standard curves. These also include statistical counting errors, but for elemental quantification at least give a more valid estimation as to the accuracy of the technique.

The approaches taken in sample manipulation were designed to minimise the effects of water loss (and water gain) from the instant when the sample is taken until analysis. It is not possible to accurately account for the possible dilution that occurs at the instant of sample extraction as a result of osmosis driven water flow. Neither is it possible to confirm categorically that the sample obtained is purely vacuolar in origin (see discussion).

A real measure of the efficacy of the system as a whole is demonstrable if repeatable conclusions can be drawn from measurements performed on separate occasions. Since the plants were grown under relatively uncontrolled conditions, absolute repeatability is not necessarily expected. The solute relations tended to follow repeatable trends and osmotic pressure tended towards constancy. Together these strengthen the argument that the quantification approaches adopted are legitimate.

### 3.3 Conclusion

Accurate measurements can be made of elemental contents and osmotic pressure after extraction. Accuracy of X-ray quantification in terms of solubilities should not be automatically assumed since X-ray microanalysis is unable to distinguish between different chemical states of elements. Nevertheless, the techniques provide a useful starting point by which the solute and water relations of the cells may be considered.

## **Chapter 4. Cellular heterogeneity in the first leaf of barley**

When using a technique capable of analysing solutes at single cell resolution, it is of value to know the variation in solutes throughout the whole leaf. Differences in concentrations across whole leaf tissue may however reflect either differences between individual cells, or different ratios of tissue. Knowledge of variation at this whole tissue level allows the results for single cells to be viewed in the context of the whole leaf and also for comparisons of these data with the literature.

Similarly, it is important to be able to distinguish real differences between cells. Large variations that might exist between individual cells would increase the apparent scatter of the data. Since the barley epidermis is composed of cells distinct in both their morphology and position relative to the vascular tissue, it provides an ideal model system to test whether heterogeneity exists between adjacent cells of a single tissue.

### **4.1 Gradients of ions and osmotic pressure along the leaf blade of barley**

Leaves of barley were excised 7 days after being transferred to nutrient solution (approximately 13 days from germination) at which point they were roughly midway through their development. Their major anions and cations were measured in bulk sap extracted from various distances along the leaf blade. Gradients were observed in the concentrations of all the ions measured. Potassium, chloride and nitrate increased in concentration towards the leaf ligules whilst the calcium concentration tended to decrease. The phosphate concentration increased initially then declined at the leaf base. (Fig 4.1 a-d).

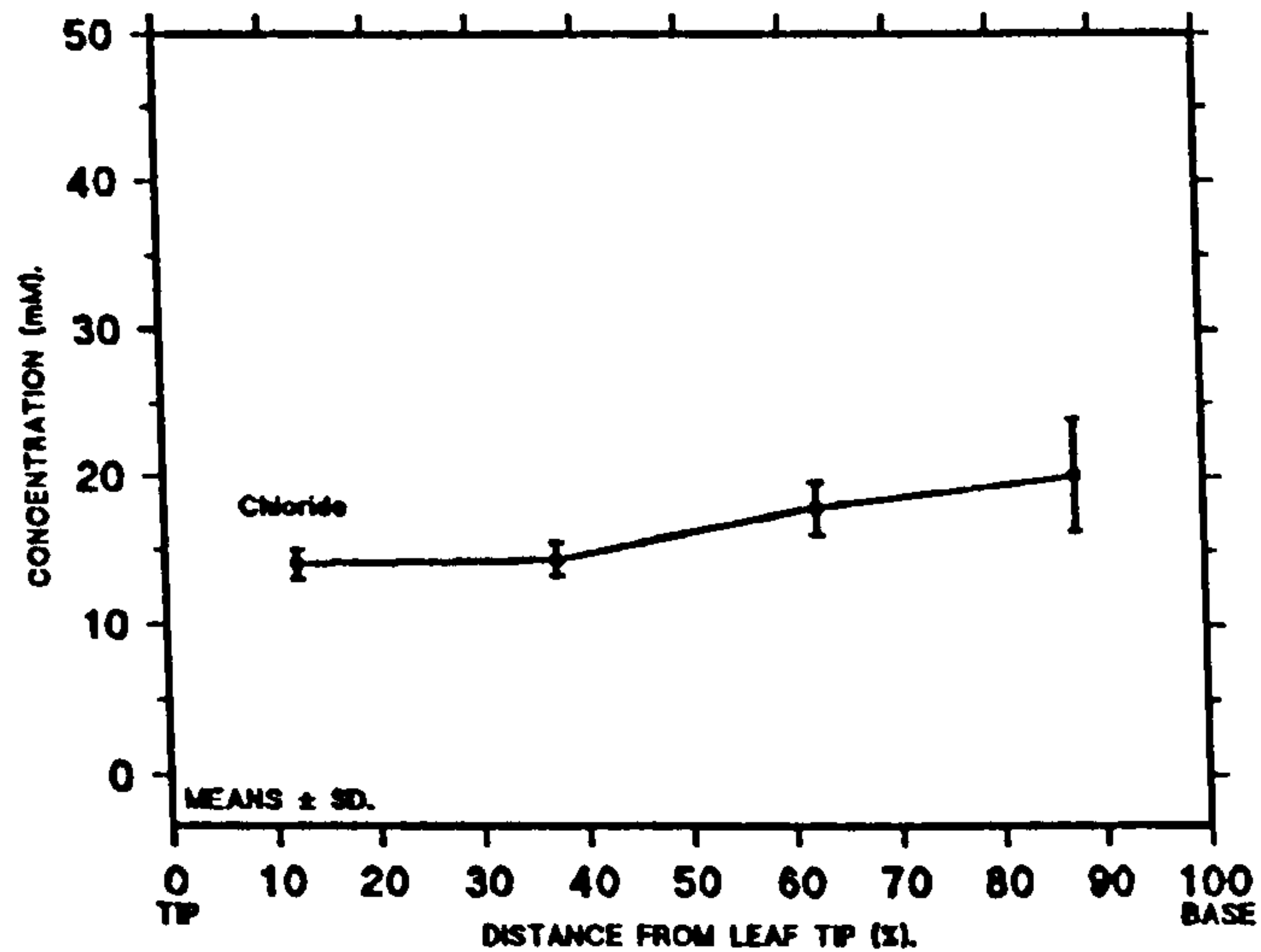
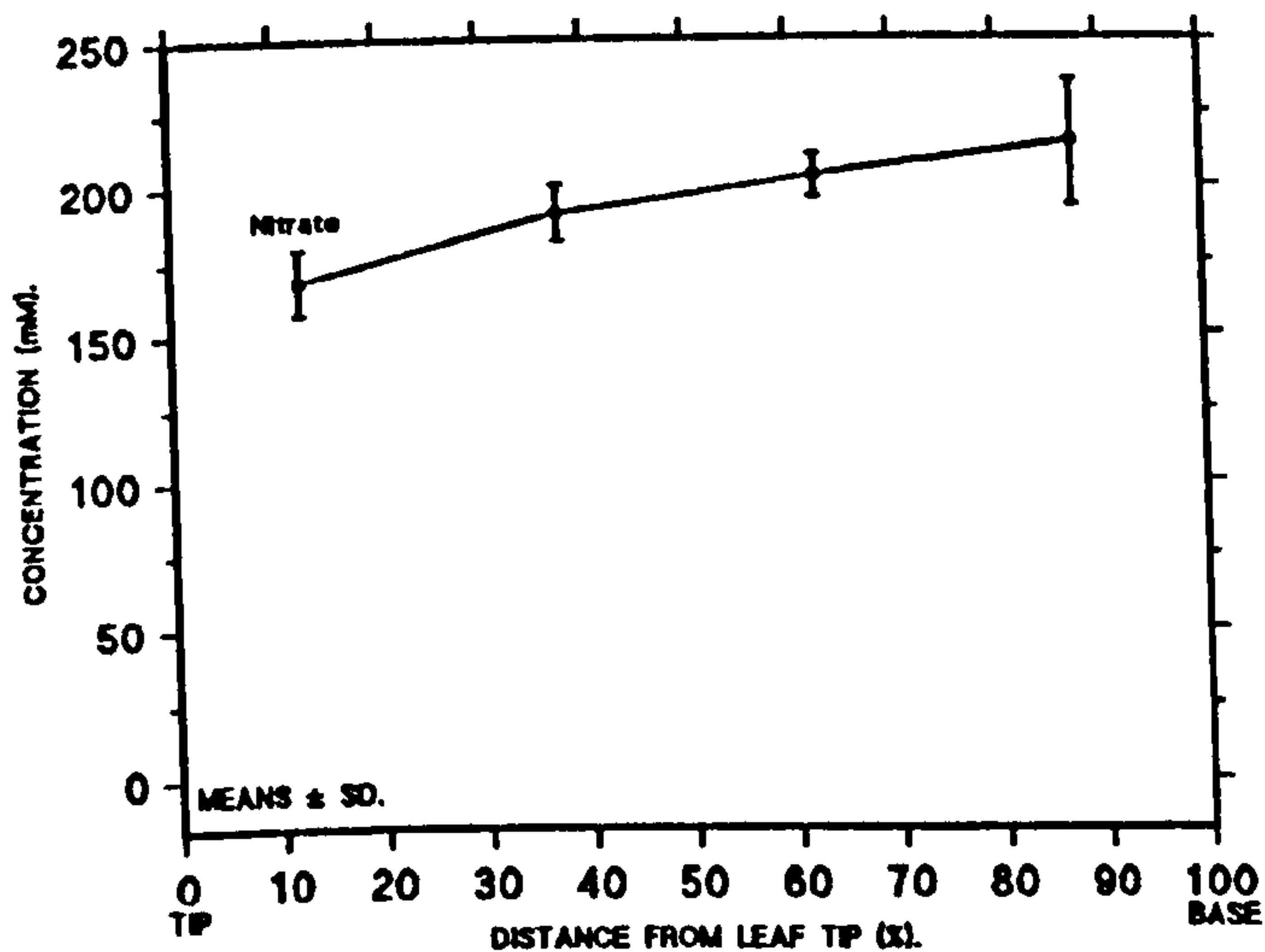
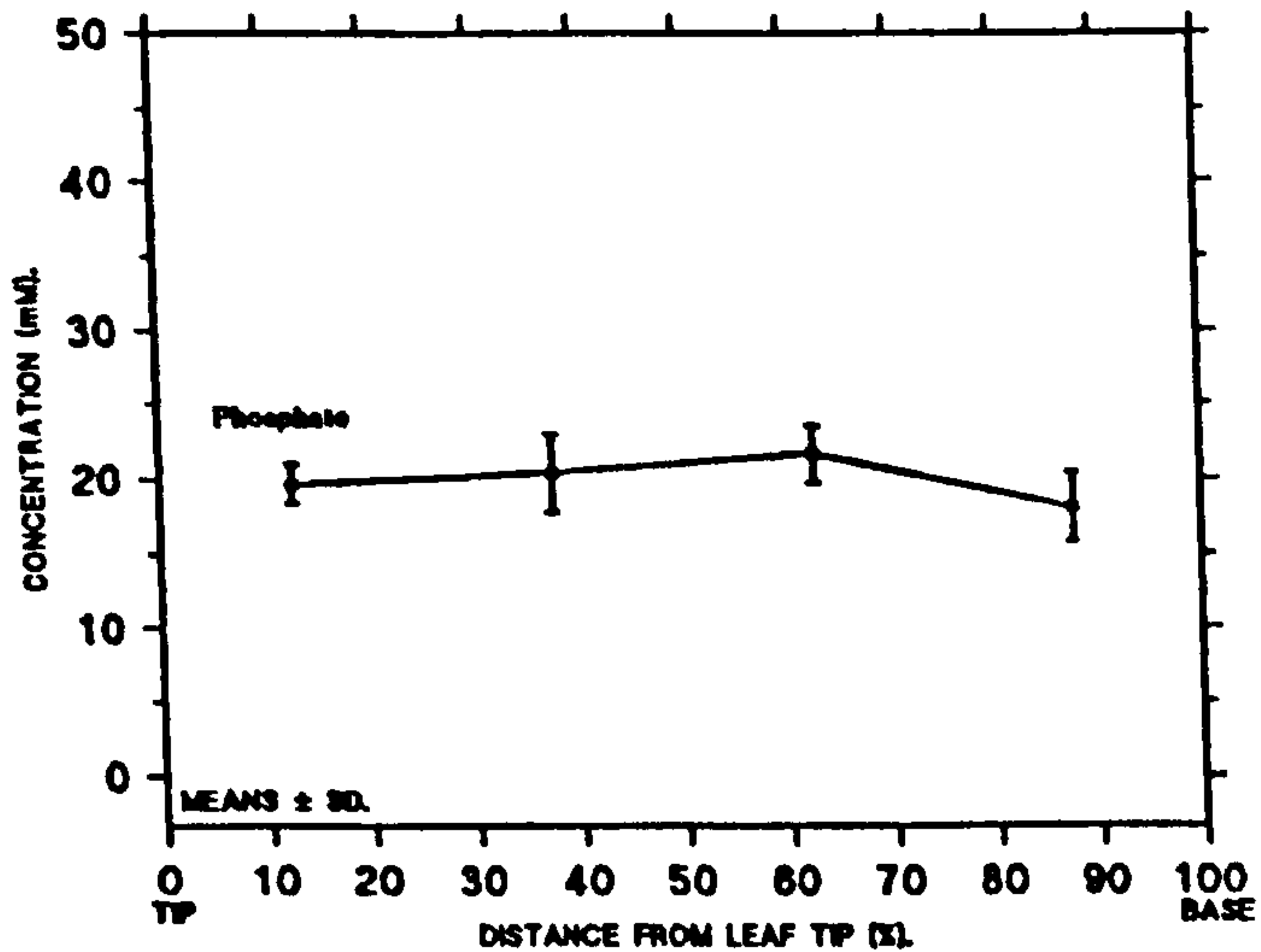
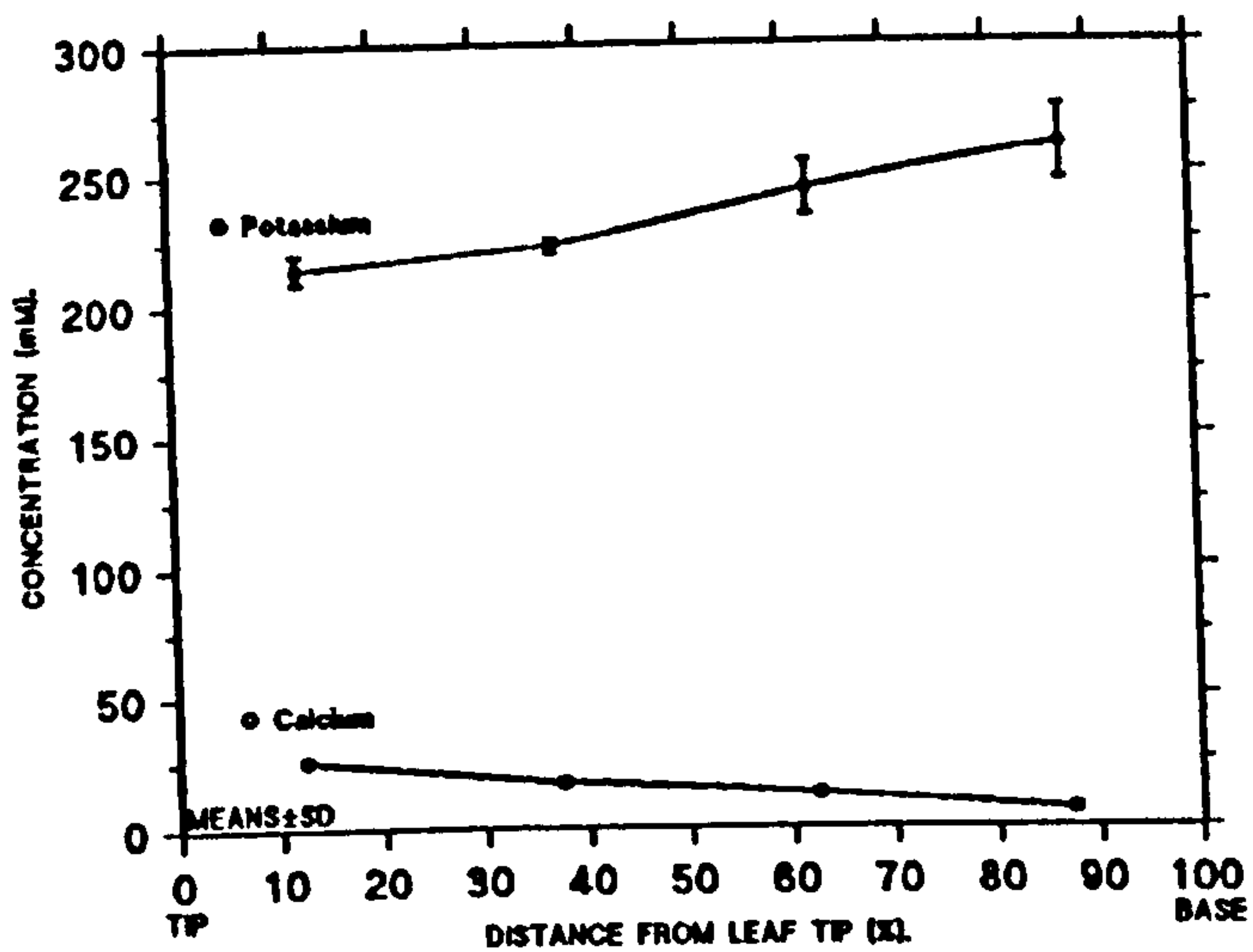


Fig 4.1 a)  $K^+$  and  $Ca^{2+}$ ; b)  $PO_4^{3-}$ ; c)  $NO_3^-$ ; d)  $Cl^-$  Variation of bulk solute content along the primary leaf blade of barley 7 days after being transferred to full strength nutrient solution (4.0 mM K). The graphs show the means and standard deviations of 5 plants

The shallow gradients of ion concentrations observed were associated with a slight variation in osmotic pressure along the leaf (Fig 4.2).



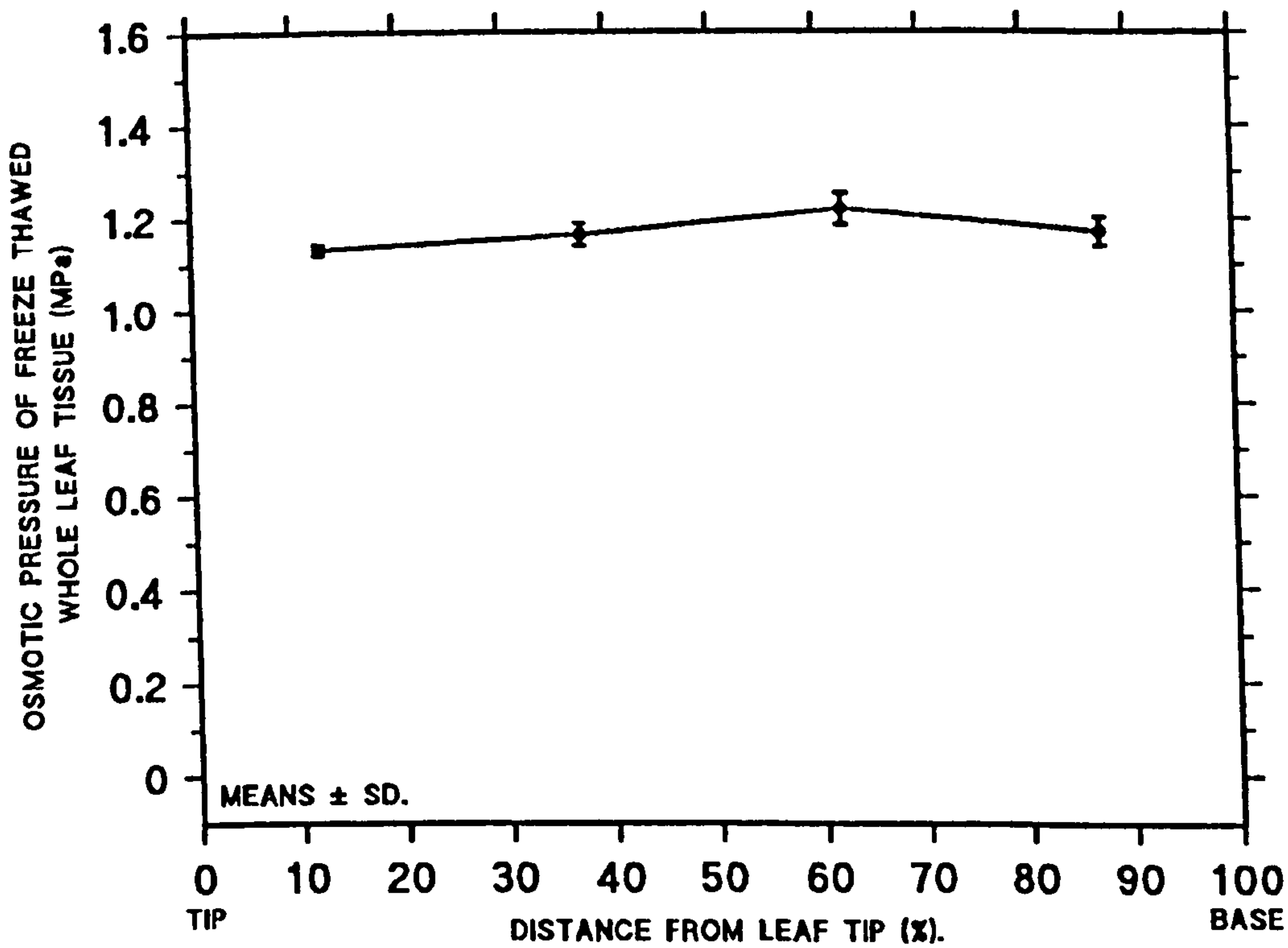


Fig 4.2 Variation of osmotic pressure along the primary leaf blade of barley 7 days after being transferred to full strength nutrient solution (4.0 mM K). Each point represents the means and standard deviations of 5 plants.

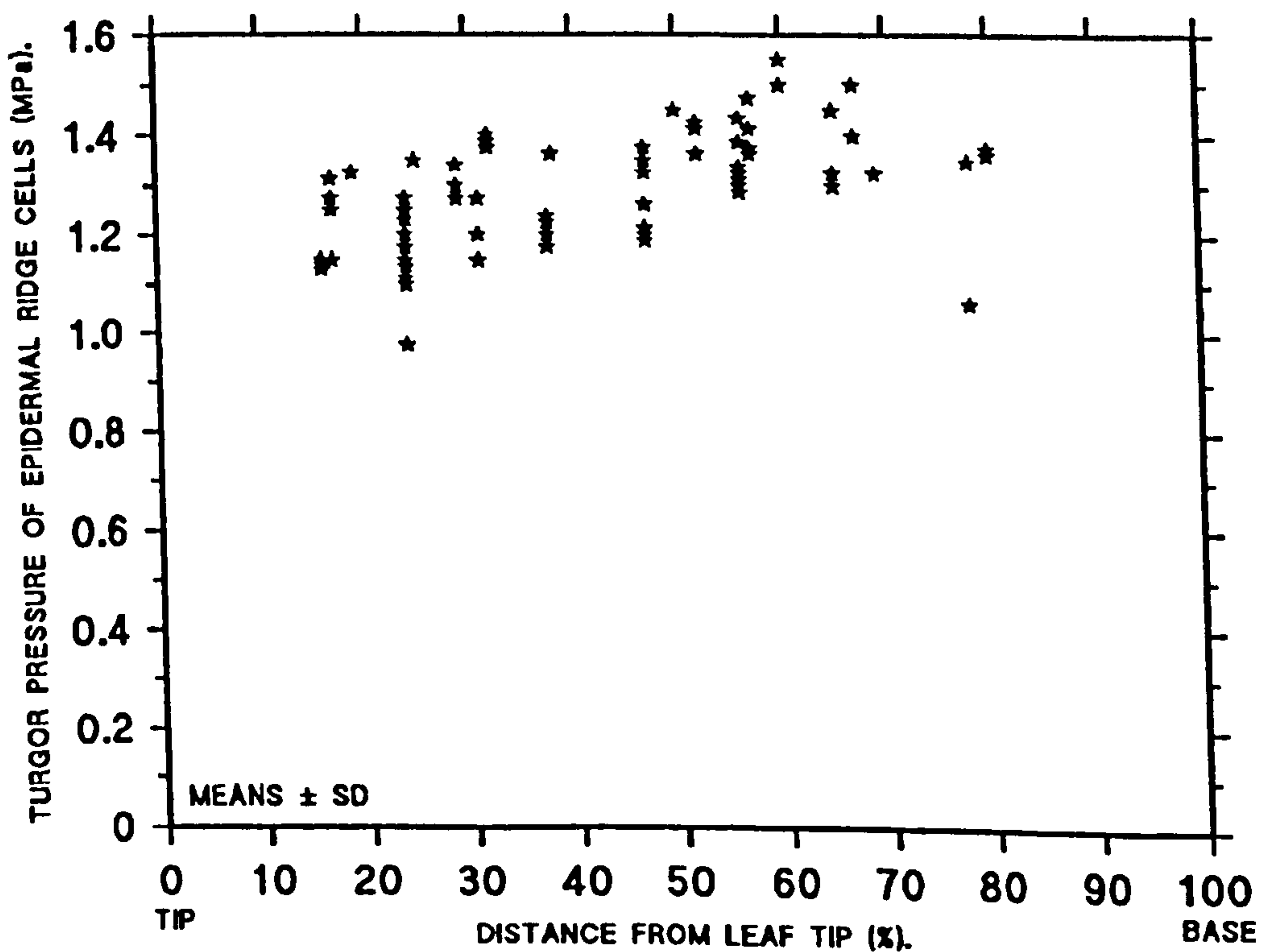


Fig 4.3 Variation of turgor in epidermal ridge cells of barley throughout the leaf blade. Plants were measured 6-8 days after transferral to 4.0 mM K nutrient solution.

## 4.2 Gradients of turgor along the leaf blade of barley

To identify whether gradients of turgor followed the same pattern as the leaf nutrients, turgor was measured in ridge cells throughout the length of the blade at days 6,7 and 8 (Fig 4.3). Despite the scatter of values, turgor apparently increases from the tip towards the proximal 25% of blade length. (A paucity of data in the final 25% of leaf blade was due to difficulty in accessing and lighting that region for microscopy and makes interpretation of turgor data at this point difficult). The slight gradient was comparable with whole tissue ion measurements measured throughout the leaf blade. This observation suggests that the basis for the turgor difference might reside in the vacuolar contents rather than in the wall water potential. It also suggests that the turgor in the epidermal cells might vary by as much as 0.3 MPa (between 20-60% of the leaf blade distance from the tip) depending upon cell position along the leaf blade.

#### 4.2.1 The resolution of the turgor heterogeneity of epidermal cells

Demonstration of a turgor gradient throughout the length of the leaf blade is in itself evidence of cellular heterogeneity. In order to more fully characterise the degree of turgor variation within the leaf, turgor pressure was measured at a much finer resolution in cells equidistant from the leaf tip. This approach was taken to eliminate errors introduced by the longitudinal variation.

Ridge and trough cells (see Plate 2.2) of the control (4.0 mM K) plants were compared at a point approximately 4 cm from the leaf tip at days 6 and 7. 25 trough cells were compared with 32 ridge cells (Table 4.1).

CELL TYPE	n	MEAN TURGOR (MPa)	STANDARD DEVIATION
TROUGH	25	1.08	0.125
RIDGE	32	1.21	0.095

Table 4.1 Turgor differences in the epidermis of the first leaf of barley measured in ridge and trough cells 4 cm from the leaf tip. A significant difference was found at the 0.001 probability level as analysed by an analysis of variance.

Barley leaf epidermal tissue shows heterogeneity of turgor pressure even across the distances of approximately 7 cells that separated the two cell types. Ideally, the cause of such differences would have been investigated by comparison of both turgor and osmotic pressure measurements. Unfortunately, however, a picolitre osmometer was not available on the date of the experiment. Variation may arise *via* changes to either vacuolar or wall solutes. The whole tissue measurements suggest that the variation might be due to the vacuolar content, but this is not conclusive since the ion gradients could have reflected a change in the proportion of tissue types along the leaf. To investigate these gradients further, the degree of variation of vacuolar solutes was investigated throughout the leaf.

### 4.3 What is the degree of heterogeneity in epidermal vacuolar solutes that exists throughout the epidermis of barley?

To establish the degree of variation of vacuolar solutes, samples for quantitative X-ray microanalyses were taken from the cells throughout the whole adaxial epidermis, and sequentially from adjacent cells. Initial investigations were of cells throughout the whole leaf blade since turgor throughout the blade displayed the greatest variation.

Three leaves were analysed on separate occasions (Fig 4.4 a,b,c,) by measuring the chloride, potassium and calcium contents of extracted vacuolar sap. Errors of measurement were reduced by taking as many subsamples as practicable from the extracted saps.

It is clear that there can be a wide variation in the composition of the extracted sap. Chloride tended to increase markedly towards the base of the leaf blade, and was generally very high in those ridge cells towards the base of the leaf. Potassium in the cells also varied. In certain instances, (particularly the trough cells 33% from the leaf tip at day 8 (Fig 4.4), cells with particularly low vacuolar potassium concentrations were found to contain correspondingly high calcium concentrations. Conversely, cells with a particularly higher than average potassium concentration were found to contain correspondingly lower calcium concentrations. If the cations were assumed to be associated with monovalent anions, then the sum of the solutes were found to be relatively constant despite this variation in composition. It appeared therefore that analysed across a wide area that the cells could contain a wide range of solute concentrations despite a relative constancy in turgor

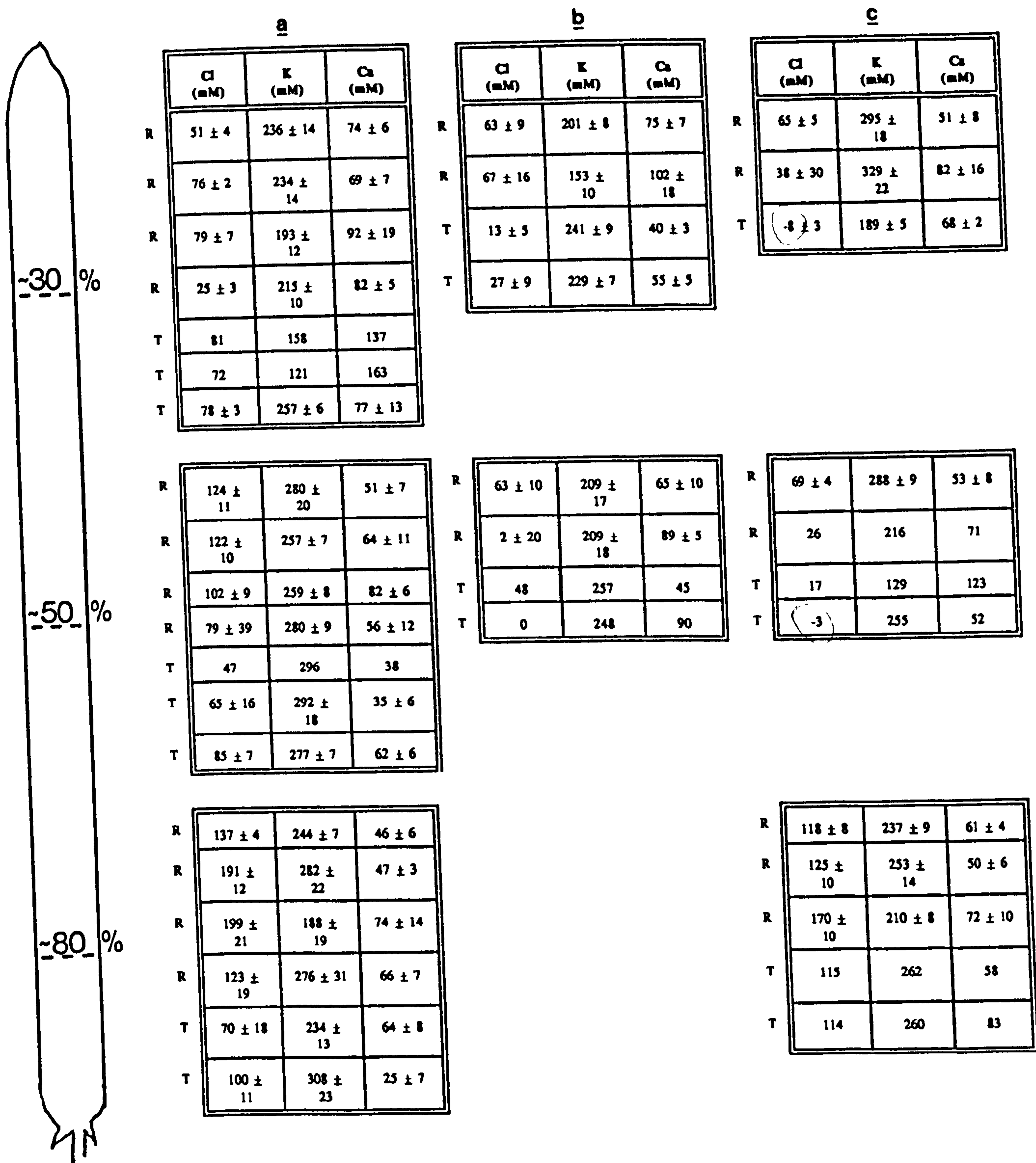


Fig 4.4 a,b,c. Vacuolar heterogeneity across the epidermis of the first leaf of barley in 3 different primary leaves. (a = 6 days after transferral to nutrient solution whilst b and c are 8 days post transferral). Where present, the error bars represent the standard deviation of the mean of 3-6 subsamples per vacuolar sap. R = ridge cells, T = trough cells. Samples were taken randomly from the leaf at various distances along its length.

### 4.3.1 The resolution of the solute heterogeneity of epidermal cells

The pressure probe allows measurement of adjacent cells. Hence, by taking sequential samples from a series of adjacent cells encompassing both ridge and trough cells, it was possible to establish whether the variation previously observed (Fig 4.4) reflected any ordered pattern. By measuring vacuolar variation it was intended to resolve whether the turgor variation observed between the ridge and trough epidermal cells (Table 4.1) was a function of obvious differences in the vacuolar composition.

Adjacent cells were sampled (without turgors) 4 cm from the tip of day 6 and day 8 control plants. The sampling position chosen (between and inclusive of ridge 3 and ridge 4) was to further investigate the earlier discovery of an apparent inverse relationship between vacuolar potassium and calcium concentrations (Fig 4.4). Since the heterogeneity of adjacent cells was being investigated, each graph is constructed from data obtained from a single leaf. The results are obtained from 3 separate batches of plants (Fig 4.5 a,b,c). Gradients in elemental concentrations are apparent in all leaves analysed at day 8. Calcium varies sinusoidally between ridges, the greatest concentration occurring in the trough cells. There is also a potassium gradient, peak concentrations occurring over the ridge cells. In one single analysis at day 8 (Fig 4.5 c) ridge cells overlying the vascular tissue were sampled sequentially towards the centre of the leaf. These cells did not appear to display an additional gradient dependent upon the cells relative position between the leaf edge and midrib.

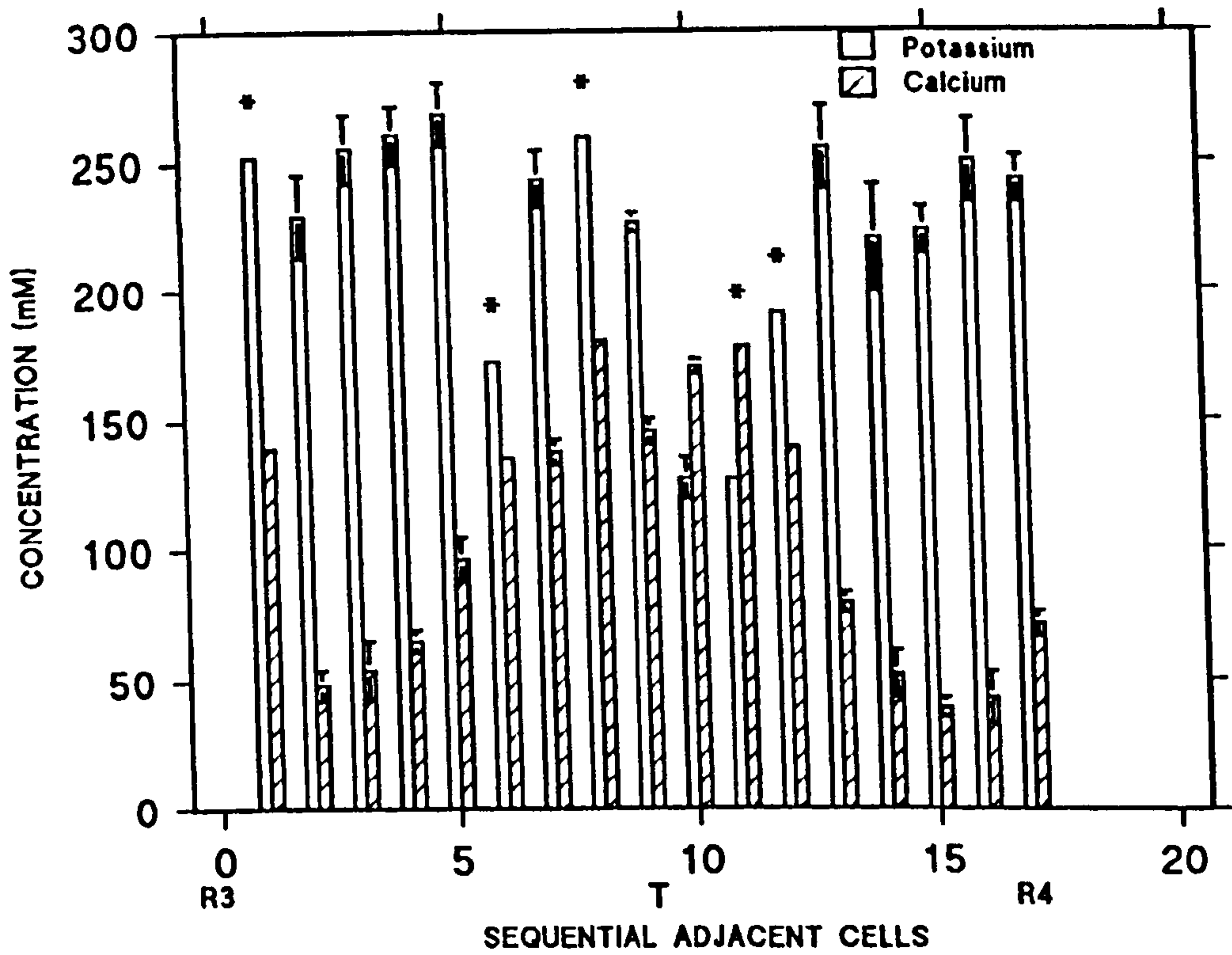


Fig 4.5 a Gradients of potassium and calcium in barley epidermal cells. R3, R4 denotes cells overlying the third and fourth ridge respectively. T denotes the trough cell region. Error bars show the standard deviation of the mean of at least 3 subsamples. (\* identifies samples subsampled less than three times).

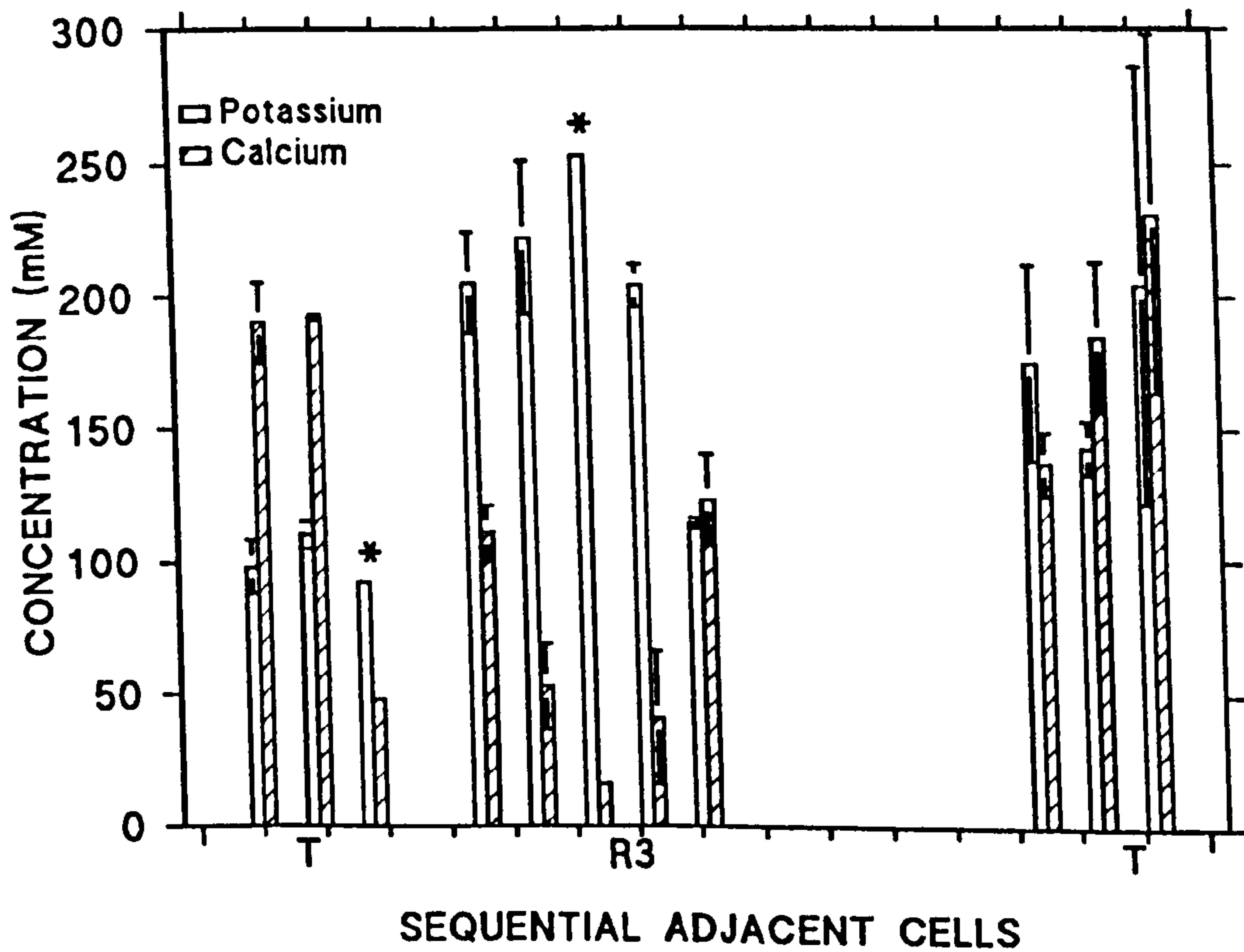


Fig 4.5 b Gradients of potassium and calcium in barley epidermal cells. Cells were sampled from one trough region to an adjacent region. (refer to Fig 4.5 a. for a more complete description of the graph).

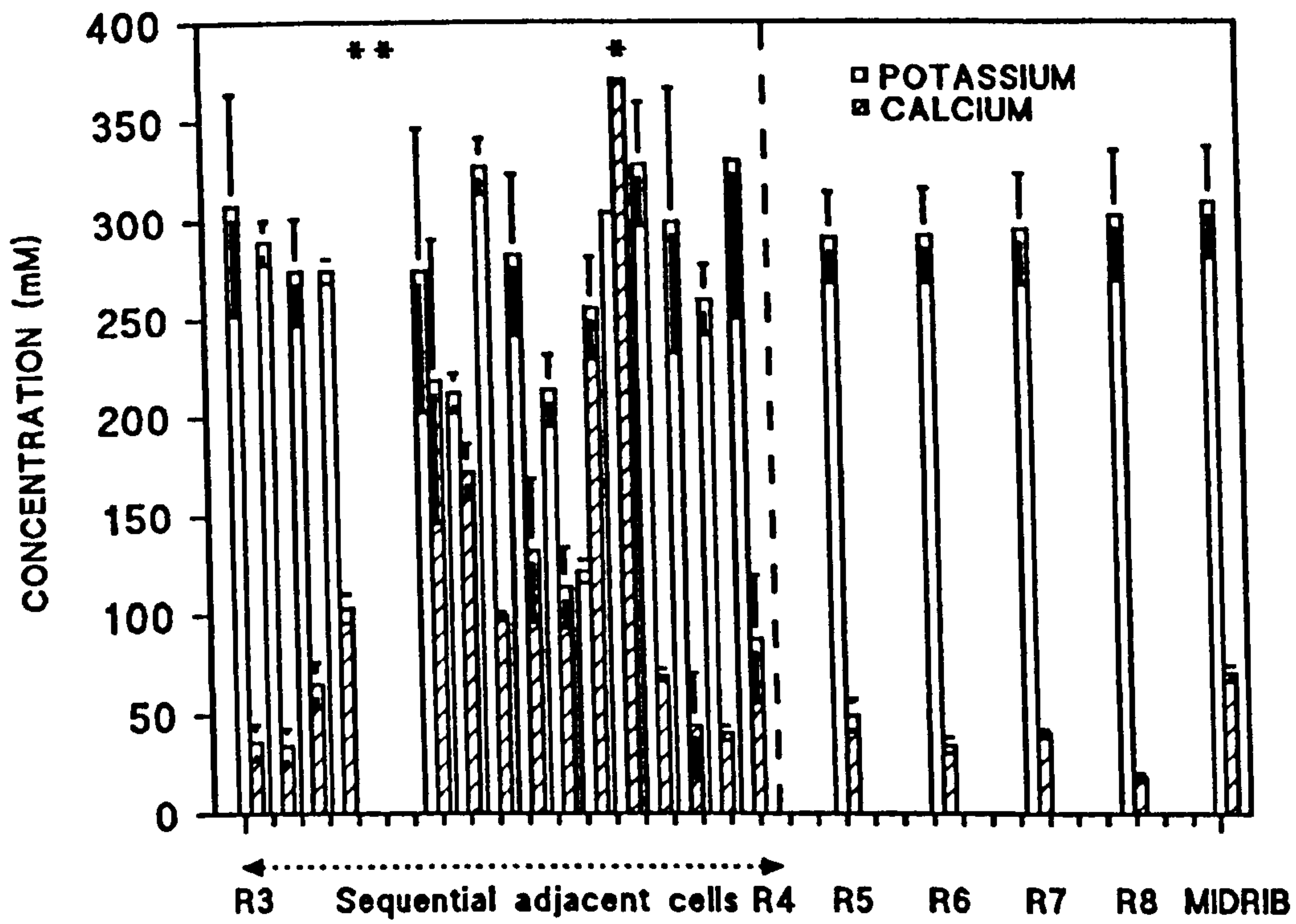


Fig 4.5 c Gradients of potassium and calcium in barley epidermal cells samples sequentially between the third and fourth ridges. Plots to the right of the vertical dotted line show the vacuolar concentrations of single ridge cells sampled from adjacent ridges towards the central midrib. (refer to Fig 4.5 a. for a more complete description of the graph).



#### 4.4 The potassium / calcium interrelationship

An apparent potassium / calcium relationship was observed in all the data analysed. Fig 4.6 is a plot of the potassium and calcium relationship of the data displayed in Fig 4.4 b. All the individual leaves displayed a similar inverse relationship (data not shown) which will be further discussed in Chapter 5.

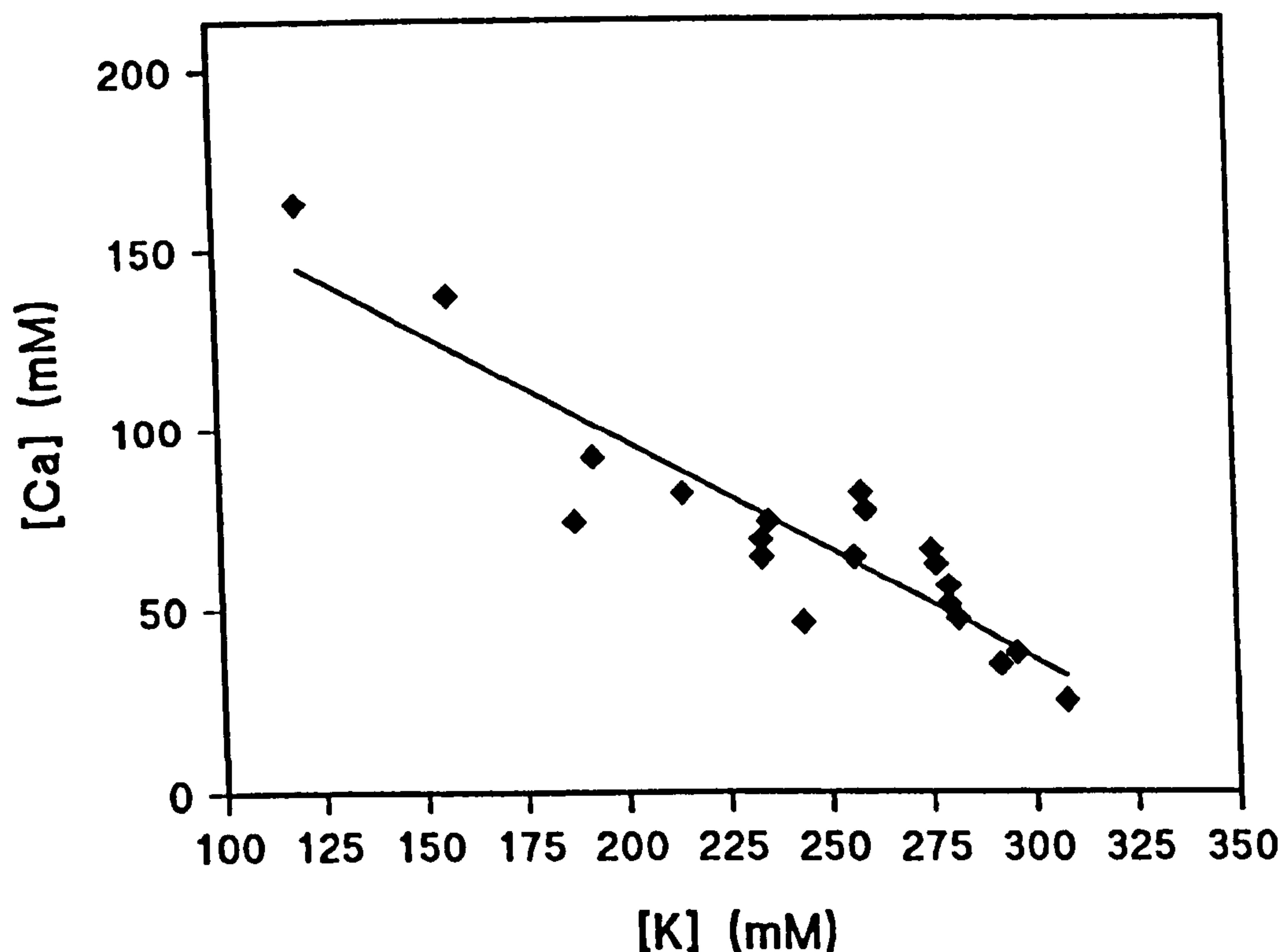


Fig 4.6 Potassium concentrations vs the calcium concentration as measured in sap extracted from barley plants 8 days after transfer to nutrient solution (4.0 mM K). Samples were taken randomly from epidermal cells throughout the length of the leaf blade (see Fig 4.4 b. for original data).

#### 4.5 Conclusion

The epidermis of barley displays heterogeneity in its vacuolar content. Gradients of both vacuolar potassium and calcium concentrations can be observed with their peak maxima apparently being related to their distance from the vascular tissue. From the data it is not clear whether this distribution is a function of the position

relative to the vascular tissue, or whether it is an innate property of the cells (themselves). A clue as to the reason for the heterogeneity is apparent in Fig 4.5 c. In addition to the sinusoidal pattern of calcium observed in Fig 4.5 a, there are two additional peaks which correspond to the positions of the stomata within the leaf. These peaks would suggest that the distribution may be related in some way to the pathway of the transpiration stream.

The observation of marked variation in epidermal vacuolar content along the leaf blade (Fig 4.4 a,b,c) might also be a consequence of either the route of solute flow within the leaf or individual cell properties. Whatever the mechanism, the variation is apparently not random. Chloride concentrations tend to be higher in the ridge cells at the base of the leaf than in those towards the leaf tip. The basis of the turgor difference between ridge and trough cells was not resolved by analysis of the vacuolar solutes although the solute composition of these cells were markedly different at 6-8 d.

Evidence that there is heterogeneity between different tissues within the leaf is suggested by the observation that phosphorus was not observed in the X-ray spectra at any stage, yet was observed as a 20 mM concentration of inorganic phosphate in a whole tissue extract (Fig 4.1 b). Such a result could only be obtained if phosphate were confined to a different area within the leaf. Data will be presented later that shows that this discrepancy is the result of compartmentation between mesophyll and epidermis (Fig 10.1).

These results illustrate the value of being able to measure water and solute relations parameters at single cell resolution. Ordered heterogeneity was observed both throughout the leaf, and in adjacent cells between the vascular tissue. It became apparent during the measurements, that the transverse variation tended to be greater in older plants. This observation suggests that the development of heterogeneity, and the accumulation of calcium may be a time dependent phenomenon. The development of heterogeneity is the subject of Chapter 5.

## **Chapter 5. Changes in solute distribution with leaf development.**

Malone et al (1991) showed that the solute concentrations of two different populations of epidermal cells (ridge and trough) were indistinguishable in 6 day old primary wheat leaves. This observation would appear to contradict the observations of solute gradients reported in the previous chapter. To resolve this contradiction the behaviour of the concentrations of epidermal solutes were measured over the lifetime of the leaf. In this way the hypothesis that the heterogeneity only develops as the leaves mature was tested.

Two methods of X-ray microanalysis were used to measure the solute composition. The micropressure-probe approach (described in Sections 2.1, 2.14) was used to analyse ridge and trough epidermal cells at 3,6,8,10 and 13 days. The frozen-tissue approach (described in 2.15) was used to measure ridge and trough epidermal cells and mesophyll cells at 0,3,7 and 13 days. The time period chosen extended from four days prior to full extension up until the appearance of senescent patches on the leaves. It therefore represented a significant proportion of the life of the leaf.

In addition to following changes in epidermal solute levels, the concentrations of the same solutes in bulk leaf extracts were measured. This approach allowed some comparison of different tissues within the leaf.

### **5.1 Changes in cell potassium, calcium and chloride concentrations**

#### **5.1.1 Epidermal cells**

Two time courses showing changes in solute concentrations in individual cells are illustrated in Figs 5.1 and 5.2. Results for Fig 5.1 were obtained using the micropressure-probe approach whilst Fig 5.2 was prepared from data obtained by the frozen-tissue approach. Several features can be seen. Both potassium and calcium concentrations change with time. At day 0, both these ions are at a concentration of approximately 25 mM. The potassium concentration, however, increases rapidly and reaches its maximum level by day 3 (see Fig 6.3 for corresponding data for whole

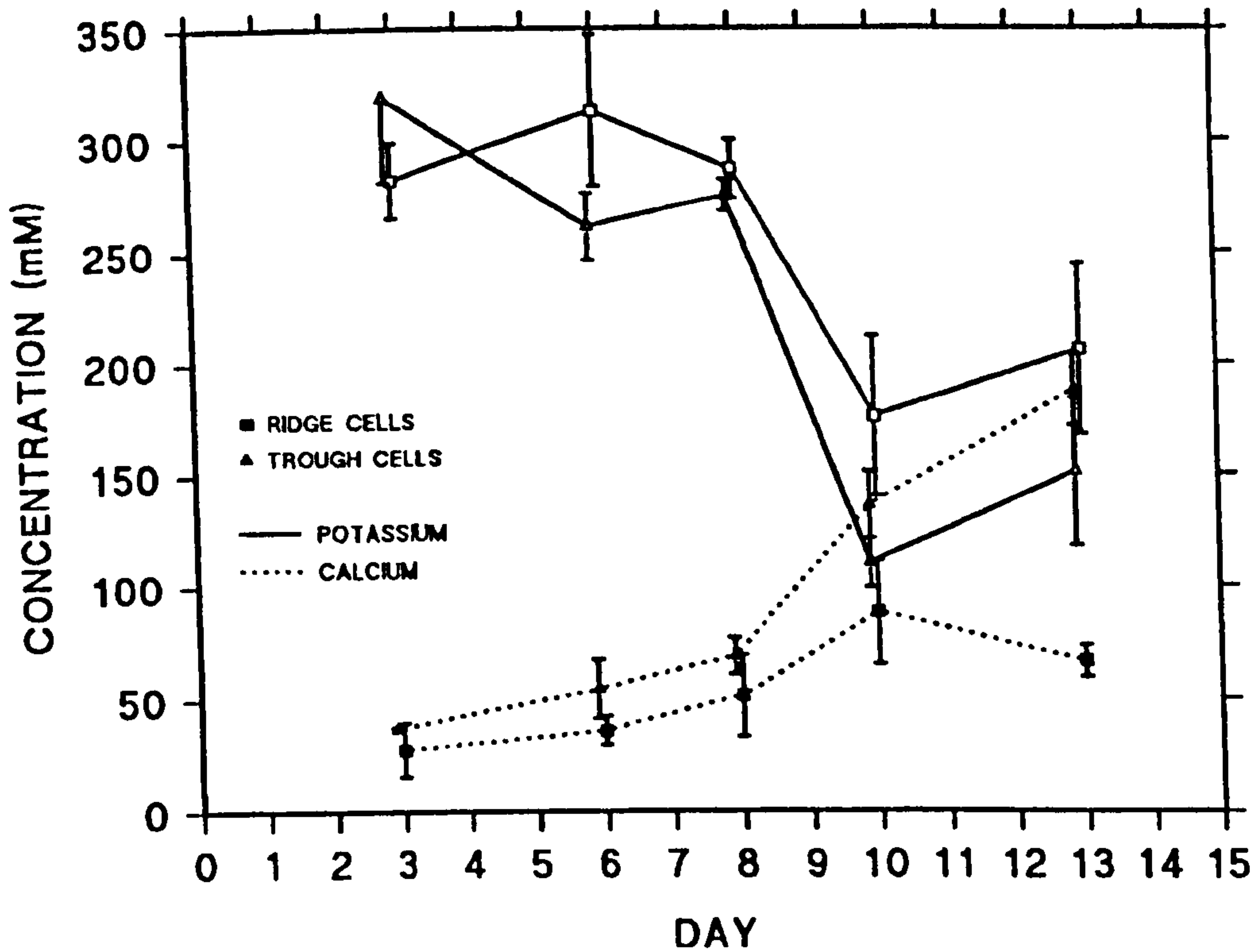


Fig 5.1. Time course of vacuolar potassium and calcium composition of barley epidermal cells. X-ray microanalysis was performed on sap extracted with the micropressure-probe. Each point represents the mean and standard deviation of at least three different cells. One plant per sampling date was analysed. This data corresponds to the chloride data illustrated in Fig 5.3.

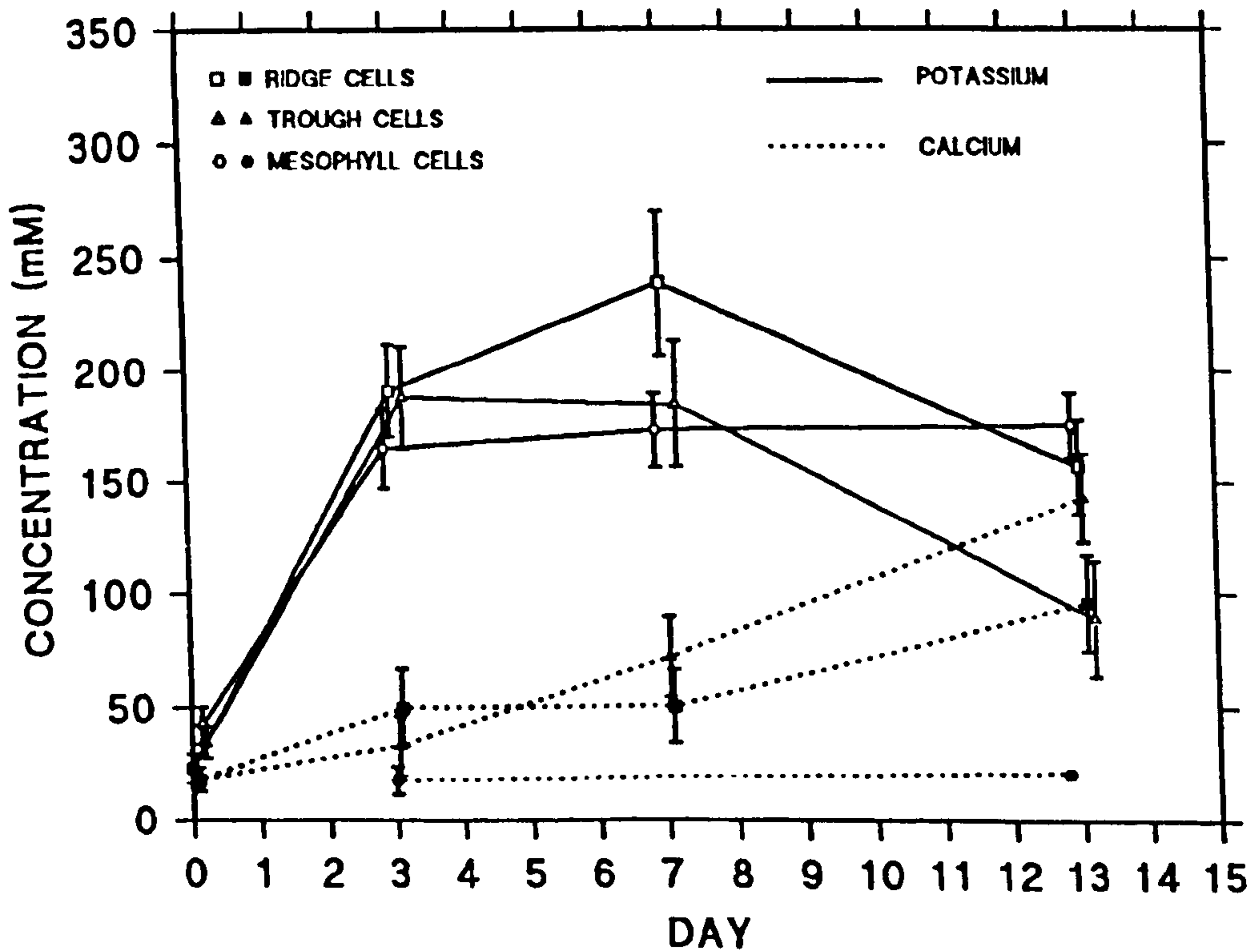


Fig 5.2. Time course of vacuolar potassium and calcium composition of barley epidermal and mesophyll cells. X-ray microanalysis was performed on frozen whole tissue. Results show the means and standard deviations of cells derived from at least 6 plants measured on two different occasions in Hannover.

tissue concentrations). In contrast, the calcium concentration increases slowly over the first three days. A decline in the  $K^+$  concentration occurs as the concentration of  $Ca^{++}$  increases. This correspondence reflects an inter-relationship between these ions that is dealt with in Chapter 9.

Most significantly, for the purpose of this chapter, is that the changes in trough cells are larger than those of the ridge cells. The heterogeneity observed in chapter 4. *does* develop with time. The measurements of Malone et al (1991) at day 6 were performed before the sinusoidal gradients became significant (unless the behaviour of wheat leaf tissue is fundamentally different from that of barley).

A more thorough 'pressure-probe' investigation of cells at 3 and 13 days confirmed this behaviour (Table 5.1).

DAY	CELL TYPE	[Chloride] (mM)	[Potassium] (mM)	[Calcium] (mM)
3	Ridge	43 ± 14	248 ± 32	26 ± 11
3 13	Trough	21 ± 9	236 ± 24	22 ± 15
13 3	Ridge	64 ± 40	258 ± 89	83 ± 11
13	Trough	69 ± **	175 ± **	113 ± **

Table 5.1. Developmental changes in epidermal vacuolar content. Concentrations of potassium, calcium and chloride were measured in sap extracted from individual epidermal vacuoles of the primary leaf of barley. Leaves were sampled at 3 and 13 days after transfer to nutrient solution (see text). The contents of trough and ridge cells are shown. The values shown are the mean and standard deviation of at least 3 cells and each cell was subsampled at least three times. At day 13 only two trough cells were measured.

The chloride concentrations corresponding to Fig 5.1 are shown in Fig 5.3. There are differences between the contents of ridge and trough cells since the latter cells consistently have lower chloride concentrations. In contrast to the cations, however, this difference does not appear to alter significantly with development. ✓

The chloride concentrations presented in Table 5.1 also show that chloride concentrations are different at day 3. Chloride concentrations at day 13 are however

higher than at day 3 and are similar in both ridge and trough cells. This observation indicates that the heterogeneity apparent at day 3 may disappear with leaf age as chloride accumulates in the epidermis. Since chloride was not accumulated significantly in an earlier experiment (Fig 5.3), proper interpretation of the behaviour of chloride partitioning throughout leaf development awaits further experiment.

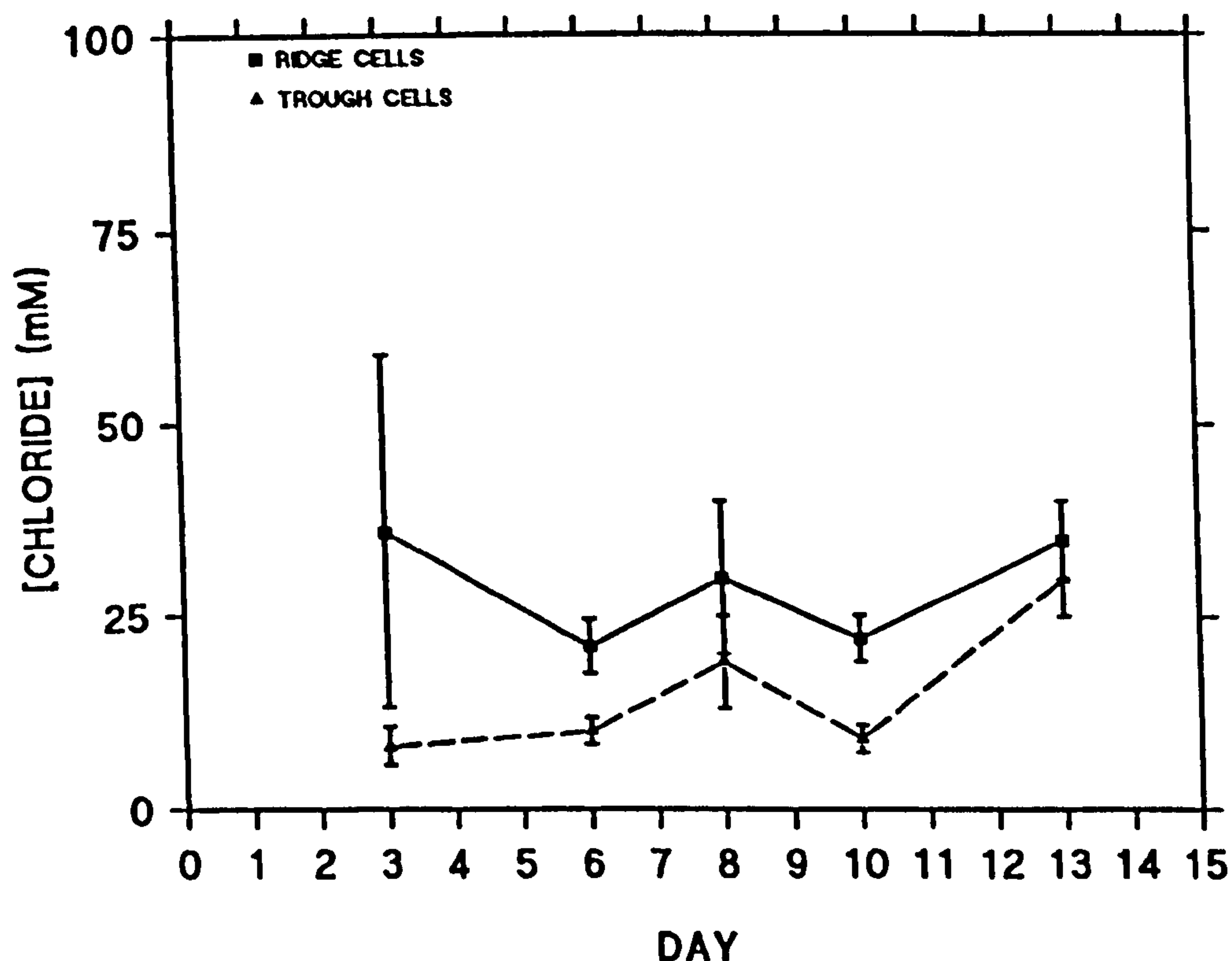


Fig 5.3 Time course of chloride content of barley epidermal cell vacuoles. Each point represents the mean and standard deviation of at least three different cells. One plant per sampling date was analysed. This data corresponds to the potassium and calcium data illustrated in Fig 5.1.

### 5.1.2 Mesophyll Cells

Concentrations of potassium and calcium were measured in barley mesophyll cells by X-ray microanalysis of frozen tissue. Similar to the epidermis, potassium accumulated rapidly in the mesophyll cells over the first three days (Fig 5.2). Unlike the behaviour of the epidermis it thereafter remained constant even when concentrations of potassium in the epidermis declined. Calcium concentrations in the mesophyll cells were low, at approximately 20 mM, and did not change with time.

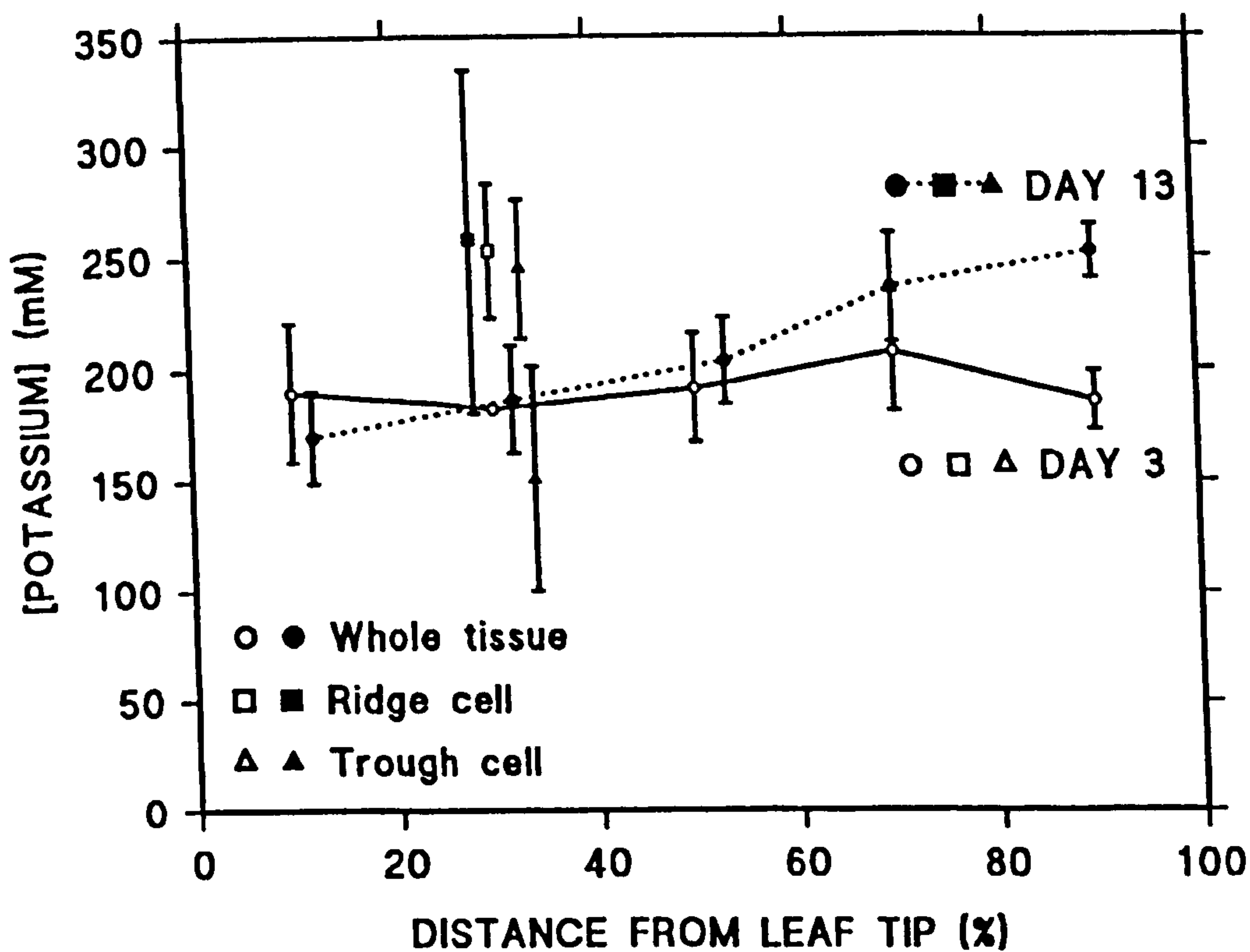
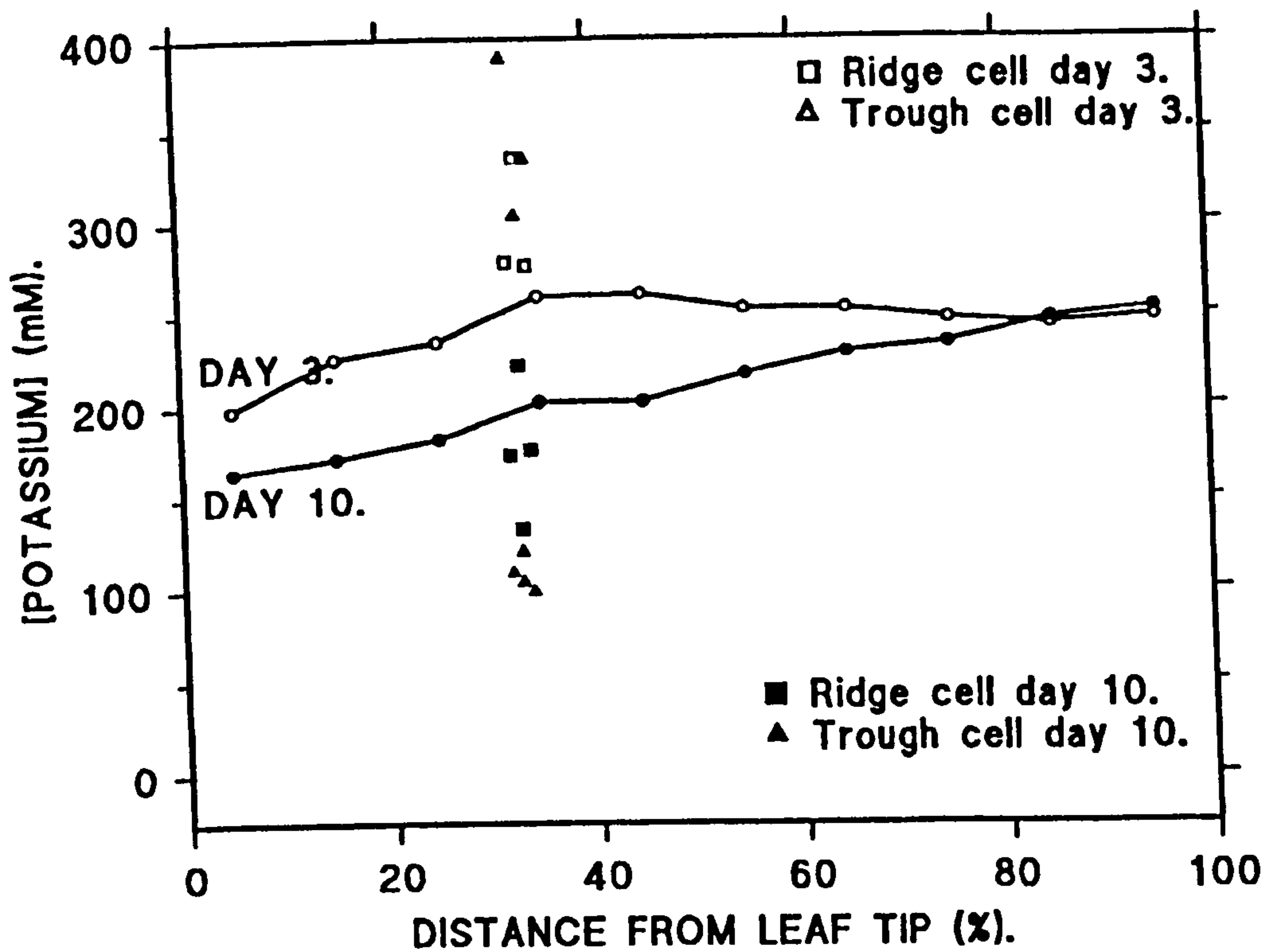


Fig 5.4 Relative changes in whole tissue vs epidermal potassium concentrations at two stages of development. a) at days 3 and 10 (In this plot the error bars of measurement have been omitted for clarity). b) at days 3 and 13 (In this plot the results show the means and SD of 3 plants. The single cell results show the mean and SD of at least 5 cells. See Fig 5.5 for corresponding data for calcium).

Mesophyll cells could not be easily measured using the micropressure-probe approach. Some idea of their behaviour relative to the epidermal cells was gained by comparing epidermal cell ion concentrations with those of whole-leaf tissue. In general the mean potassium and calcium concentrations of the leaf were different to those in the ridge and trough cell vacuoles of the upper epidermis.

## 5.2 A comparison of epidermal and whole tissue ion concentrations

### 5.2.1 Potassium

Potassium concentrations were measured in epidermal cells (approximately 30% of the leaf length from the tip), and (directly after) in the whole leaf. Samples were taken at days 3 and 10 (Fig 5.4a) and at days 3 and 13 (Fig 5.4b). Potassium concentrations in whole tissue tended to remain higher at the base of the leaf as the leaf aged. At 3 d each epidermal cell type had a potassium concentration higher than the local leaf mean concentration. By days 10 and 13, however, the potassium concentration in the trough cells had declined to below the mean leaf concentration.

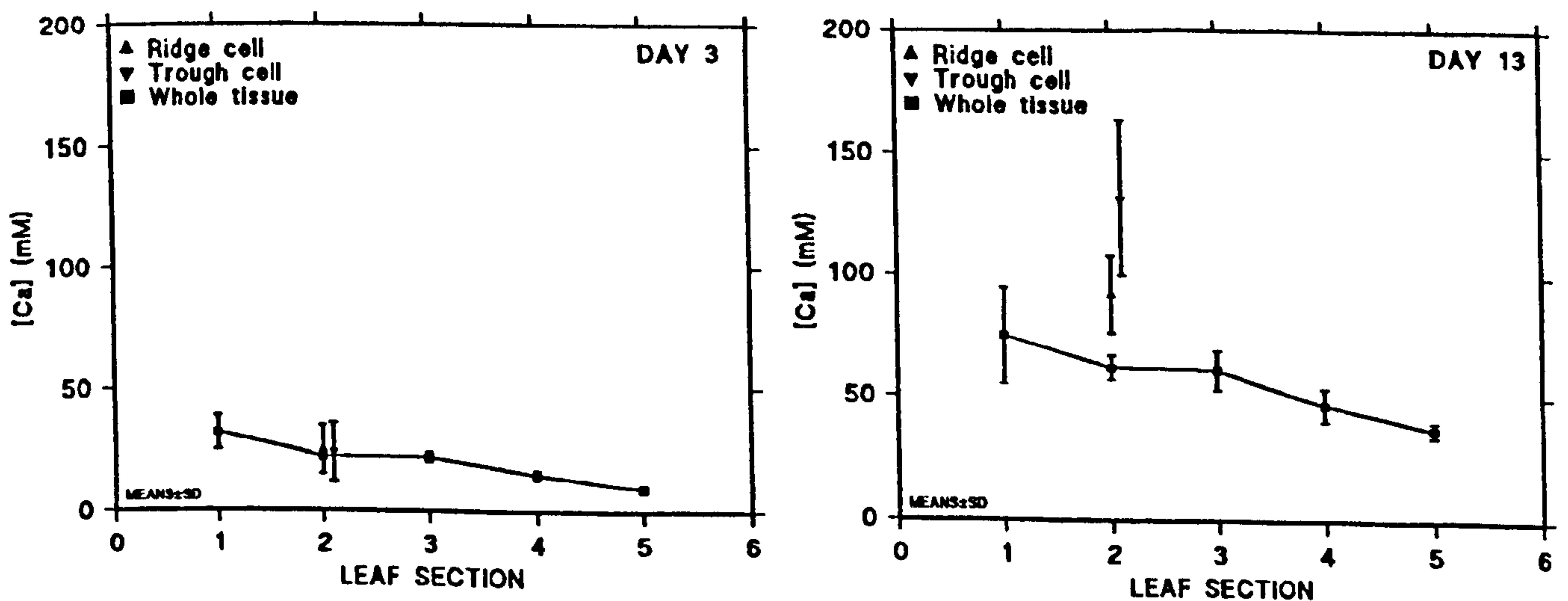


Fig 5.5. Relative changes in the whole tissue calcium content vs the epidermal calcium content at days 3 and 13. The results shown are the mean and SD of three plants. The single cell results represent the mean and SD of at least 5 cells. (See Fig 5.4 b for corresponding data for potassium).



The ridge cells lost less of their potassium relative to the rest of the leaf than the trough cells. This behaviour was confirmed in investigations of frozen tissue (Fig 5.2).

### 5.2.2 Calcium

Fig 5.5 illustrates the corresponding behaviour of calcium. At day 3, ridge, trough and local mean-leaf concentrations are the same. By day 13, however, both epidermal cell types have higher calcium concentrations than that measured in the whole leaf. The relative concentrations of ridge and trough cells are the reverse of their potassium concentrations and most calcium is accumulated in the trough cells. The data show that calcium is preferentially accumulated in epidermal cells rather than mesophyll cells (Fig 5.2).

### 5.3 Conclusion

Different cell types within the first leaf of barley have different vacuolar solute concentrations and these change with time. The sigmoidal gradients described in chapter 4. are therefore not contradictory to the observations of Malone et al (1991). Whilst changes in the distribution of potassium and calcium between ridge and trough cells depends on the developmental state of the leaf, the behaviour of chloride is less well understood. The physiological implications of these gradients and inter-relationships between potassium and calcium are dealt with in Chapters 9 and 10. It certainly means that the solute relations of the first leaf of barley are dynamic. It may well be that the first leaf is un-characteristic of subsequent leaves. Further measurements alone will determine this. However, the first leaf, with its relatively short life time, may indeed prove to be a useful model for studying development and senescence in a conveniently short period. The heterogeneity within the epidermis, however, dictates that such studies of leaf development must take some account of the single-cell resolution required.

The various cells of the epidermis, moreover, are not the only source of heterogeneity within the leaf. Upper-epidermal cell content is generally different from the mean leaf concentration indicating that various tissues within the organ are also different. In fact, direct comparisons of the epidermis and mesophyll cells show that the differences between their potassium and calcium concentrations develop with time. Such a conclusion has been reached for other solutes by the analysis of protoplasts obtained from different tissues within leaves by Deitz *et al.* (1992a,b). Indeed in recent investigations, Fricke *et al.* (1994a) have directly measured solutes in mesophyll and (probably) bundle-sheath cells of 3rd and 4th leaves of barley as well as comparing upper and lower epidermal layers (Fricke *et al.* 1994b). As expected from the data presented in this chapter, a non-uniform distribution of all solutes measured (including also nitrate and malate) was found.

The dramatic changes in the concentrations of individual solutes confirmed by this chapter clearly have implications for the control of solute transport. These implications are dealt with in Chapter 9. They also, however, have implications for the water relations of the tissues. If individual solute concentrations change, what is the response of cell osmotic and turgor pressures? These questions are dealt with in Chapter 7. First, the effects of withholding potassium from the plant are investigated to show whether heterogeneity within the leaf is influenced by its potassium nutrition.

## Chapter 6. The effects of potassium-deficiency.

At a concentration of 250 mM, potassium is the major osmoticum in barley leaves (see introduction). In order to investigate the solute relations of the epidermis further, a potassium-deficit stress was imposed upon the plant. The data included in this section describe the plant characteristics; firstly as observed in the whole plant and secondly at the level of the single cell when nutrient solution potassium concentrations were reduced from 4.0 mM to 0.2 mM. The 4.0 mM potassium control treatments are described in Chapters 4 and 5.

### 6.1 Growth

Growth of both barley roots and shoots was retarded when plants were grown in 0.2 mM potassium. The decreased shoot growth first became visible as a retarded development of the 2nd leaf. Retarded growth was noticeable at day 8 and obvious by day 13 at which point plants grown in 4.0 mM potassium were beginning to tiller. The growth rate of the primary leaf were similar in both control and potassium-deficient treatments. Moreover, senescence of the primary leaf as intimated by the development of brown necrotic regions towards the tip of the leaf was also apparently unaffected by potassium nutrition.

In those plants grown in Hannover for the frozen tissue X-ray analyses the differences between the treatments was less obvious. This may have been due to a slower rate of potassium depletion in the nutrient solution which could have arisen as a consequence of early removal of plants that led to a lower plant density. These plants were destructively sampled and were not replaced.

### 6.2 Leaf nutrient concentrations

Comparison of the inorganic contents of control and potassium-deficient leaves at day 7 illustrates the effect of potassium deficiency on the ionic composition of the plant (Fig 6.1). Except for calcium, there were differences in the accumulation of all the ions measured, with the potassium and nitrate concentration being approximately

75 mM less in the low potassium treatment. Phosphate was similar in both treatments at approximately 20 mM, whilst the chloride concentration in the potassium-deficient treatment was double that of the control at approximately 30 mM. The most significant effect of deficiency was a reduction in the concentration of potassium nitrate of approximately 150 mOsmol (0.4 MPa) in the plant. Although the actual levels attained were different from control values, the pattern of ion distribution along the leaf blade was similar. For instance, the slight increase in the nitrate concentration towards the base of the leaf occurred in both potassium treatments (Fig 6.1c).

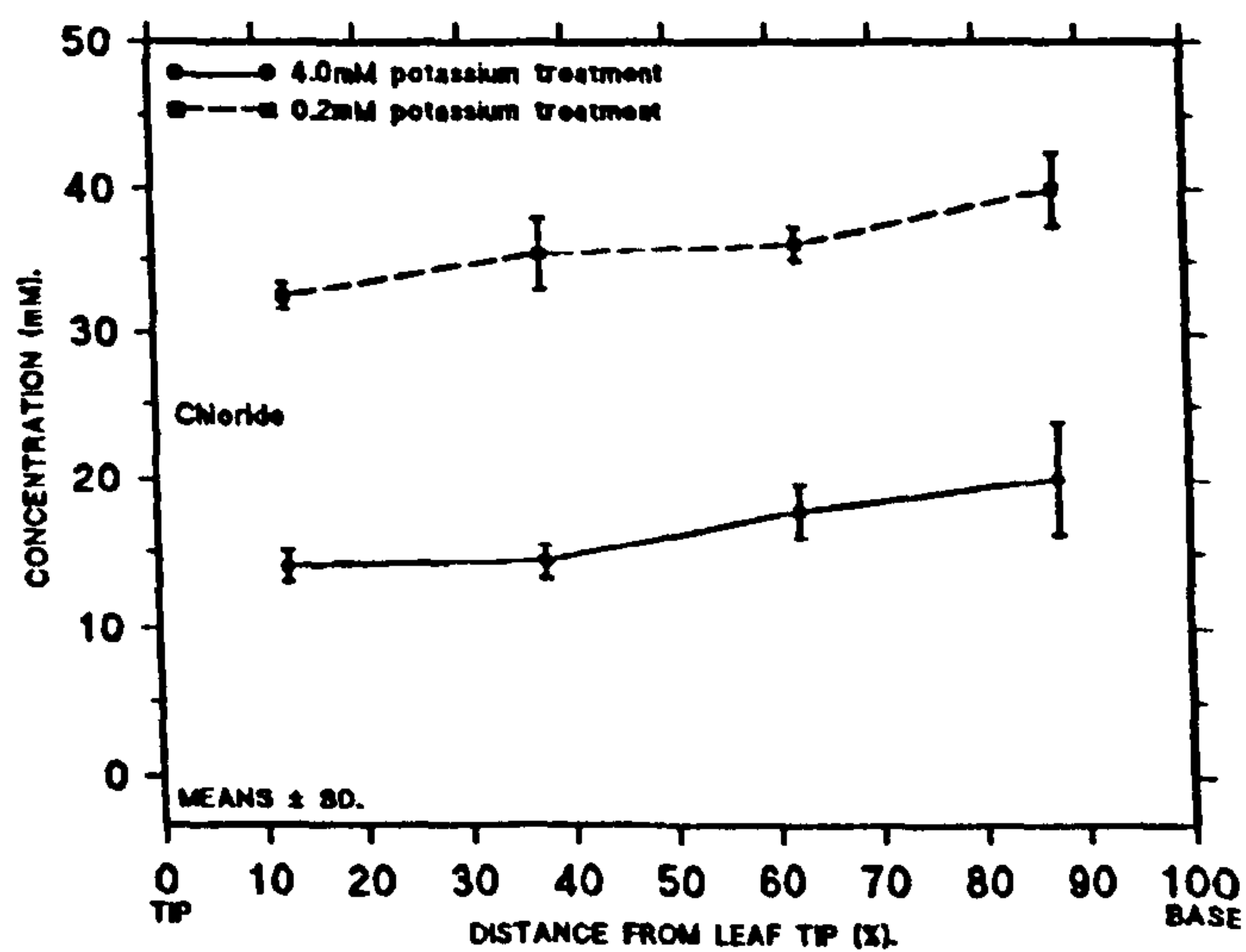
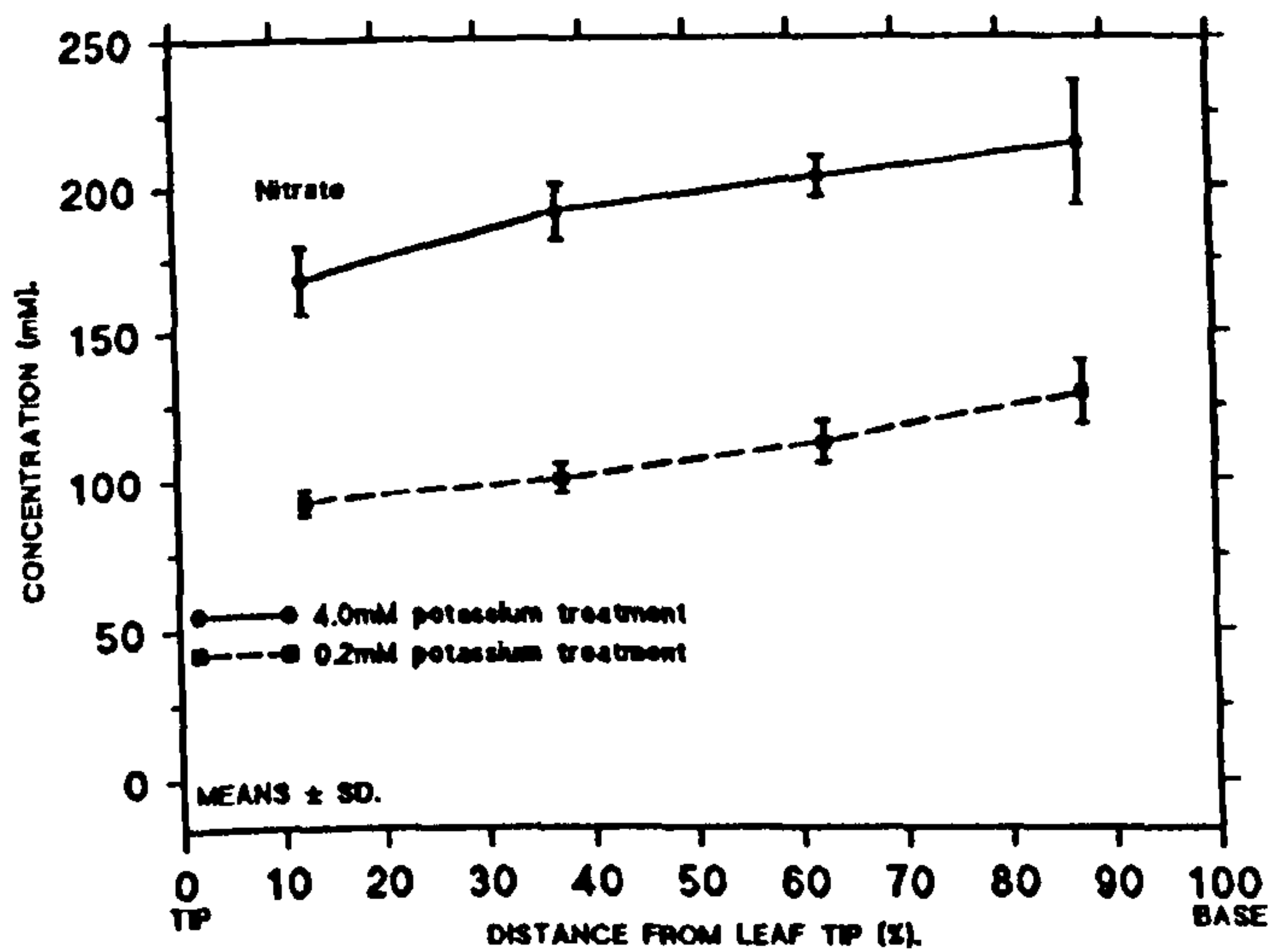
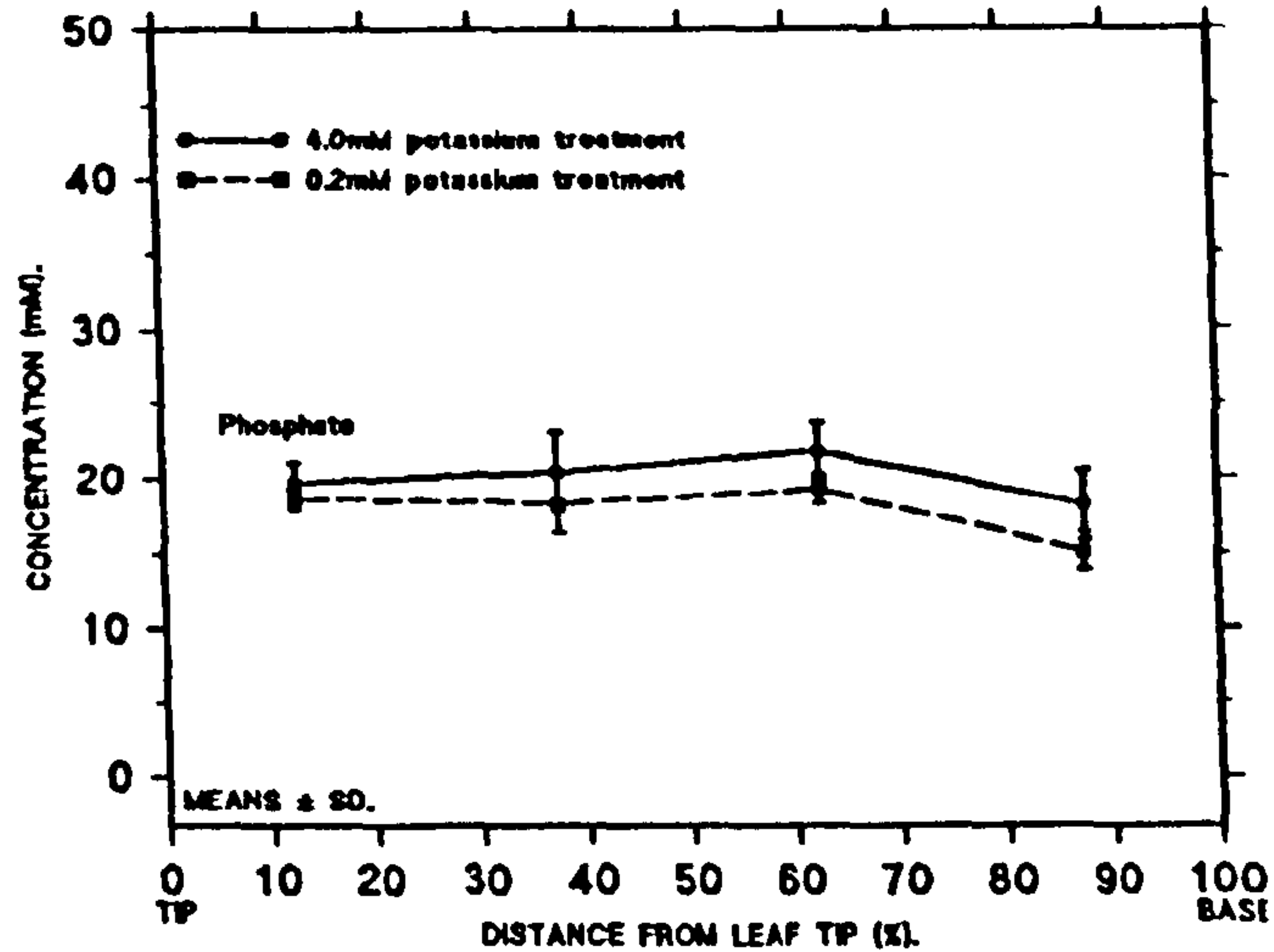
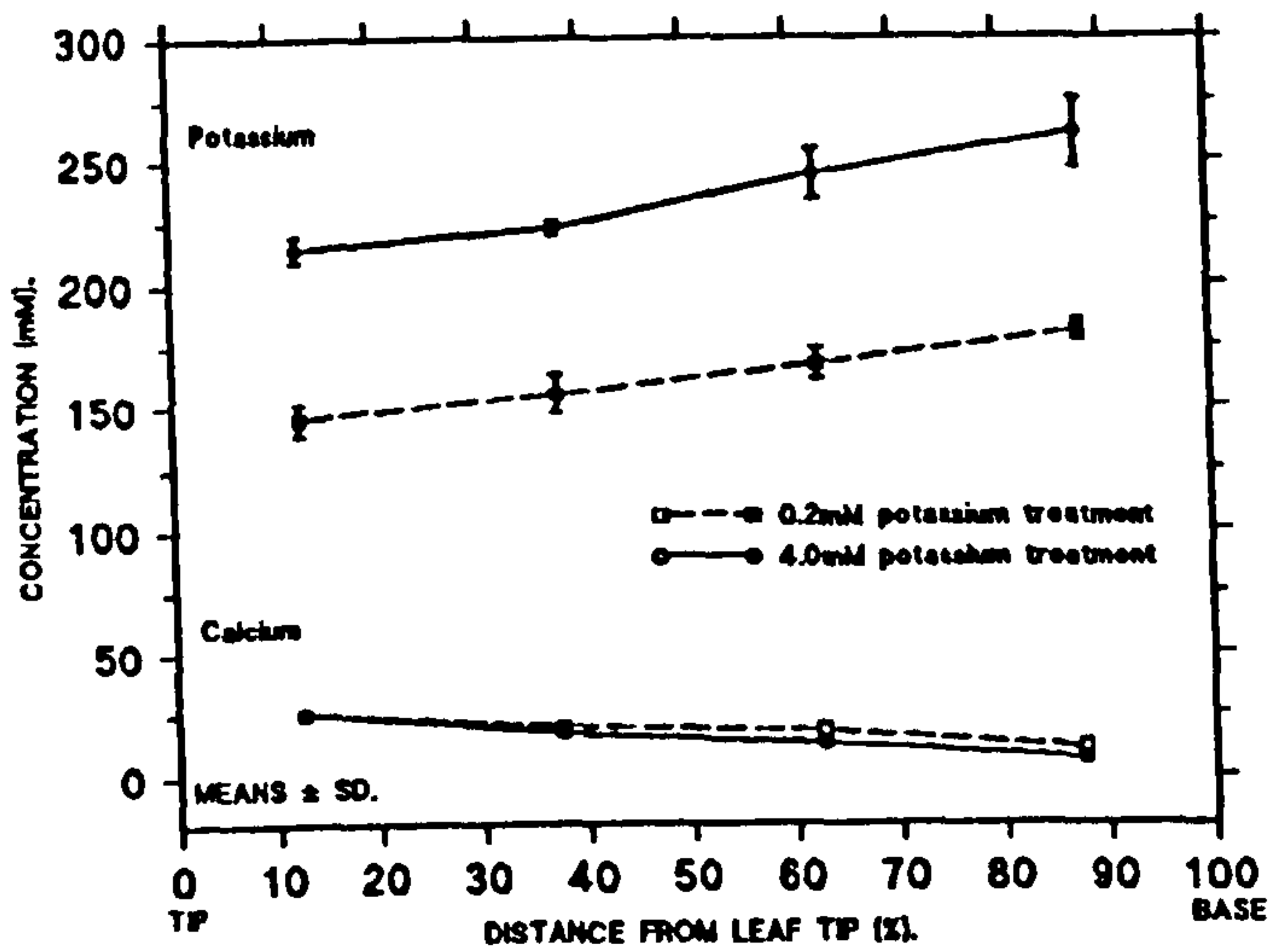


Fig 6.1 The effect of potassium deficiency on the nutrient concentrations along the primary leaf blade of barley 7 days after transferral to nutrient solution containing 0.2mM K (the control plants were grown on 4.0 mM K). The graphs show the means and standard deviations of 5 plants for (a) potassium and calcium; (b) phosphate; (c) nitrate and (d) chloride.

### 6.3. Leaf-sap osmotic pressure.

Whole-tissue osmotic pressure increased slightly away from the leaf tip until the basal 25% of both treatments whereupon it declined (fig 6.2). The osmotic pressures were observed to be approximately 0.1 MPa lower in the plants grown in 0.2mM K.

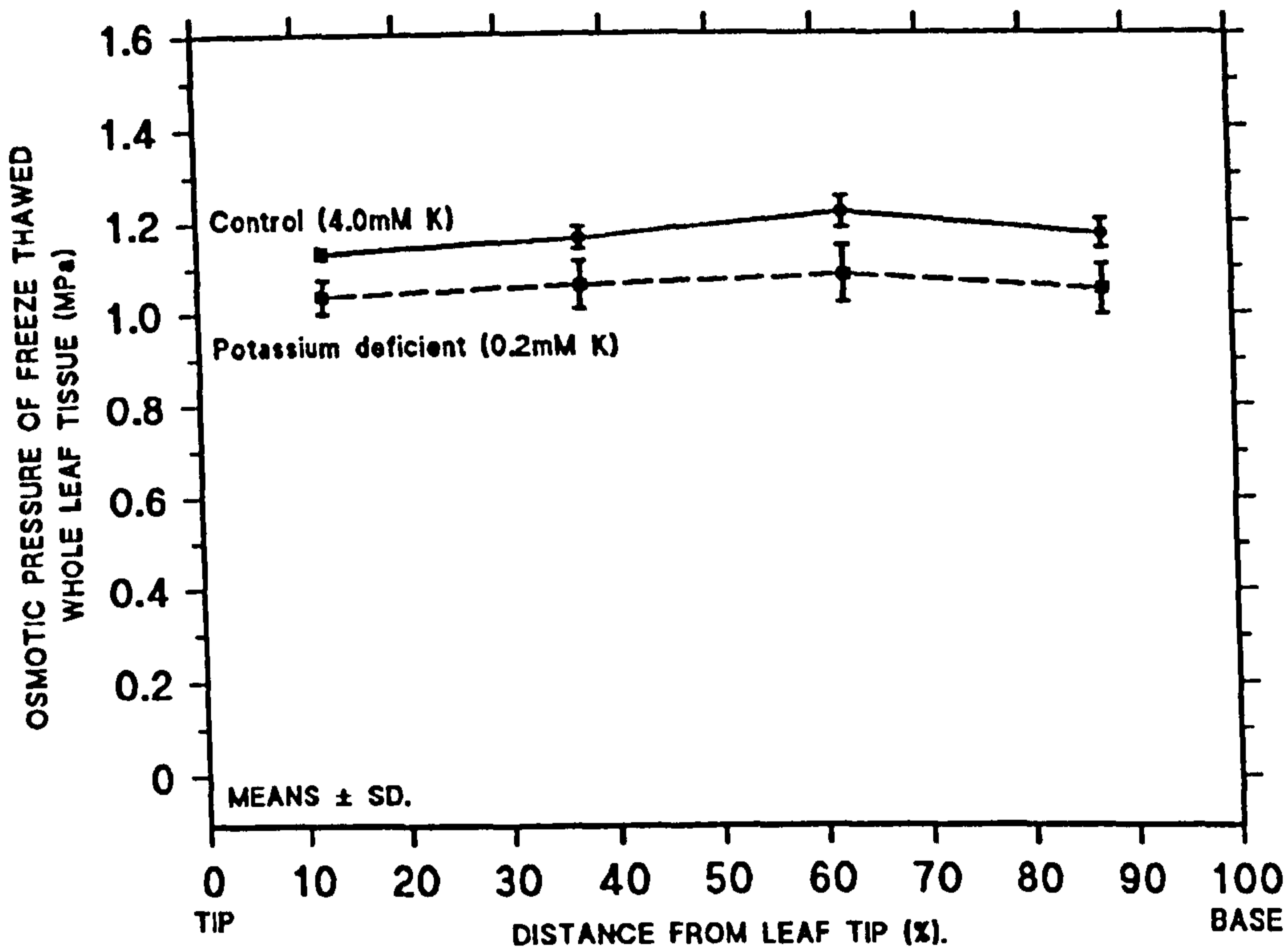


Fig 6.2. The effect of potassium-deficiency on the osmotic pressure of leaf sap extracted from the primary leaf blade of barley 7 days after transfer to nutrient solution. Control plants were grown on 4.0 mM K whilst the potassium deficient plants were grown on 0.2mM K. The graph shows the means and standard deviations of 5 plants.

Although the distribution of osmotic pressure along the leaf blade increased towards the base of the leaf similar to potassium and nitrate (fig 6.1 a,c), the difference in measured osmotic pressure from high and low K leaves did not reflect the differences in their ion levels between the treatments. Even with a reduction of tissue potassium nitrate of 0.4 MPa the osmotic pressure only declined by 0.1-0.15 MPa. This implies the accumulation of 0.25-0.3 MPa of alternative solutes. An alternative explanation is that precipitation of solutes incurred by the mixing of solutes

from two different compartments in the freeze-thaw process artificially lowered the leaf-sap osmotic pressure. To explain the data such precipitation would have to occur to a greater extent in the 4.0 mM K extract.

Because of the similarities of osmotic pressure between treatments despite differences in ion composition, the hypothesis was proposed that the increase in osmotic pressure observed was for the process of turgor maintenance in a regulated system. This hypothesis was one which could be further investigated by means of the pressure probe.

#### 6.4 Turgor pressure

The observed difference in whole tissue osmotic pressure of approximately 0.1 MPa might imply that turgor be maintained to within 0.1 MPa of the control in the potassium-deficient treatment. This was not the case. The turgor pressure from days 3-10 remained at approximately 0.7 MPa despite a whole tissue osmotic pressure of approximately 1.0 MPa. The reduced turgor was detectable at the whole plant level as a markedly reduced stiffness and turgidity of the plant. In combination with the thinner stem this led more easily to 'lodging'. Turgor pressures measured with the pressure probe in individual epidermal cells of the first leaf were always initially lower in the potassium-deficient treatment and were observed to remain low over the first 10 days (Table 6.1). However, a later experiment showed that turgor did increase with time (Fig 7.3b). These differences must be a consequence of the variable growth conditions. It is difficult to conclude more from the data except that reduced turgor was always apparent in potassium-deficient leaves during the first week of growth.

DAY	TURGOR PRESSURE (MPa)	TURGOR PRESSURE (MPa)
	4 mM K <sup>+</sup>	0.2mM K <sup>+</sup>
3	1.35 ± 0.07	0.71 ± 0.02
10	1.22 ± 0.12	0.72 ± 0.12

**Table 6.1 Relationship between nutritional state of plants and epidermal cell turgor pressure at days 3 and 10, illustrating a large difference in turgor pressure between the applied treatments (mean ± SD). The data was compiled from several experiments.**

Whole tissue osmotic pressure does not reflect the turgor values within single component cells of that tissue. This observation further illustrates the value of measuring cell water and solute relations at its component resolution. The reduced turgor in the 0.2mM K treatment could occur via partitioning of solutes away from the epidermis, or by increasing the concentration of wall solutes. These can be distinguished by measurements of the water and solute relations parameters of single cells. If the former were true, then large differences in solute concentrations and osmotic pressure might be observed within individual vacuoles. If the latter were true then comparison of turgor and osmotic pressure values would provide a value for the cell water potential.

To further investigate the effect of potassium-deficiency the solute and water relations of individual epidermal cells were studied. Whole tissue solute measurements were also made to provide information on the whole plant nutritional status. Sections 6.5 - 6.8 describe these solute relations whilst Chapter 7 addresses the water relations.



### 6.5. Leaf composition with age.

The whole-tissue potassium and calcium contents were determined in 2 cm sections of leaf cut 3 cm from the leaf tip and concentrations expressed based on tissue water (fig 6.3). The time course was measured under reproducible growth conditions in Hannover.

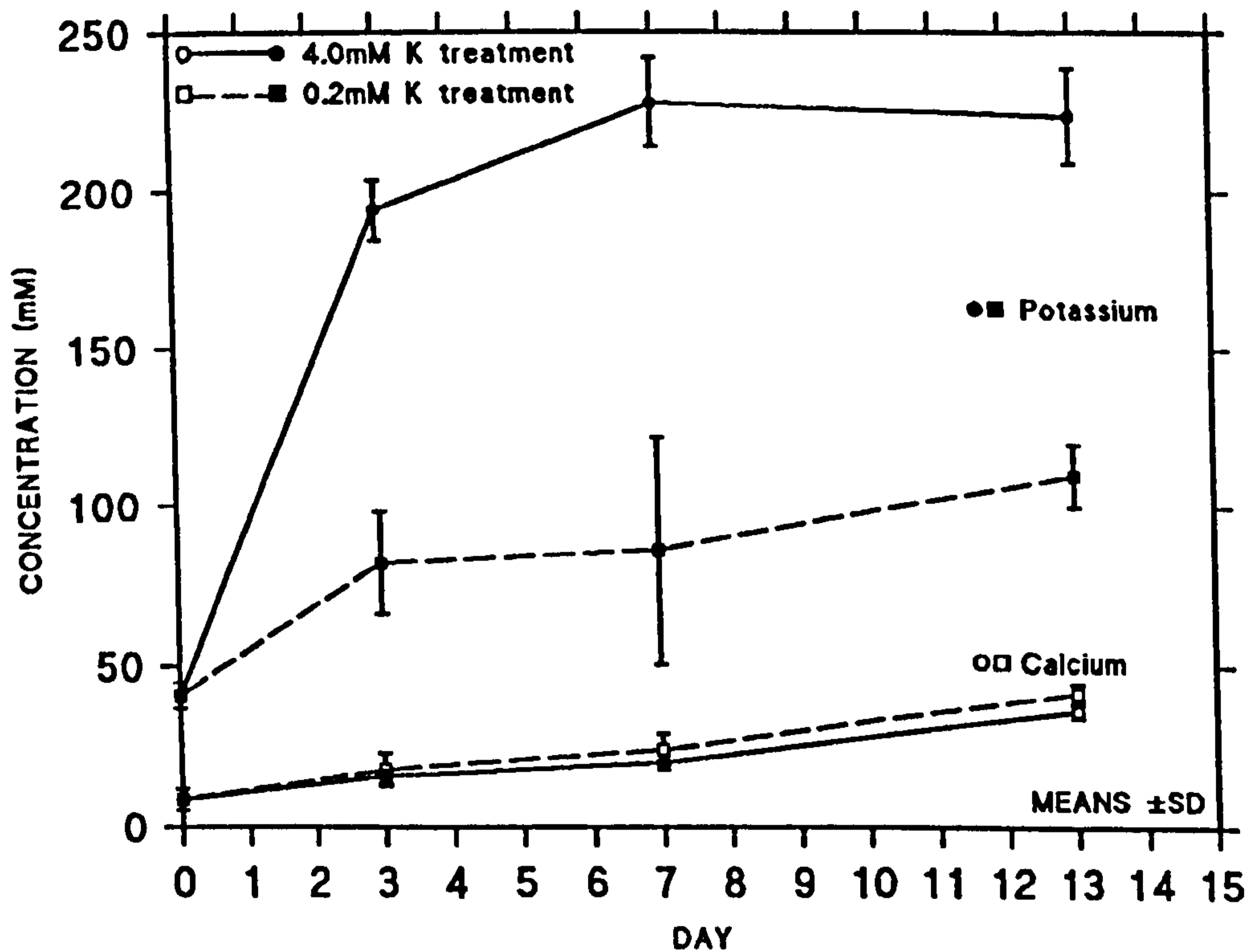


Fig 6.3. Leaf potassium and calcium concentrations throughout leaf development in control (4.0 mM K) or potassium-deficient (0.2mM K) primary leaves of barley. The results show the mean elemental concentrations of 5 plants  $\pm$  SD.

In the control (4.0 mM K) treatment, potassium increased rapidly over the first three days to nearly 200 mM. From days 7 to 13, the leaf potassium content was approximately constant at approximately 250 mM. In contrast, potassium in the potassium-deficient treatment rose to approximately 80 mM at day 3, and thereafter increased slowly to reach a concentration of 120 mM at day 13. It never rose to the value of over 200 mM reached in plants grown in 4.0 mM K (fig 6.3).

In contrast to the potassium concentration, the calcium concentration in leaves of both treatments were similar. Concentrations of calcium increased steadily to reach a leaf content of approximately 40 mM 13 days after transfer to nutrient solution. Since the osmotic pressure of the whole leaves at day 7 was similar between treatments (Fig 6.2) despite a markedly reduced potassium concentration (Fig 6.3), this suggests that calcium is *not* being taken up in whole leaf tissue to increase the osmotic pressure of the low potassium treatment (cf Leigh *et al.* 1986).

## 6.6 Vacuolar composition of leaf cells throughout development

### 6.6.1 Epidermal cells

A reduction of potassium in the nutrient solution potassium from 4.0 to 0.2 mM results in a reduction in the leaf potassium concentration of over 100 mM (Fig 6.3). If the epidermis also exports potassium as the leaf ages in the same manner as plants grown in 4.0 mM potassium (Fig 5.4) this could have serious implications for osmotic pressure maintenance. In order to investigate the affect of potassium-deficiency on potassium export from the epidermis, solutes in the epidermis were measured throughout leaf development. Two analytical techniques were used. The pressure-probe was used to analyse vacuolar solutes in ridge and trough epidermal cells (Fig 6.4) whilst the frozen-tissue approach was used to analyse ridge and trough epidermal cells and mesophyll cell vacuoles (see 6.6.2).

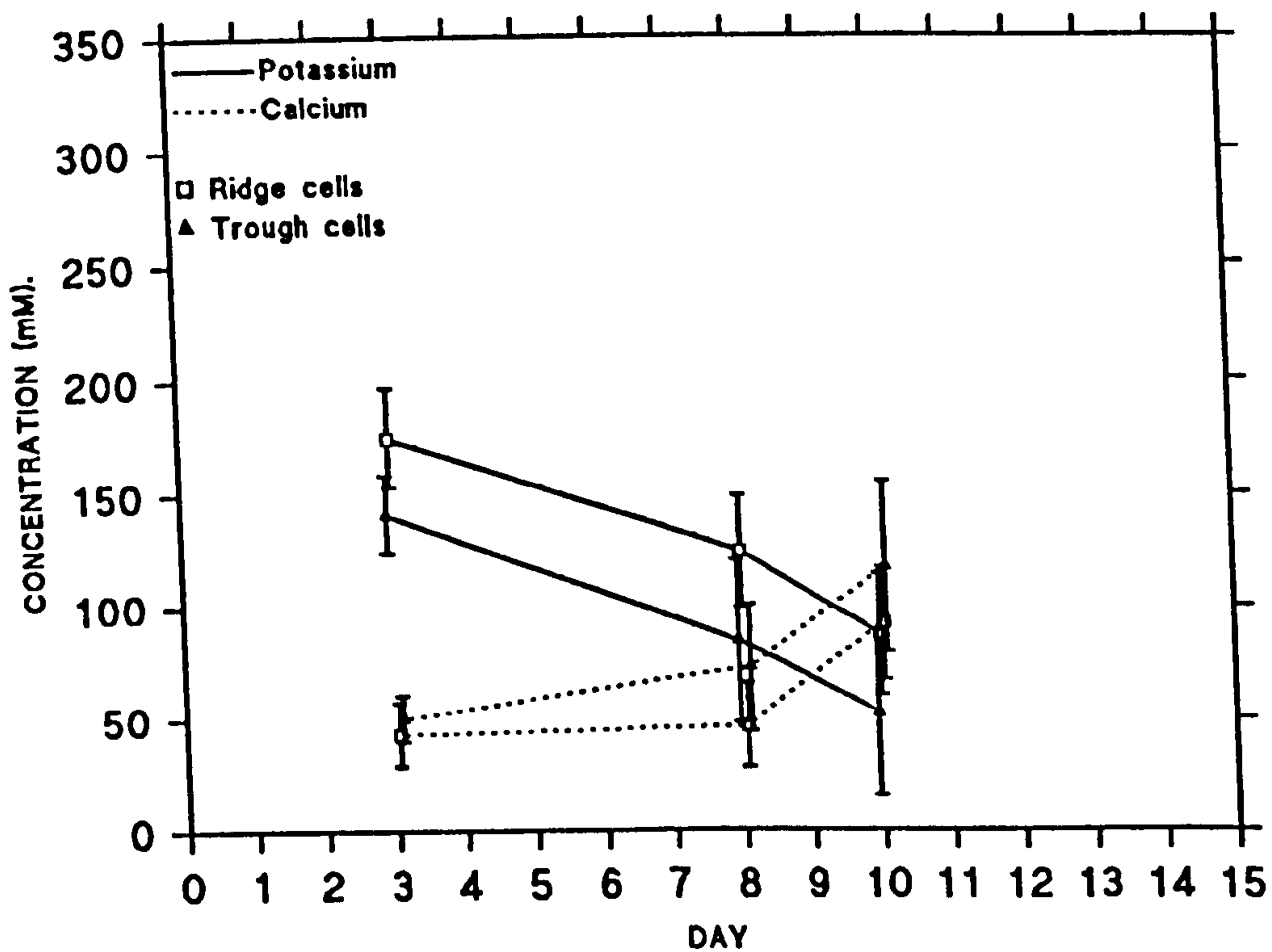
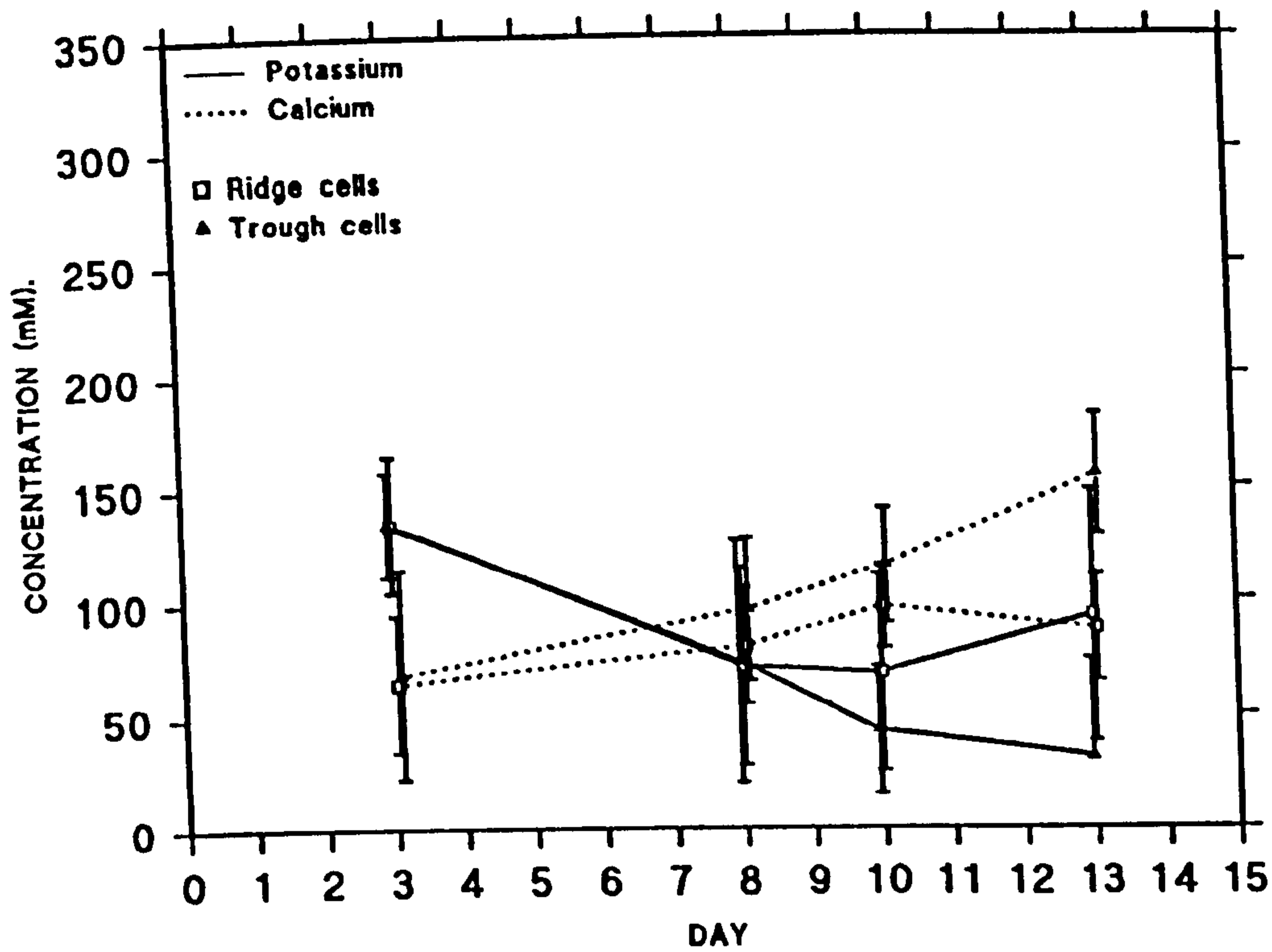


Fig 6.4 The influence of time on the potassium and calcium concentrations within ridge and trough cells of the epidermis of the barley primary leaf grown with 0.2 mM potassium. Two independent experiments are illustrated, a) and b). The single cell values represent the means and standard deviations of 5 cells taken variably from three plants. Each cell measurement was derived from up to 6 subsamples.

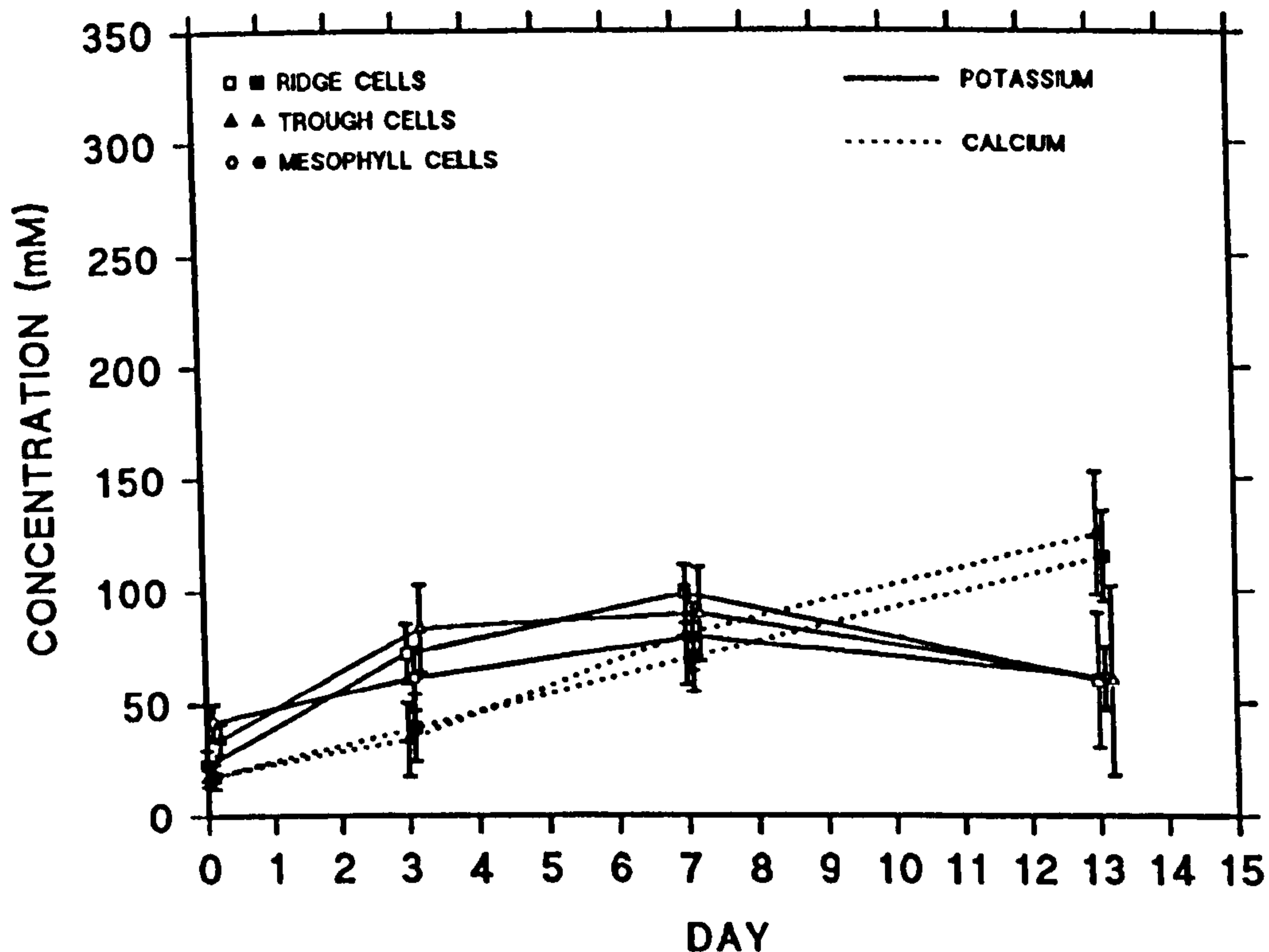


Fig 6.5 Time course of vacuolar potassium and calcium concentrations in barley primary leaf epidermal and mesophyll cells. X-ray microanalysis was performed on frozen whole tissue. Results show the the means and standard deviations of cells derived from at least 6 plants measured on two separate occasions.

The time courses are qualitatively, but not quantitatively, the same as the high K plants. Calcium is accumulated preferentially in epidermal trough cells, whilst potassium shows the reverse trend. Absolute concentrations of vacuolar potassium were lower than in the 4.0 mM K treatment (see chapter 5) although calcium concentrations were similar. Instead of maintaining a fairly steady vacuolar potassium concentration up to day 8, the potassium concentration on both occasions declined. This decline was associated with an increase in the calcium concentration which exceeded the potassium concentration at day 10. Once again the differences were dependent upon cell-type with the trough cells consistently displaying the most extreme changes in vacuolar content (In certain instances, individual trough cells completely emptied of potassium (see table 9.3)). This behaviour would suggest that the gradients of potassium and calcium which occur between ridge cells of plants grown in 4.0 mM K (fig 4.5) also develop with time under conditions of potassium-deficiency.

### 6.6.2 Mesophyll cells

The concentration of inorganic vacuolar solutes within mesophyll cells were measured directly using the frozen-tissue approach of X-ray microanalysis (Section 2.15). In general, potassium concentrations within the mesophyll were similar to the concentrations in the epidermis and were approximately half that of plants grown in 4.0 mM K. (cf Fig 5.2). Unlike these plants, however, the concentration of potassium in the mesophyll apparently declined after day 7 by approximately 30 mM. Calcium could not be detected in the mesophyll cells.

## 6.7 The relationship between the solute concentrations of the epidermis and whole tissue

### 6.7.1 Potassium

The frozen-tissue approach can be used directly to provide values for the inorganic solute content of the mesophyll. In plants measured using the pressure-probe approach these were estimated by comparing epidermal with whole tissue solute concentrations (cf Fig 5.4).

Similar to plants grown in 4.0 mM K (Figs 5.2, 5.4), potassium levels in the epidermal cells decline whilst the levels in the leaf remain constant. This decline is particularly evident in the trough cells (Fig 6.6) where potassium dropped from approximately 130 mM to 50 mM or less. Potassium concentrations also declined in the ridge cells, but to a lesser extent (decreasing by approximately 30 mM). The nature of the approach taken means that it is difficult to interpret where the epidermal potassium is exported to. The early decline of potassium in the potassium-deficient treatment may be linked to the potassium requirements of other cells within the leaf, or to be ready for re-export to other growing organs.

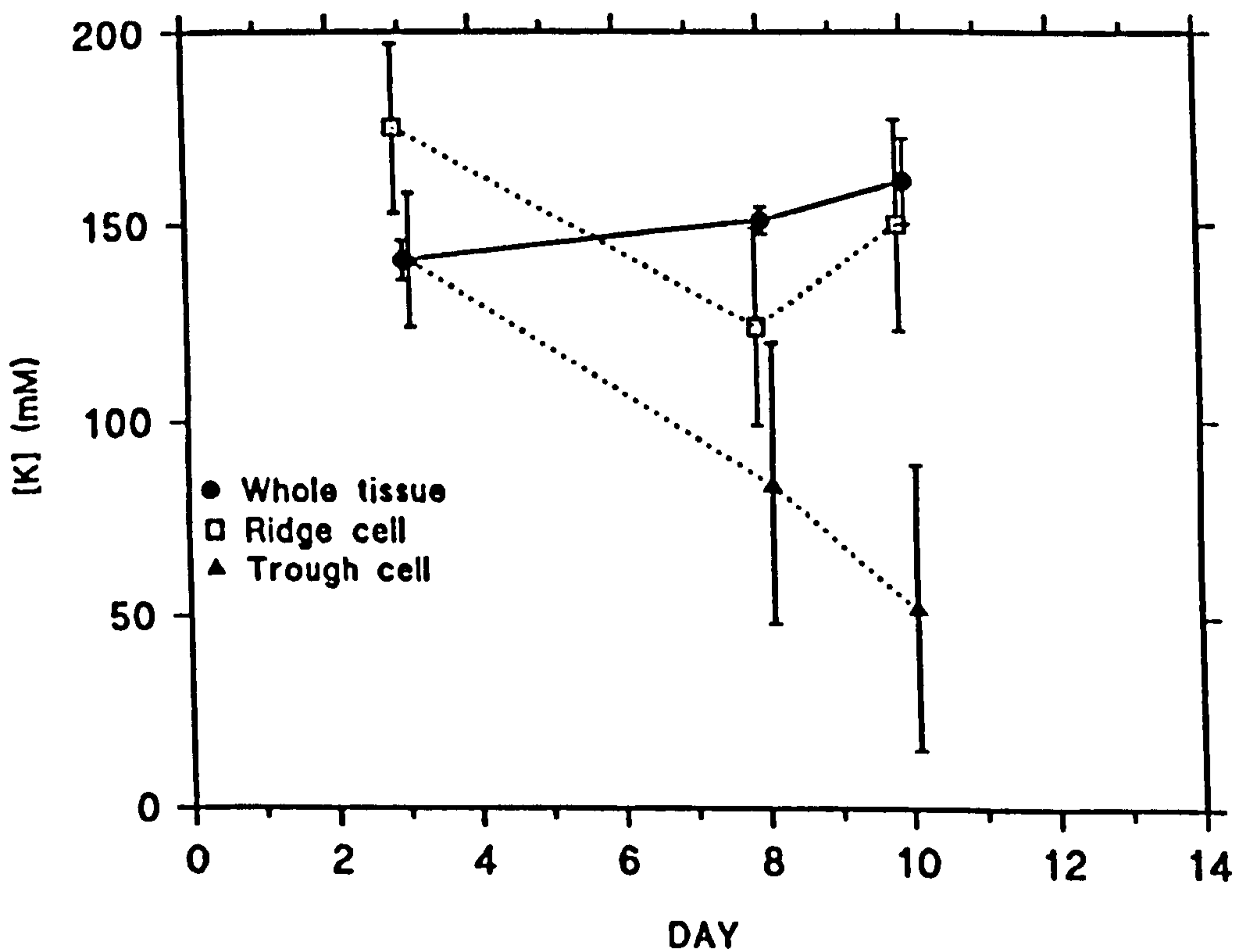
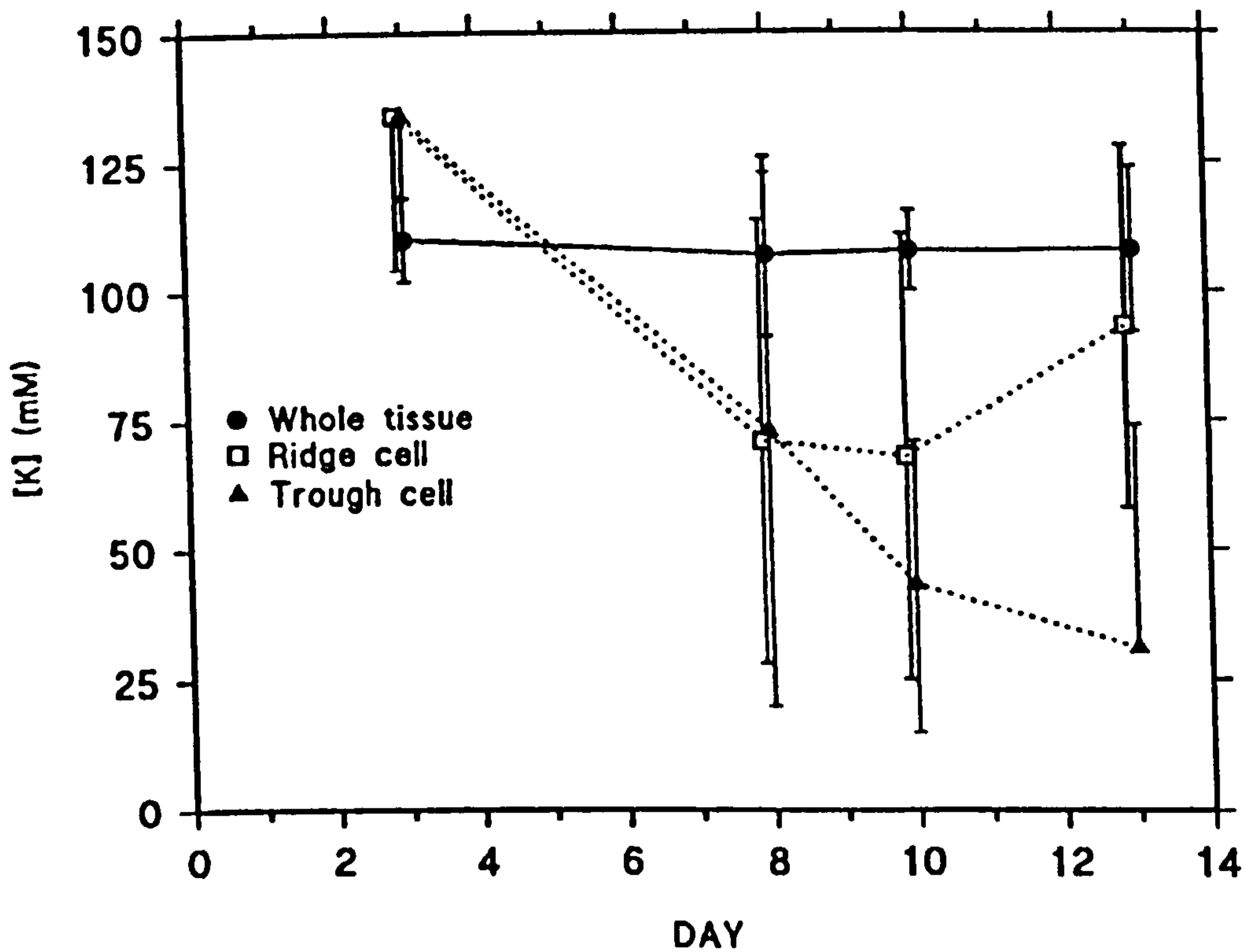


Fig 6.6. Relative changes in whole tissue potassium vs epidermal potassium concentrations throughout the development of the first leaf of barley grown under potassium deficient conditions. Two independent experiments are shown. The whole tissue values were derived from the means of 3 plants. The single cell readings represent the means of 5 cells and each cell value was derived from up to 6 subsamples. All error bars are  $\pm$  SD.

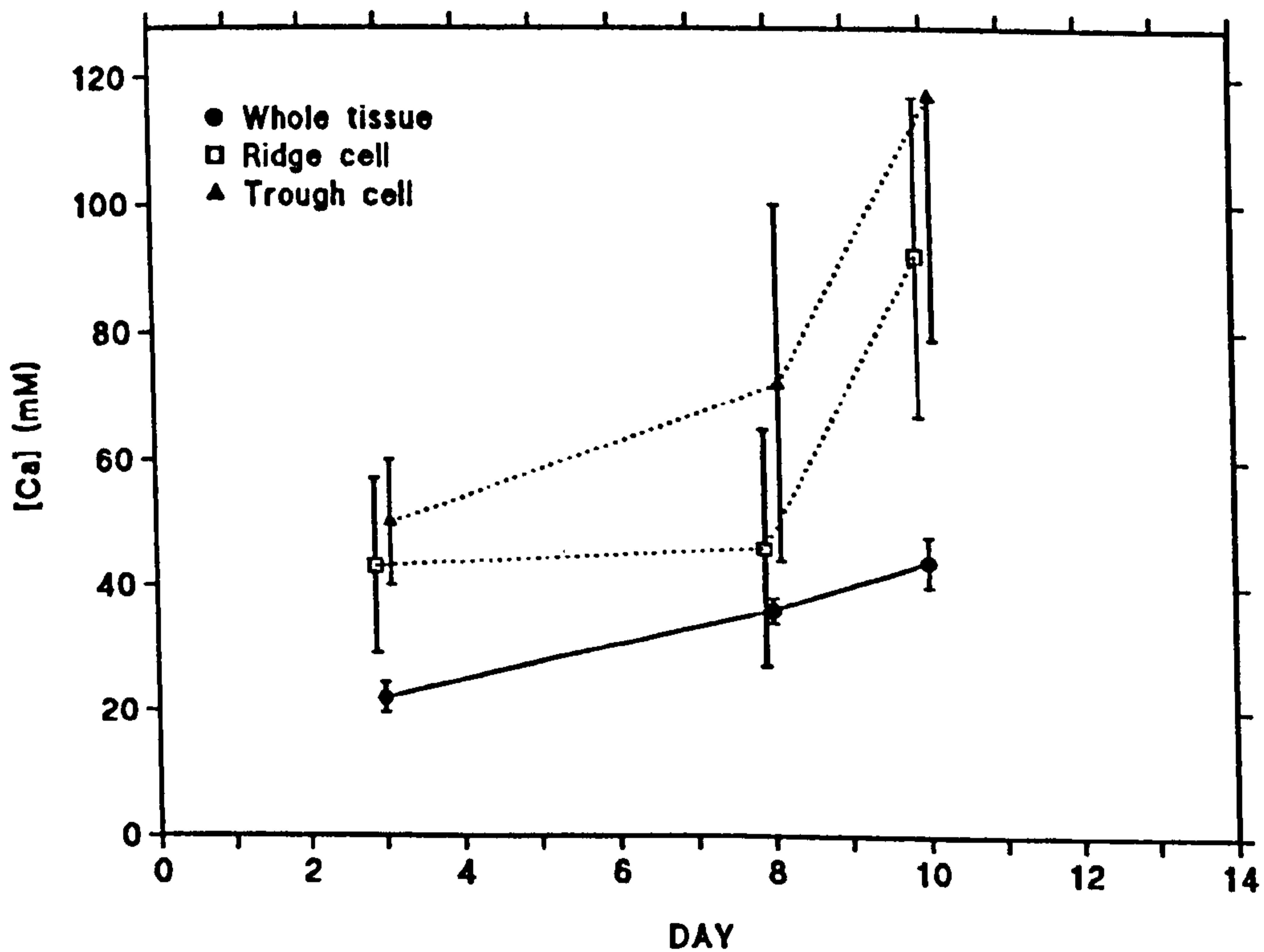
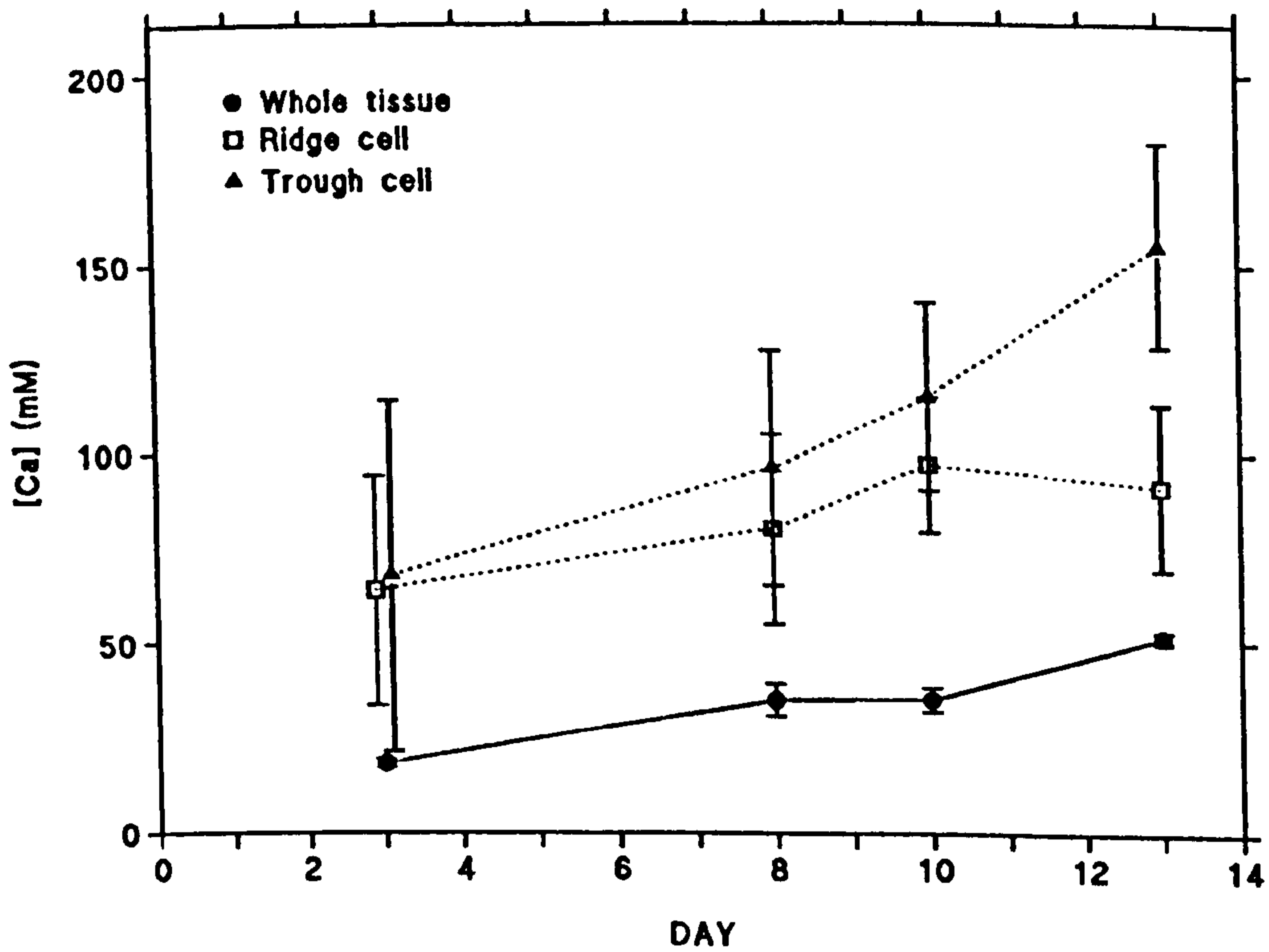


Fig 6.7. Relative changes in whole tissue calcium vs epidermal calcium contents throughout the development of the first leaf of barley grown in 0.2 mM K. Two independent experiments are illustrated. The whole tissue measurements were derived from the means of 3 plants. The single cell readings represent the means of 5 cells and each cell measurement was derived from up to 6 subsamples. All error bars are  $\pm$  SD.

### 6.7.2 Calcium

Similar to the behaviour of plants grown in 4.0 mM K, the calcium concentration in the epidermis increased steadily throughout the development of the leaf. It was always higher in the epidermal cells than the whole tissue, which was consistent with the preferential partitioning of calcium into this tissue (Figs 6.5, 10.1).

### 6.8 Conclusion

The potassium deficiency treatment imposed led to a reduction in leaf potassium. This decline was not accompanied by a corresponding decrease in leaf osmotic pressure showing that osmotic adjustment was occurring. Turgor pressure in epidermal cells was markedly reduced despite this osmotic adjustment.

The reduction in whole leaf potassium concentrations occurred as a result of changes in vacuolar concentrations of potassium in both epidermal and mesophyll cells. This reduced accumulation apparently occurs within most (if not all) cells of the leaf since the pattern of potassium distribution in the epidermis compared to the rest of the leaf remains unchanged. These data show that the mesophyll does not have priority for potassium receipt during leaf development.

Despite having less than half the potassium concentration of the control treatment, the leaf epidermis still exports potassium in the same manner as plants grown in 4.0 mM K. The potassium exported from the epidermis is not, however, accompanied by a net decrease in the whole tissue potassium concentration suggesting that the potassium is merely being redistributed within the leaf. The potassium released does not remain in the apoplast since this would lead to a reduction in turgor over time (see 1.3). If it is transferred into other cells this would suggest that certain cells do indeed have prioritised receipt of potassium, but only at later stages of development. These cells may be involved in transporting rather than accumulating potassium and do not appear to be mesophyll cells since their potassium content also declines with time. It is concluded that potassium export from the epidermis is a consequence of normal plant development.



Calcium accumulation in the leaf was unaffected by potassium nutrition suggesting that it is not able to substitute for potassium as an osmoticum *per se*. The relationship between potassium and calcium throughout leaf development is the subject of Chapter 9.

## **Chapter 7. Vacuolar osmotic pressure and its relationship to turgor pressure**

Potassium-deficiency leads to a reduction in leaf turgor (Table 6.1) greater than the reduction in leaf sap osmotic pressure (Fig 6.2). Furthermore, the epidermis exports potassium from its vacuole during development regardless of its potassium nutrition (Figs 5.1, 6.4) whilst calcium accumulates in the leaf at a constant rate (Fig 6.3). Unless compensated for, movement of any solute within a leaf will produce a corresponding change in osmotic pressure (see Section 1.2.1). The changes in the concentrations of calcium and potassium observed in the vacuole may therefore affect the osmotic pressure of the vacuolar sap. Similarly, changes in the apoplastic concentrations of solutes could lead to changes in cell turgor. To investigate the effect of these solute movements on the cell water relations, the osmotic and turgor pressures of both control and potassium-deficient plants were measured throughout leaf development.

The single-cell water relations data presented in this chapter relate to the single-cell solute concentrations already presented in Chapters 5 and 6. Later, in Chapter 8, the relationship between the measured solutes and osmotic pressure is explored.

### **7.1 Osmotic pressure throughout leaf development**

#### **7.1.1. Plants grown in 4.0 mM K**

The osmotic pressure of sap extracted from ridge and trough cells was analysed throughout the leaf development. On 3 occasions, osmotic pressure was measured in Bangor, whilst a fourth and fifth time course were measured independently in Hannover.

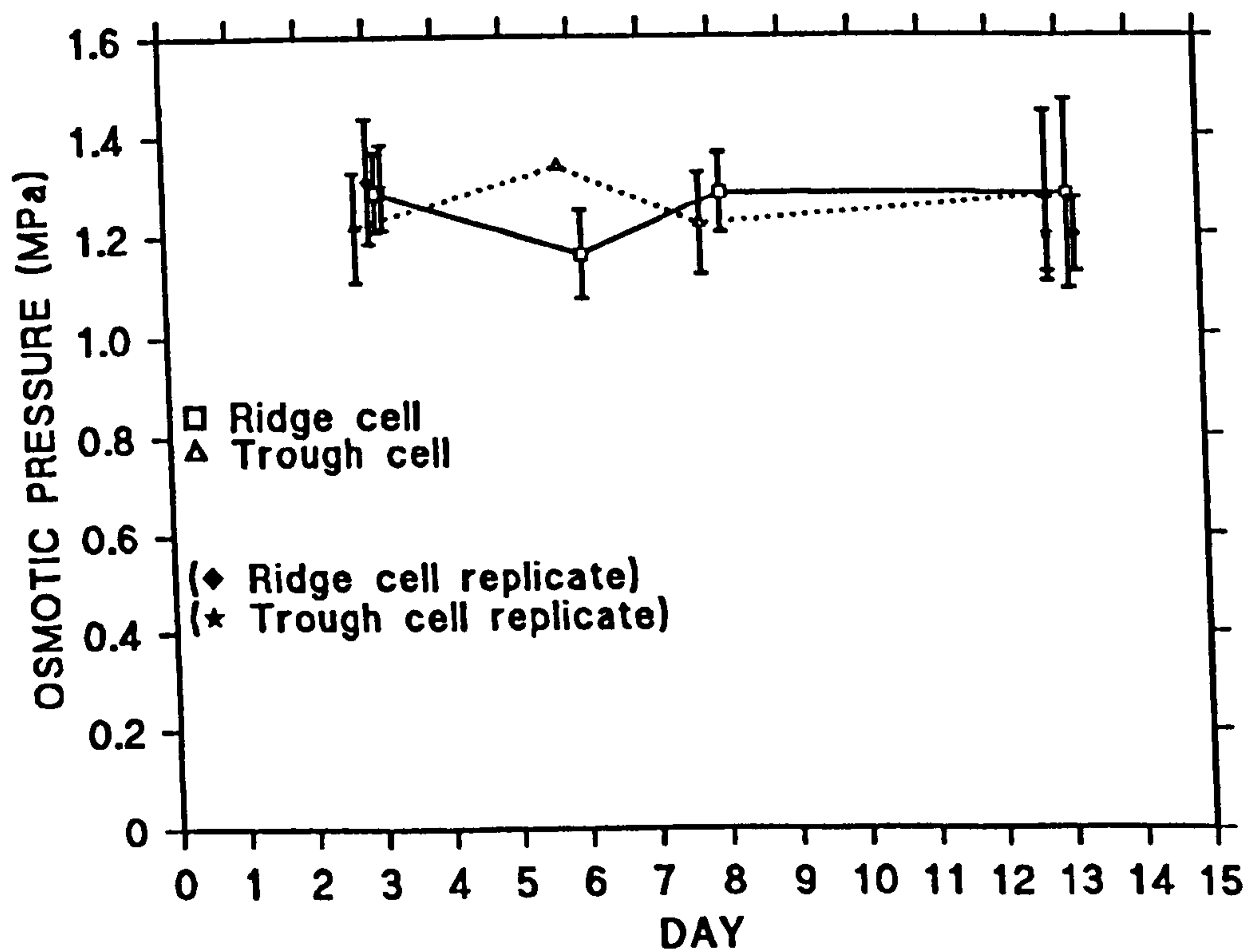
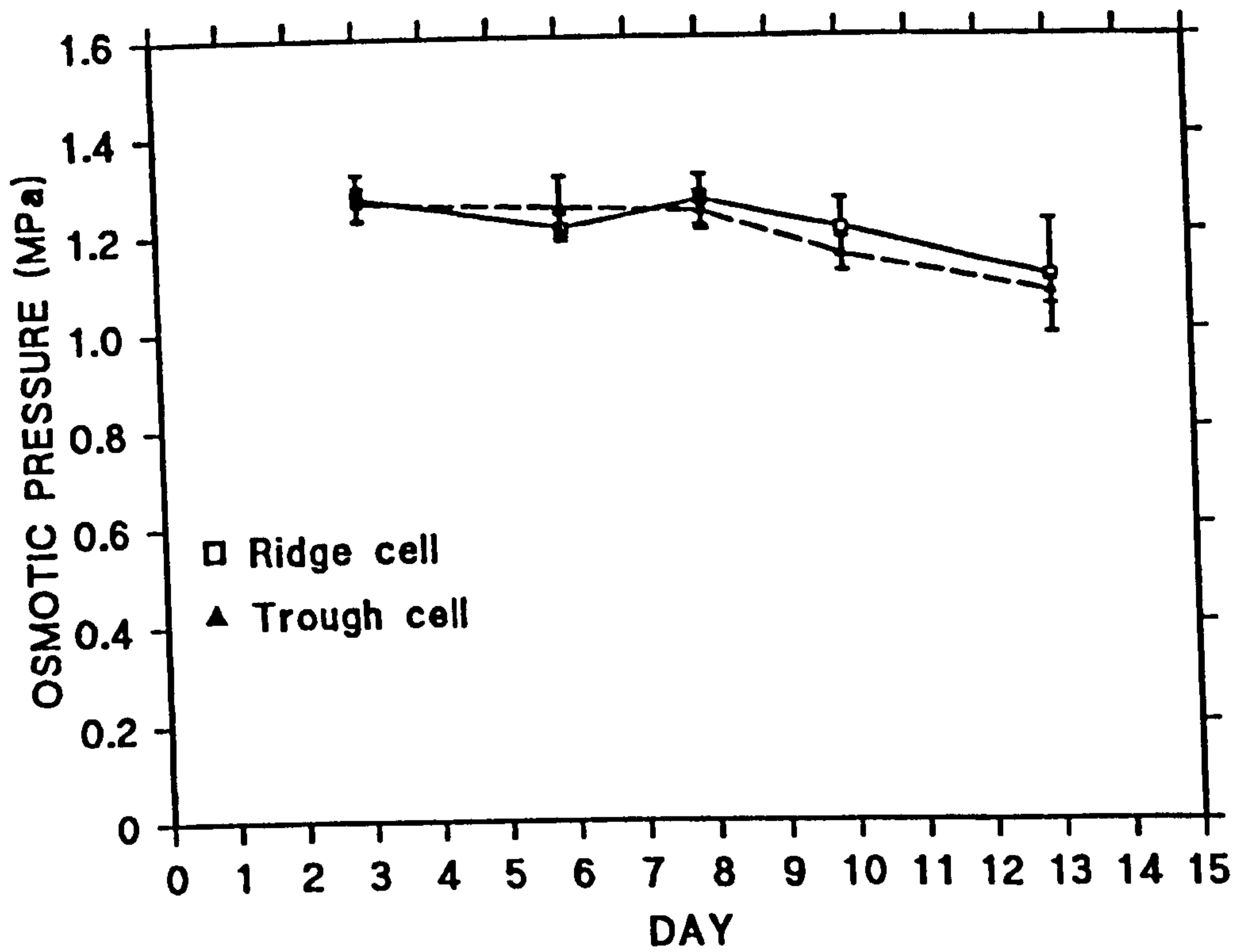


Fig 7.1 Variation of osmotic pressure over time in epidermal cells of barley. In (a), only one plant per measurement date was measured. (b) The experiment was repeated twice with samples being selected from at least three plants per sampling date. Error bars show the SD of the mean of at least 5 cells per sampling date.

A further investigation of osmotic pressure was undertaken in Hannover where growth conditions were more controlled.

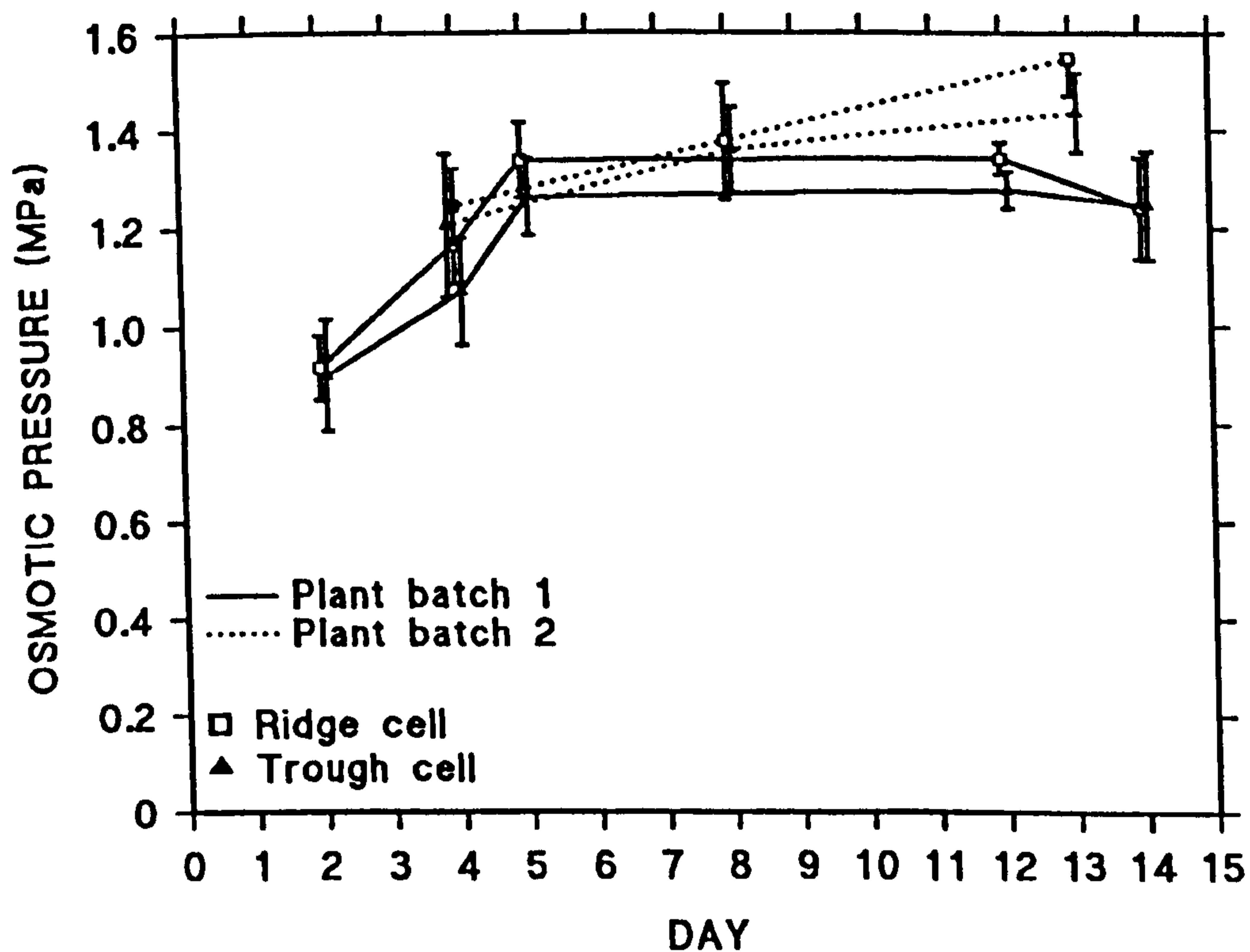


Fig 7.1 c Variation of osmotic pressure over time in epidermal cells of barley plants grown under higher light intensity in Hannover (see text). 3 plants per measurement date were taken. Error bars show the standard deviations of the mean of at least three cells.

The osmotic pressure remained fairly constant throughout development and was close to its maximum value by day 3 (one day before full blade expansion) (Fig 7.1). Osmotic pressure does not decline in the manner suggested by the reduction of potassium from day 8 to day 10 (Fig 5.1). Maintenance of a high osmotic pressure suggests that another solute is being accumulated to replace potassium.

### 7.1.2 Potassium-deficient treatment

The effect of leaf development on osmotic pressure was also investigated in potassium-deficient barley plants. Twice in Bangor (Fig 7.2 a), and twice in Hannover (Fig 7.2 b).

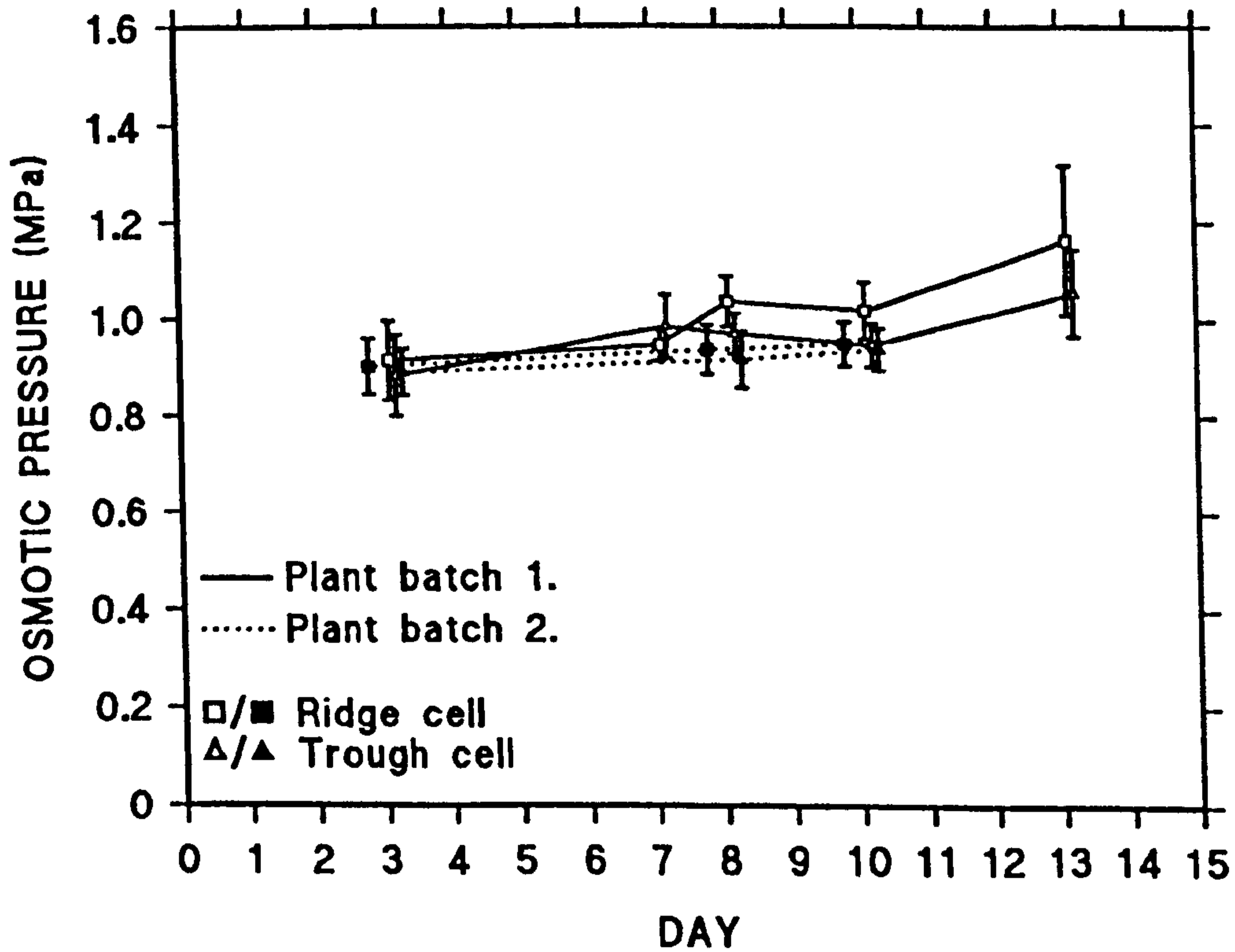


Fig 7.2 a Variation of vacuolar osmotic pressure throughout development in epidermal cells of barley grown under potassium deficient-conditions in Bangor. At least 3 plants were measured with 6 cells per sampling date. The results shown are the mean  $\pm$  SD.

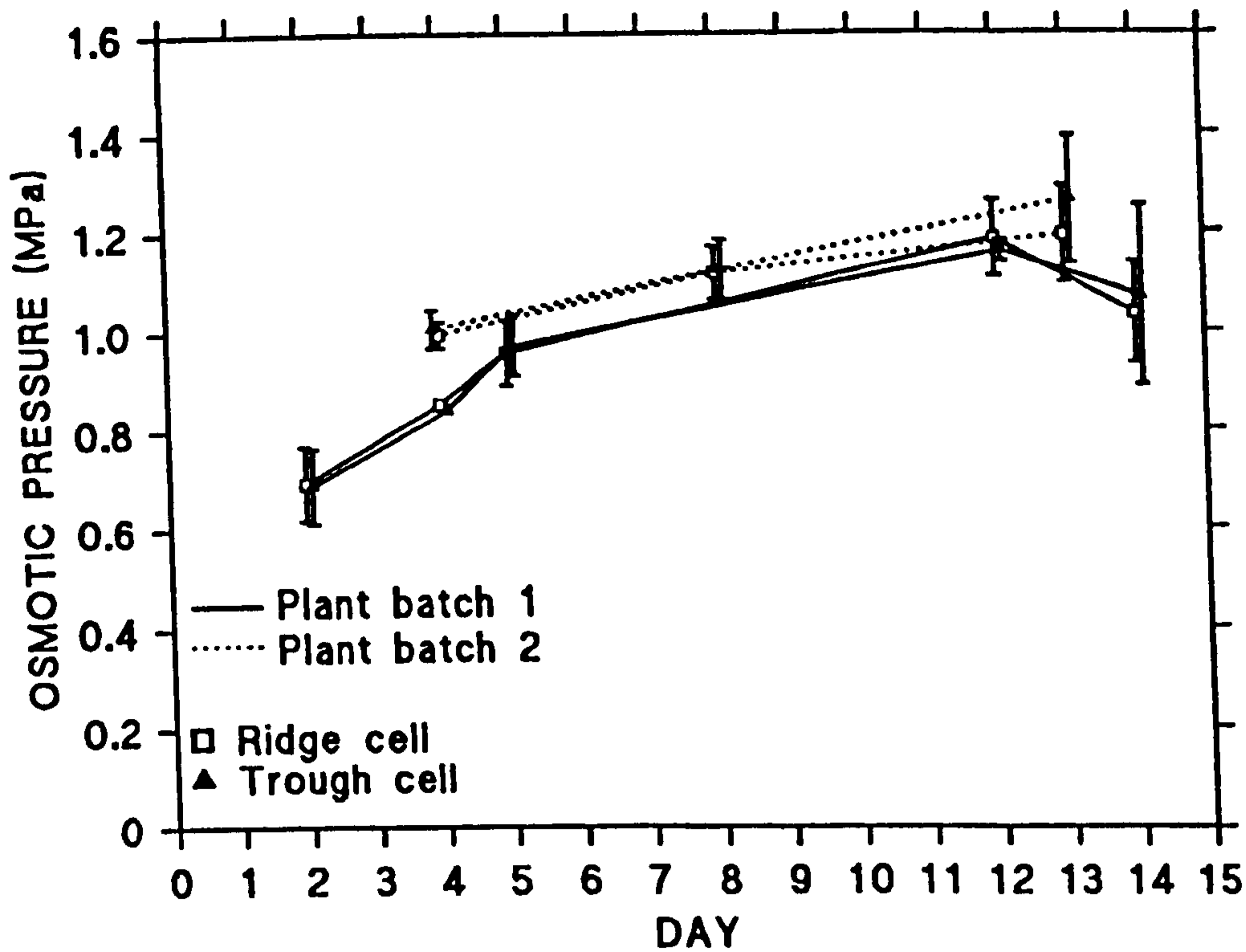


Fig 7.2 b Variation of vacuolar osmotic pressure throughout development in epidermal cells of barley grown under potassium-deficient conditions in Hannover. At least 3 plants were measured with 6 cells per sampling date. The results shown are the mean  $\pm$  SD.

In all measured time courses of osmotic pressure in the potassium-deficient treatment, the osmotic pressure apparently increased throughout leaf development. Hence differences between the control and potassium-deficient treatments were most marked at the early stages of growth. Towards the end of development, the values of osmotic pressure were somewhat comparable, and extrapolation of the graph suggests that they would have equalled the controls within one or two days (senescence permitting).

## 7.2 The relationship between vacuolar osmotic pressure and turgor

Vacuolar osmotic pressure is only one parameter that determines the level of turgor. In order to investigate the relationship between them, turgor and osmotic pressure were measured throughout leaf development. The experiments were performed once in both the high potassium and the low potassium treatments (Fig 7.3).

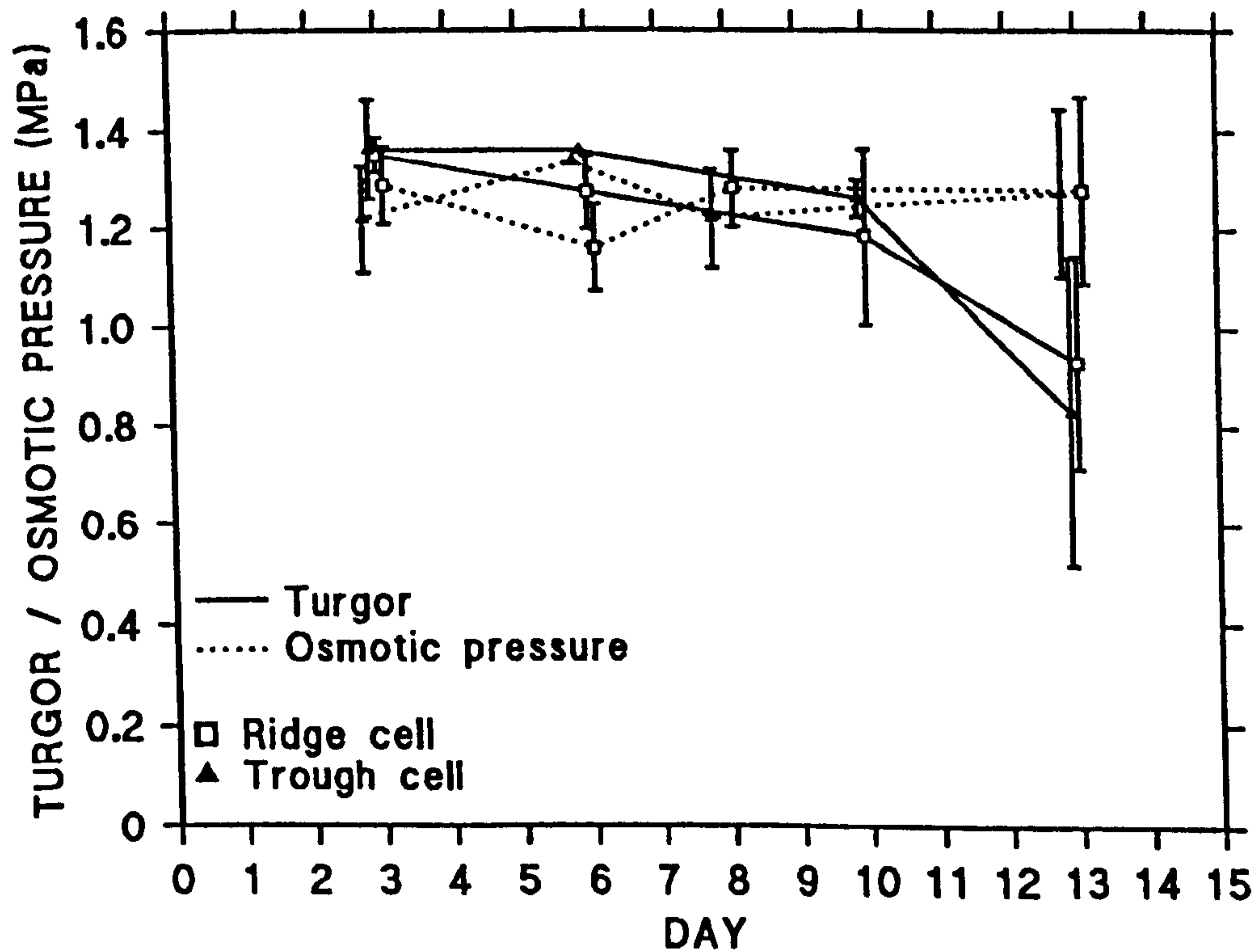


Fig 7.3 a Time course of turgor and osmotic pressure in epidermal cells of primary leaves of barley plants grown in 4.0 mM K. Osmotic pressure values were derived from the same cell in which turgor was measured by using the instant sampling device of the pressure probe (see materials and methods). At least three plants per sampling date were measured. Error bars show the SD of the mean of at least 3 cells.

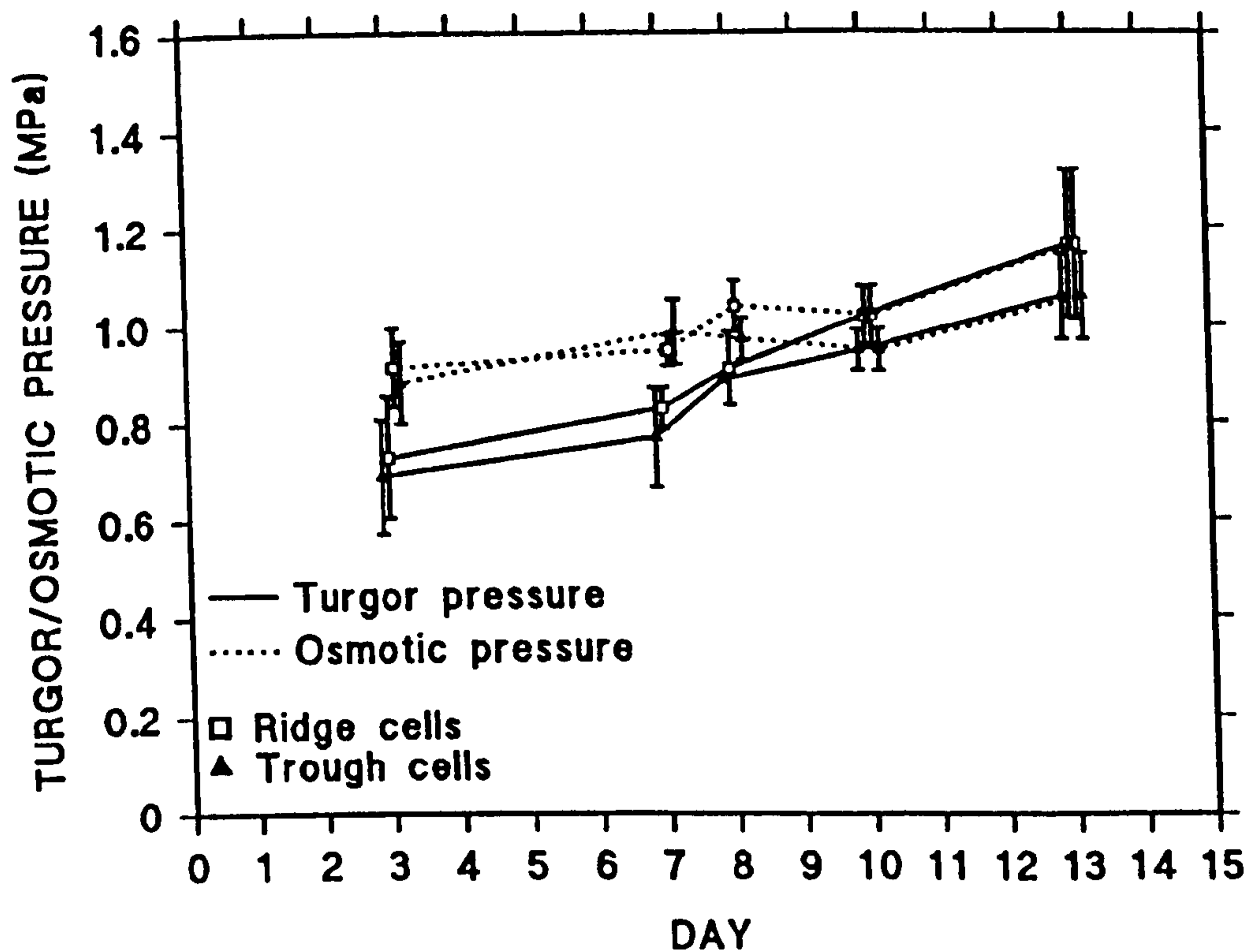


Fig 7.3 b Time course of turgor and osmotic pressure in epidermal cells of primary leaves of barley grown in 0.2 mM K. Osmotic pressure values were derived from the same cell in which turgor was measured by using the instant sampling device of the pressure probe (see materials and methods). At least three plants per sampling date were measured. Error bars show the SD of the mean of at least 3 cells.

The turgor in the control plants was comparable to the osmotic pressure from days 3-10 and was occasionally greater. This is indicative of a degree of sample dilution at the point of sap extraction. Although such dilution is likely to be minimised due to the use of the rapid sampling pressure probe, measured half times were at least as fast as the response of the pressure probe motor/transducer/chart recorder combination and possibly faster than 0.5 s (see Fig 3.1). Even with rapid half times, the errors caused by water influx at the instant of extraction are likely to be small (see 3.1.1). Recorded half times of water influx into epidermal cells of the potassium-deficient treatment were also very fast, but yielded values of turgor which were lower than the measured sap osmotic pressure indicating a cell water potential of 0.1-0.15 MPa.

At day 13, several of the cells of the epidermis had turgor pressures which were lower than osmotic pressure. This decreased wall water potential was possibly a result of solutes leaked into the apoplast by dying cells. The large scatter in the data apparent at day 13 is possibly due to the random nature of cell selection, which in



certain instances led to cells being measured which were adjacent to the necrotic areas. Electron microscopy of frozen leaf tissue at day 13 (Plate 7.1) reveals that turgid cells can exist alongside apparently dead tissue implying complete closure of plasmodesmata. Clearly the actual turgor of these cells may be strongly influenced by their proximity to areas of dead tissue. Cell water potential would decrease if vacuolar solutes were released into the apoplast and would also decrease if distortion of the tissue leads to stomatal malfunction and increased transpiration (Plate 7.1).

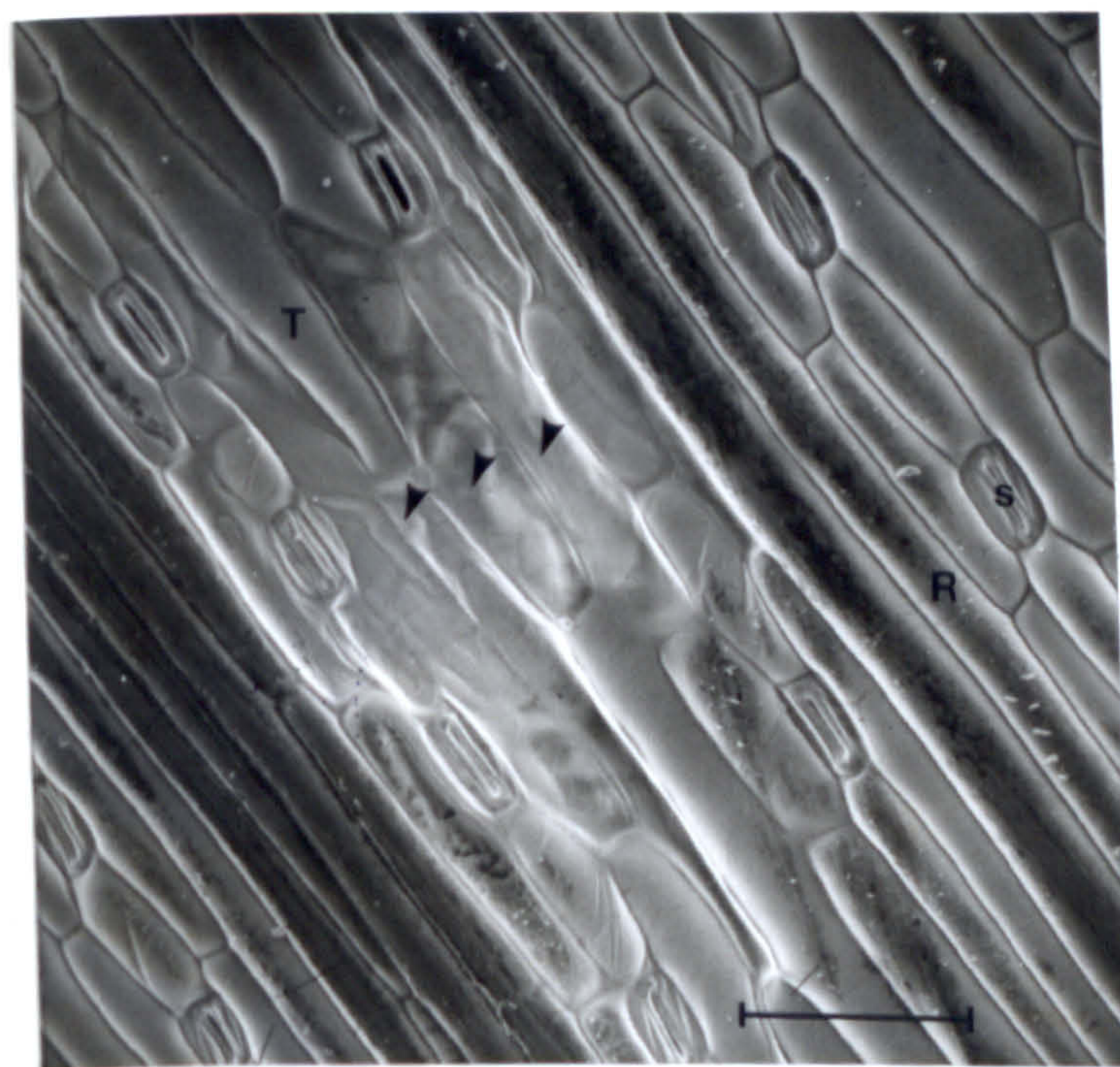


Plate 7.1 Scanning electron micrograph of adaxial epidermis of barley primary leaf at day 13, illustrating the close proximity of apparently turgid cells to dead tissue. Pressure-probe data confirm that these convex cells do indeed have positive turgor pressures (data not shown). Leaf tissue was fixed for photographic purposes by plunging into liquid nitrogen. R = ridge cell, T = trough cell, s = stomata. (dead and dried epidermal cells are arrowed). Scale bar = 100  $\mu\text{m}$ .

Because the osmotic pressure does not increase when the turgor is lowered by apoplastic flooding, this might suggest that osmotic pressure rather than turgor is regulated in the epidermis. Such a conclusion is drawn with some caution however since it ignores the effects of senescence on regulatory systems. The question of whether turgor or osmotic pressure is regulated is further discussed in Section 10.2.1.

In the potassium-deficient treatment turgor increased concomitantly with the increase in vacuolar osmotic pressure. The turgor was always lower than in the control implying that potassium is important for turgor generation and cannot be substituted for early during leaf development. The subsequent increase in osmotic pressure during the period of potassium export suggests the accumulation of an alternative solute. Turgor, however, increased at a faster rate than the osmotic pressure indicating that the water potential of the cell also changed with time. At days 3-7, the water potential of the cell was approximately -0.2 MPa. The water potential subsequently increased to reach zero by day 10. The reduced wall water potential may be a consequence of either increased transpiration or wall solutes. Whatever the cause, in young leaves this acts to limit turgor to below its theoretical maximum for the first 8 days of development. The increasing water potential may be interpreted in several ways. If wall solutes are the cause of the diminished turgor this could be indicative of transport from the apoplast. If, however, transpiration is the cause of the reduced turgor it would suggest that increasing turgor correlates with reduced transpiration. Increased transpiration under potassium-deficiency has been observed in both *Triticum* and *Pisum* (Brag 1972). It may result from inefficient functioning of stomata when the turgor of the epidermis is low (Meidner 1990).

## **Chapter 8. The components of vacuolar osmotic pressure**

The developmental decrease in potassium concentrations (Chapters 5 and 6) without corresponding decreases in osmotic pressure (Chapter 7) shows that the potassium is replaced in its osmotic role. It was hypothesised that this role was played by the calcium accumulated because calcium was observed to increase as the potassium concentration declined. The contribution of potassium and calcium to the vacuolar osmotic pressure depends both upon their concentration and the nature of the anions with which they are associated.

Osmotic pressure due to potassium and calcium would be maximised if these cations were associated with fully soluble monovalent anions such as nitrate and chloride. In fact, these anions have been shown to accumulate to high concentrations in whole tissue (Figs 4.1, 6.1) and therefore support the hypothesis that the potassium and calcium accumulated are associated with monovalent anions. However, tissue heterogeneity (Chapters 4,5 and 6) would suggest that whole tissue solute measurements are not necessarily a good indicator of individual cell solute measurements thus an investigation was made of the inorganic ion complement (K,Ca,Cl,NO<sub>3</sub><sup>-</sup>) in vacuolar samples from the epidermis (Table 8.1). In addition, the role of calcium as an osmoticum in cells was specifically investigated (Tables 8.2, 8.3).

### **8.1 The inorganic anion composition of vacuolar sap**

The inorganic ion complement of epidermal vacuolar sap at day 3 and 13 was measured by a combination of X-ray microanalysis and microfluorescence techniques. To ensure that a sufficient volume of sap was available for analysis up to 4 saps from each cell type were pooled. Osmotic pressure was measured immediately thereafter, and then the samples were diluted approximately 1:1 with water and their osmotic pressures were remeasured. Subsamples were taken for immediate preparation for X-ray microanalysis whilst other subsamples were stored for subsequent nitrate

determinations over the following two weeks<sup>5</sup>. This meant that for a certain number of cells both potassium and calcium could be accounted for together with the osmotic pressure and monovalent anion complement of chloride and nitrate (Table 8.1).

### DAY 3

CELL TYPE	OSMOTIC PRESSURE (mOsm kg <sup>-1</sup> )	[NO <sub>3</sub> ] (mM)	[Cl] (mM)	[K] (mM)	[Ca] (mM)	[Cl + NO <sub>3</sub> ] (mM)	[K + Ca] (mM) monovalent cation equivalents
R	509	248 ± 34	32 ± 10	232 ± 11	29 ± 16	280 ± 44	290 ± 43
R	511	226 ± 30	36 ± 25	210 ± 7	10 ± 13	230 ± 37	297 ± 34
R	513	195 ± 53	64 ± 6	271 ± 20	32 ± 8	262 ± 55	230 ± 33
R	569	270 ± 26	39 ± 8	277 ± 13	34 ± 17	309 ± 34	345 ± 45
T	524	207 ± 29	23 ± 8	223 ± 14	37 ± 10	230 ± 37	297 ± 34
T	529	258 ± 40	29 ± 7	264 ± 17	23 ± 13	287 ± 47	310 ± 43
T	521	190 ± 81	12 ± 12	222 ± 19	7 ± 10	202 ± 93	236 ± 39

### DAY 13

CELL TYPE	OSMOTIC PRESSURE (mOsm kg <sup>-1</sup> )	[NO <sub>3</sub> ] (mM)	[Cl] (mM)	[K] (mM)	[Ca] (mM)	[Cl + NO <sub>3</sub> ] (mM)	[K + Ca] (mM) monovalent cation equivalents
R	549	208 ± 42	110 ± 46	330 ± 77	73 ± 17	318 ± 88	476 ± 111
R	528	233 ± **	40 ± 8	285 ± 51	95 ± 46	273 ± **	475 ± 143
R	525	262 ± 57	41 ± 7	158 ± 14	80 ± 25	303 ± 64	318 ± 64
T	528	198 ± 53	59 ± 26	198 ± 30	105 ± 28	257 ± 79	408 ± 86
T	521	229 ± 53	79 ± 6	152 ± 9	120 ± 12	308 ± 59	392 ± 33

Table 8.1 Analyses of the vacuolar composition of barley epidermal vacuole samples at (a) day 3, and (b) day 13 in plants grown in 4.0 mM K. R = ridge cells, T = trough cells. Results show the means and standard deviations of at least three subsamples per measurement. Nitrate measurements were performed by Mr Paul Richardson, UCNW Bangor. (For method see Richardson, 1993).

<sup>5</sup> To allow for possible evaporation over this period, samples had their osmotic pressures remeasured immediately prior to each nitrate assay. If any change in the osmotic pressure was observed nitrate and X-ray microanalysis derived concentrations were adjusted to correspond to the original osmotic pressure.

Potassium and nitrate are the dominant anions in the barley epidermal vacuole hence potassium is generally balanced by a monovalent anion. At day 3 the net measured charge balance is close to zero showing that the measured anions balance the measured cations. By day 13 calcium has accumulated in all cells and the positive and negative charges no longer balance. The difference between charges suggests that calcium accumulation is not accompanied by a proportional increase in monovalent anions.

The data can be further interpreted. Firstly, because the anion balance was larger than the potassium concentration, it is possible that some calcium is balanced by monovalent anions and the rest balanced by an ion not measured here (possibly malate). A second interpretation is that the data is misleading. This stems from the large additive errors associated with the measurements themselves. Two different approaches were taken in order to resolve the problem. Firstly, experimental errors were reduced (Table 8.2), and secondly, calcium was demonstrated to be an osmoticum (Table 8.3).

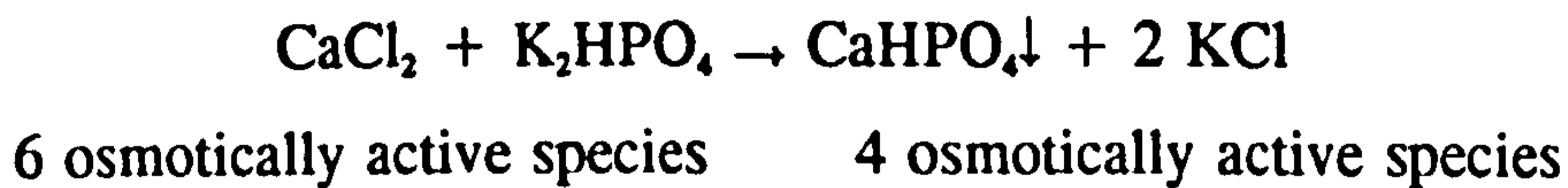
Table 8.2 illustrates several examples of the vacuolar composition of several cells grown in 0.2 mM K. By day 10 these cells have exported the majority of their potassium (Fig 6.4) and in several instances the potassium concentration in several cells declines to zero whereas the calcium concentration increases to over 100 mM. In addition, substantial quantities of chloride can be observed. This chloride cannot be balanced by potassium and it would therefore seem likely that it is in association with the calcium. Measurement of K, Ca and Cl from the same cell in this instance has the advantage that errors are not introduced by cell to cell variation. Similarly, additive measurement errors are reduced. The chloride concentration measured does not necessarily represent the maximum amount of soluble calcium present because this will also depend on nitrate which was not measured.

Cell Type (day)	[Cl] (mM)	[K] (mM)	[Ca] (mM)
R (10)	156 ± 20	53 ± 10	135 ± 20
R (10)	129 ± 9	11 ± 4	117 ± 10
T (10)	158 ± 15	4 ± 2	151 ± 8
T (10)	216 ± 17	53 ± 4	133 ± 10
T (10)	96 ± 13	18 ± 9	109 ± 21
T (10)	112 ± 12	43 ± 8	98 ± 16
T (10)	151 ± 21	44 ± 6	122 ± 21
T (10)	193 ± 18	4 ± 7	146 ± 22
T (13)	218 ± 16	14 ± 5	149 ± 15
T (13)	206 ± 33	-1 ± 7	173 ± 28
T (13)	144 ± 9	-2 ± 5	181 ± 13
T (13)	185 ± 11	2 ± 5	165 ± 13

Table 8.2 Surplus of chloride over potassium concentrations in vacuoles of epidermis at days 10 and 13. It is proposed that the chloride is in association with the calcium in the vacuole, making it fully soluble. R = ridge cells, T = trough cells. Results show the mean and standard deviation of 3-6 subsamples per sample analysed.

## 8.2 Further evidence that soluble calcium is accumulated

If soluble calcium is accumulated in the epidermal vacuole as an osmoticum, it follows that removal of that calcium from the vacuolar sap will decrease its osmotic pressure. This reasoning formed the basis of the second experiment (Table 8.3). Calcium was removed from solution by precipitation as calcium phosphate<sup>6</sup> according to the following reaction scheme:



Using micropipettes, phosphate solutions of known osmotic pressure were added to equal volumes of vacuolar sap of known osmotic pressure. The resulting osmotic pressure (assuming no reaction) would simply be the average of the two original osmotic pressures. Any decrease in osmotic pressure below that predicted would indicate the presence of calcium in a soluble form in the sample. Calcium chloride and 'non-reactive' potassium nitrate were used as controls. In order to maximise the reduction of osmotic pressure the phosphate concentration was chosen to be as close as possible to the predicted calcium concentration (approx 120 mM at day 11 in trough cells of the potassium-deficient treatment).

Vacuolar sap from plants of the low potassium treatment was collected from trough cells at day 11 and pooled.

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<sup>6</sup> The solubility of  $\text{CaHPO}_4$  in cold water is 0.001 g /100 ml (Weast 1980).

SOLUTION MIXED WITH $K_2HPO_4$	ORIGINAL OSMOTIC PRESSURE (mOsm $kg^{-1}$ )	PREDICTED OSMOTIC PRESSURE (assuming no reaction with added $K_2HPO_4$ )	MEASURED OSMOTIC PRESSURE (mOsm $kg^{-1}$ )
$K_2HPO_4$	347	347	341
$KNO_3$	258	303	298
$CaCl_2$	310	329	265
Sample	443	395	337

**Table 8.3** Demonstration of the solubility of calcium present within the vacuole of trough cells of 13 day old barley. Precipitation of calcium phosphate leads to a decline in the osmotic pressure where calcium is present in a soluble form.

The mean osmotic pressure was close to that predicted when the potassium phosphate was mixed either with itself or with potassium nitrate. This observation shows that precipitation has not occurred in these cases which is expected. When potassium phosphate was added to the calcium chloride solution a reduction in the mean osmotic pressure of 64 mOsmoles was obtained. This reduction is consistent with the precipitation of insoluble calcium phosphate. Similarly, a reduction of 58 mOsmoles is obtained upon adding the potassium phosphate to the vacuolar sample. This shows that a significant proportion of the calcium accumulated inside the vacuole is in an osmotically active soluble state. It is difficult to further quantify the extent of calcium solubility from the information given. Earlier results suggest that the trough cell vacuolar sap measured would have a calcium concentration of approximately 120 mM (this would have an osmotic pressure of 288 mOsm $kg^{-1}$  if fully soluble and associated with monovalent anions). The osmotic pressure of the cell at this date was measured to be 440 mOsm  $kg^{-1}$ . Thus at a maximum, 65% of the measured osmotic pressure at this date will be due to calcium, and 35% to other solutes. Clearly, mixing equal volumes at equal concentrations may lead to complete precipitation of calcium, yet the extent of the decrease in osmotic pressure observed will be influenced by the concentration of the other solutes present. The situation is further complicated if the



phosphate concentration does not exactly equal the calcium concentration. In this instance, unreacted salts will lessen the decrease in osmotic pressure that may be obtained, even after full precipitation of either. Because the precipitation method will never overestimate the quantity of soluble calcium, the data show that substantial quantities of soluble calcium can be accumulated in epidermal vacuoles.

Collectively the data presented in Tables 8.1-8.3 show that soluble calcium can exist in cells. It also shows that some of this calcium will be balanced by monovalent anions. In order to understand the importance of potassium and calcium as osmotica, it was assumed that all the potassium and calcium were balanced by monovalent anions and the *maximum* osmotic pressure that could be generated was calculated. It is therefore possible to calculate the proportion of vacuolar osmotic pressure that could maximally be accounted for by potassium and calcium throughout leaf development.

### 8.3 Determination of the osmotic-coefficient of mixed solutions

The van't Hoff equation relating osmotic pressure to the concentration of a solute has been described in Section 1.2.3. The equation includes a constant that accounts for the deviation of solutions from ideal behaviour. This constant, termed the osmotic-coefficient provides a measure of the osmotic pressure that a given solution of a salt of known concentration (expressed as its molality) will impart. The value is concentration dependent and temperature dependent, (although the effects that these factors have is negligible in comparison to the errors associated with measurement).

After having assumed that the measured cations are balanced by monovalent anions, it is desirable to be able to predict the osmotic pressure that a given mixed solution of salts (representing the vacuolar constituents) will impart. A value for a quasi-osmotic coefficient (qoc) was determined empirically. It does not represent the true osmotic-coefficient for the solutions because the concentration units on which it

was calculated are millimolar rather than millimolal<sup>7</sup> hence the calculated coefficient also includes a general correction for the units of concentration. With the given assumptions, this correction factor allows for direct translation of salt concentrations into osmotic pressure.

A range of values for vacuolar concentrations of potassium, calcium and chloride were obtained from plants varying in age from day 3 to day 13. Using as extensive a range of these values as possible, a set of artificial vacuolar saps was prepared, containing chloride at the concentration measured by X-ray microanalysis with the anionic balance being met by nitrate. The osmotic pressure of these solutions was measured.

The qoc was determined by measuring the real osmotic pressure of the artificial sap on the stage of the nanolitre osmometer, and expressing this as a percentage of that expected if no corrections were necessary. (For instance, if a 100 mM solution of potassium nitrate had an osmotic pressure of 180 mOsmoles, its calculated qoc would be 0.9). The saps measured had K/Ca ratios ranging from 0.7-9.1. (see Fig 8.1).

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<sup>7</sup> If potassium nitrate and calcium chloride are considered, the difference in concentration between a 200 millimolar solution and a 200 millimolal solution is less than 3% hence the qoc used here approximates closely to the real osmotic coefficient.

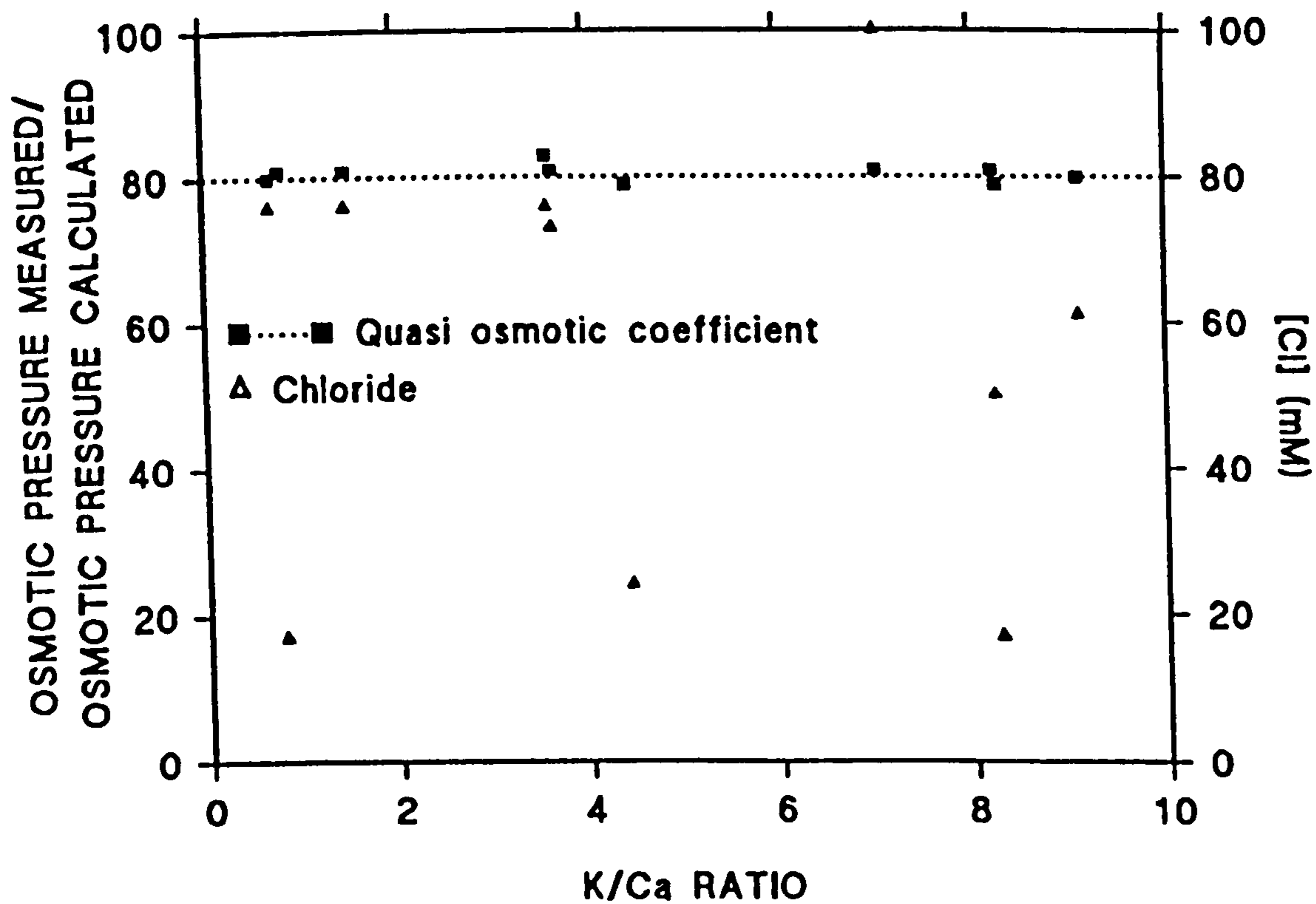


Fig 8.1 Determination of the quasi-osmotic coefficient for mixed monovalent solutions representing artificial sap. Solutions were prepared according to the range encountered within the barley epidermis throughout its development, and contained chloride and nitrate concentrations of varying proportions. The chloride concentration used is shown on the graph, and demonstrates that the quasi-osmotic coefficient is apparently little influenced by the potassium/ calcium ratio or the chloride/ nitrate ratio.

The qoc is fairly constant over the range of K/Ca ratios investigated. The second y axis shows the varying proportion of chloride associated with the samples and shows that the nature of the monovalent anion accumulated is not an important determinant of osmotic pressure insofar as nitrate and chloride are concerned.

#### 8.4 Are the assumptions justified?

If the assumption of association with monovalent anions were correct, then the calculated osmotic pressure values should closely match the real values measured at the point of sap extraction. Fig 8.2 shows the difference in osmotic pressure between real vacuolar saps and artificial vacuolar saps prepared so as to contain only monovalent anions.

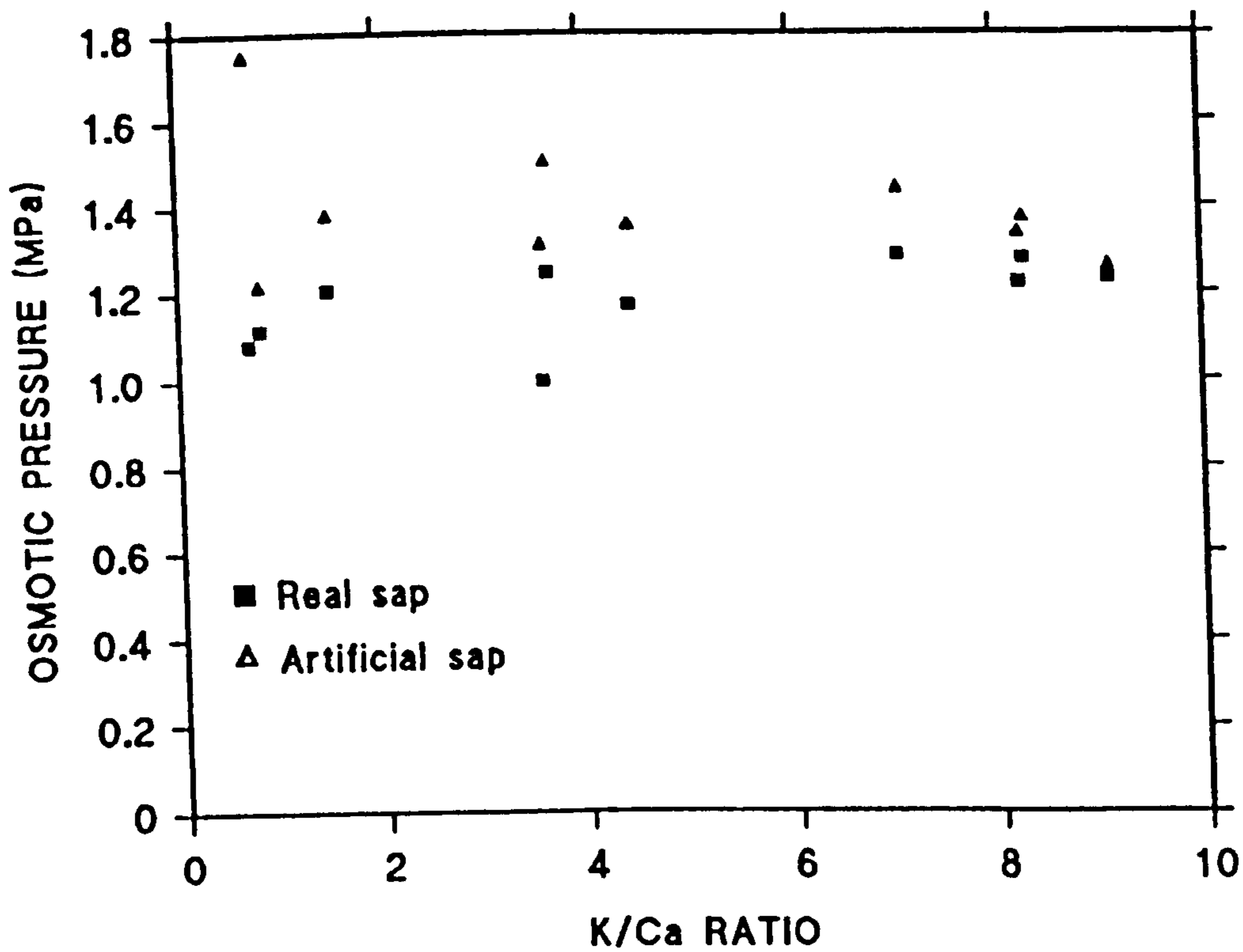


Fig 8.2. Discrepancy between measured osmotic pressure of real and artificial vacuolar saps. Artificial saps were prepared using chloride and nitrate salts of potassium and calcium.

The osmotic pressures of the artificial saps were close in value to the osmotic pressures of the real sap, but in general slightly higher. This discrepancy would suggest that a proportion of the vacuolar cations were not balanced by monovalent anions. This proportion must be small (approximately 10 to 15%). It is not possible to draw certain conclusions from the data since the results are influenced by errors introduced when the real vacuolar sap was analysed for their potassium and calcium content. Although these errors are generally less than 10% for each ion, they are additive and could easily have accounted for the 10 to 15% discrepancy. Generally the data support the hypothesis that vacuolar potassium and calcium *are* the main cationic osmotica contained within the epidermis and that they *are* generally balanced by monovalent anions. It has therefore proved possible to calculate the maximum osmotic pressure that potassium and calcium can generate. This calculated osmotic pressure has been expressed as a percentage of the real osmotic pressure. Hence the contribution of potassium and calcium to the vacuolar osmotic pressure has been studied throughout leaf development.

## 8.5 The maximum contribution of potassium and calcium to the vacuolar osmotic pressure

The maximum possible contribution of potassium and calcium to the vacuolar osmotica was calculated from the measured vacuolar concentrations by assuming that these cations were balanced by monovalent anions (Sections 8.1, 8.2) and an osmotic coefficient of 0.805 (Section 8.3). The data were expressed as a percentage of the osmotic pressure actually measured in the same samples. It was not possible to obtain simultaneous single cell osmotic pressure measurements in those plants analysed by the frozen tissue approach (Figs 8.3c, 8.4c) hence osmotic pressure values for these calculations were obtained from other plants at the same sampling date.

Fig 8.3 shows the maximum percentage osmotic pressure that can be accounted for by potassium and calcium in plants grown in 4.0 mM K. The corresponding data for plants grown in 0.2 mM K are shown in Fig 8.4.

In general, the osmotic pressure has been fully accounted for by the vacuolar potassium and calcium the epidermal cell vacuoles in plants grown in both 4.0 mM and 0.2 mM K. There is no consistent difference between the ridge and trough cells.

The osmotic pressure accounted for in those plants grown for analysis by the frozen-tissue approach was slightly less than in those analysed by the pressure-probe. It is not clear whether this difference is real and a consequence of differing growth conditions or whether this is a consequence of underestimation of the vacuolar potassium and calcium concentrations. These arguments are further addressed in Chapter 10.

## 8.6 Summary

Both potassium and calcium can accumulate in barley epidermal cells. Potassium accumulates initially, but thereafter is replaced by calcium as potassium is exported from the cell. The evidence suggests that the potassium and calcium within the epidermal cell vacuole are largely balanced by monovalent anions. This being so,

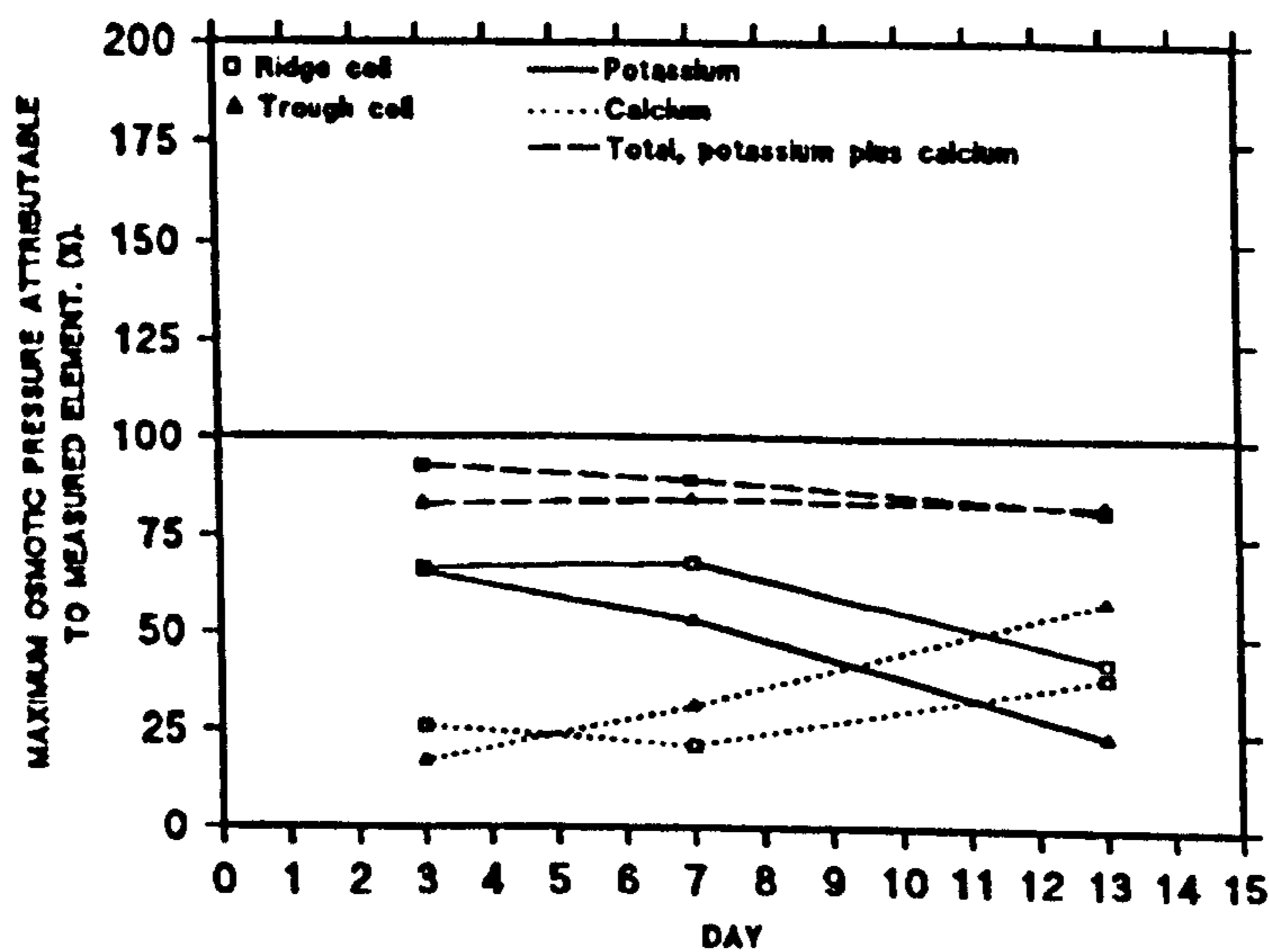
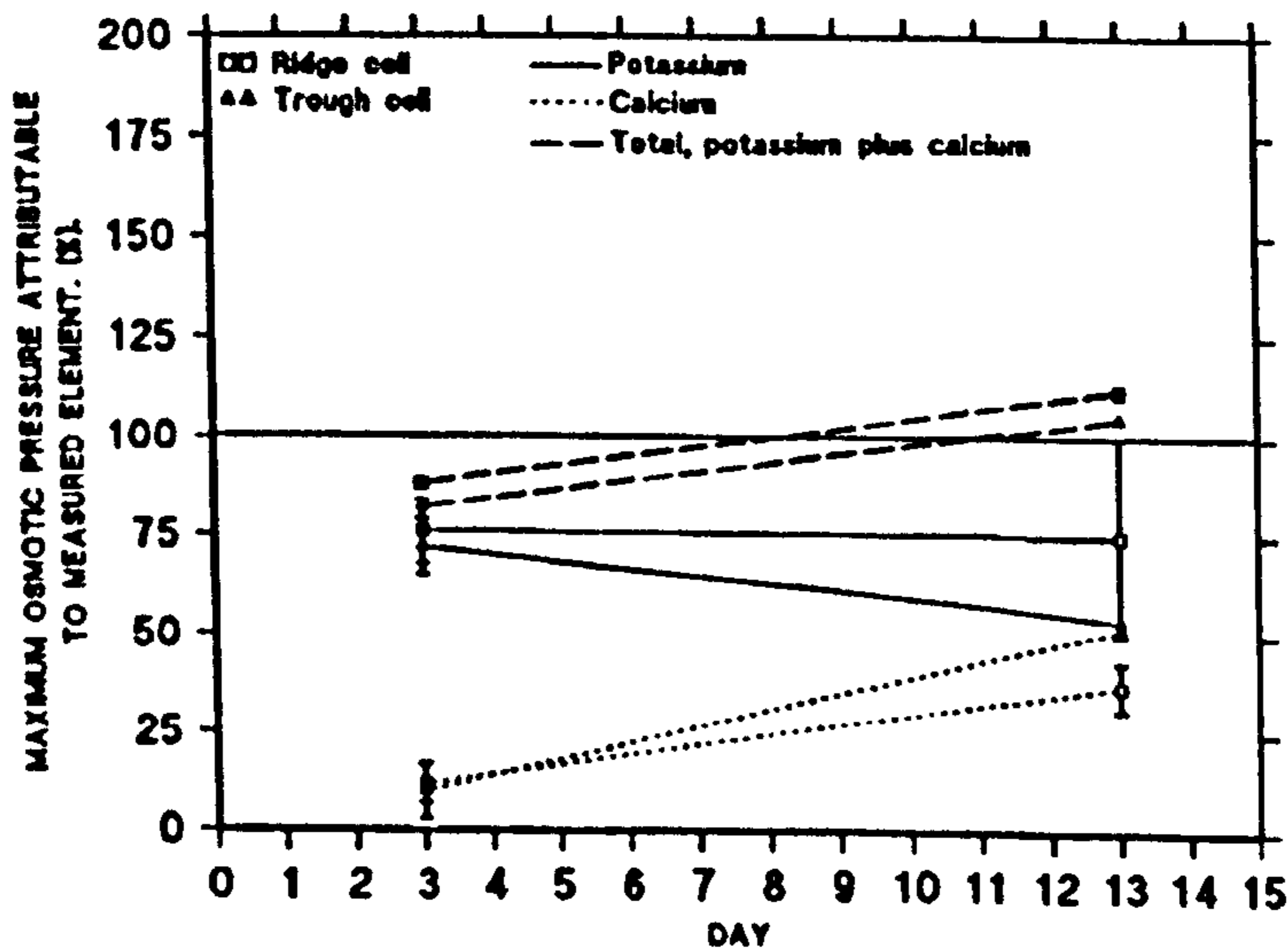
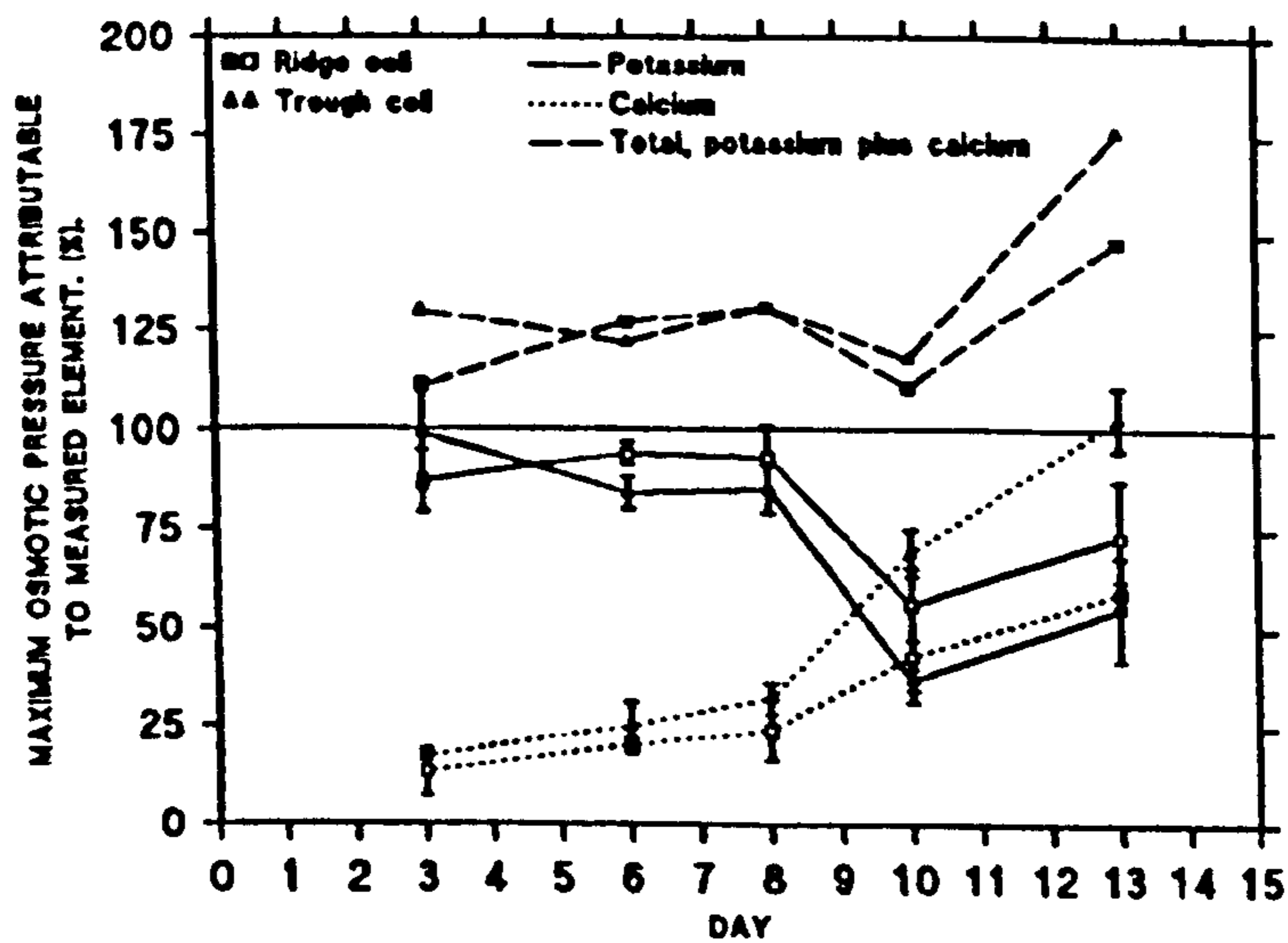


Fig 8.3 Maximum possible contributions of vacuolar potassium and calcium concentrations to the vacuolar osmotic pressure of the mesophyll. Barley plants were grown in 4.0 mM K. In a) and b) the plants were analysed using the pressure-probe approach. In c), the plants were analysed using the frozen-tissue approach whilst representative osmotic pressure values were obtained from different plants to those analysed. Datapoints show the means and SD of at least 3 cells per sampling date.

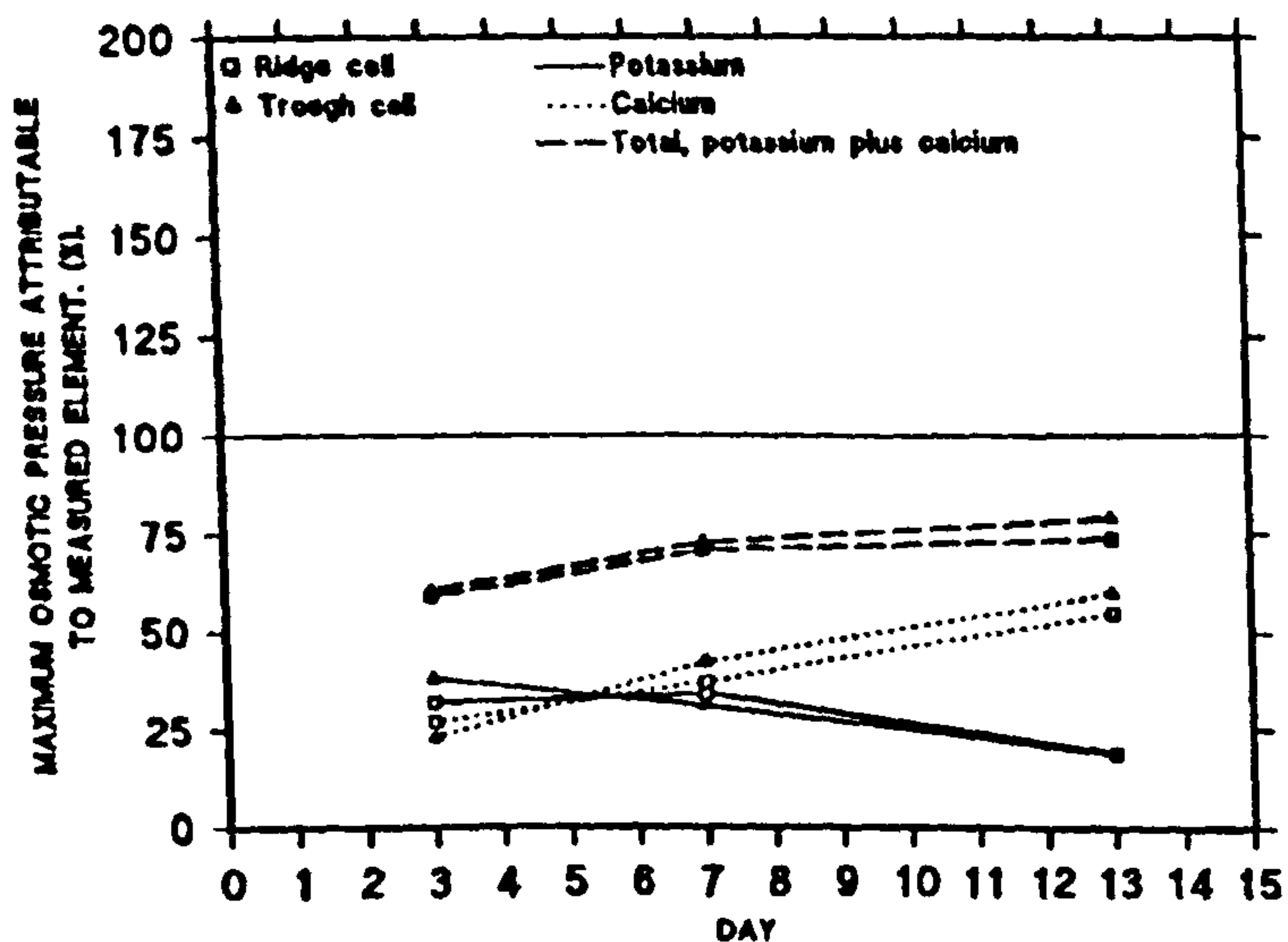
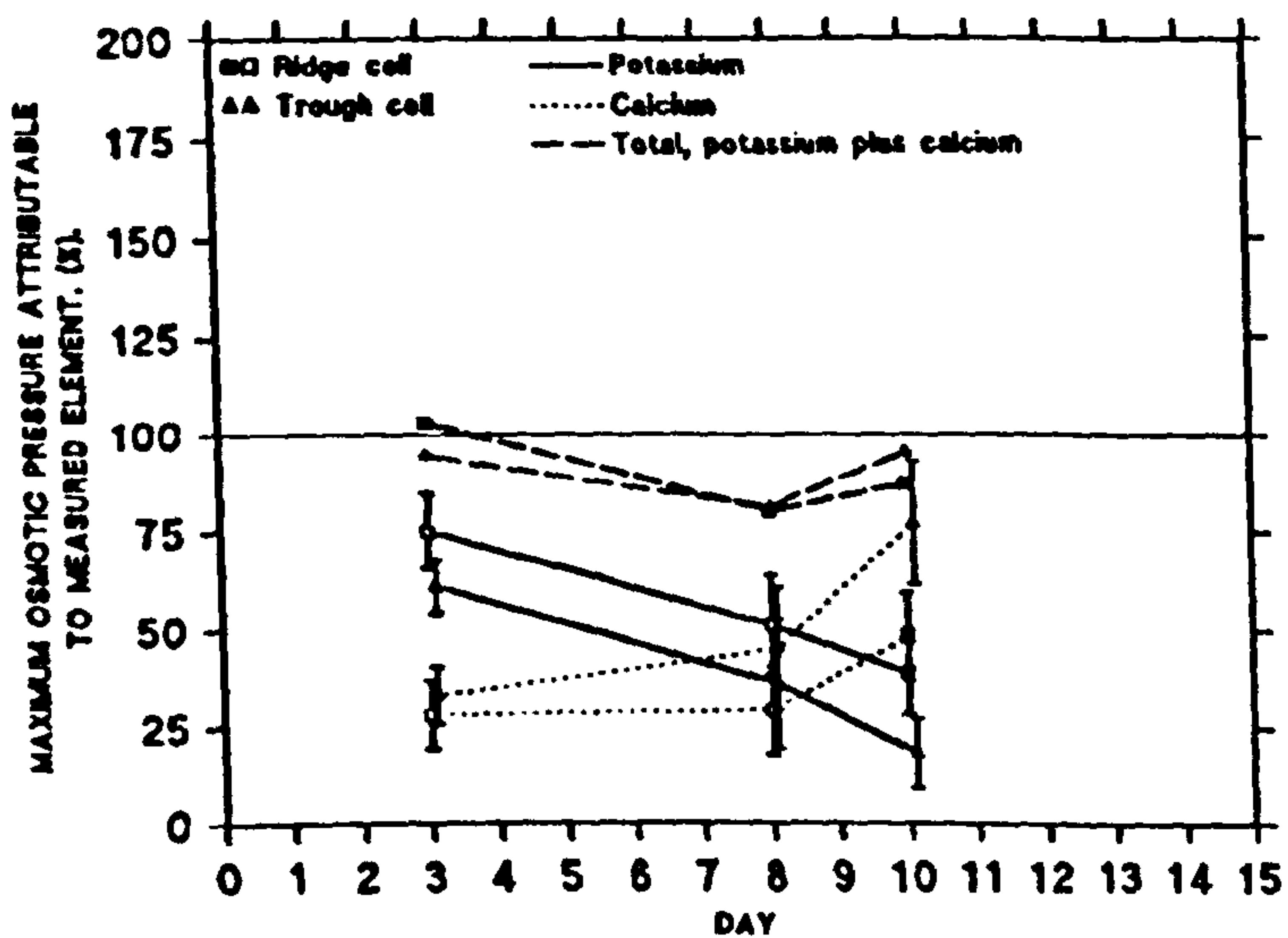
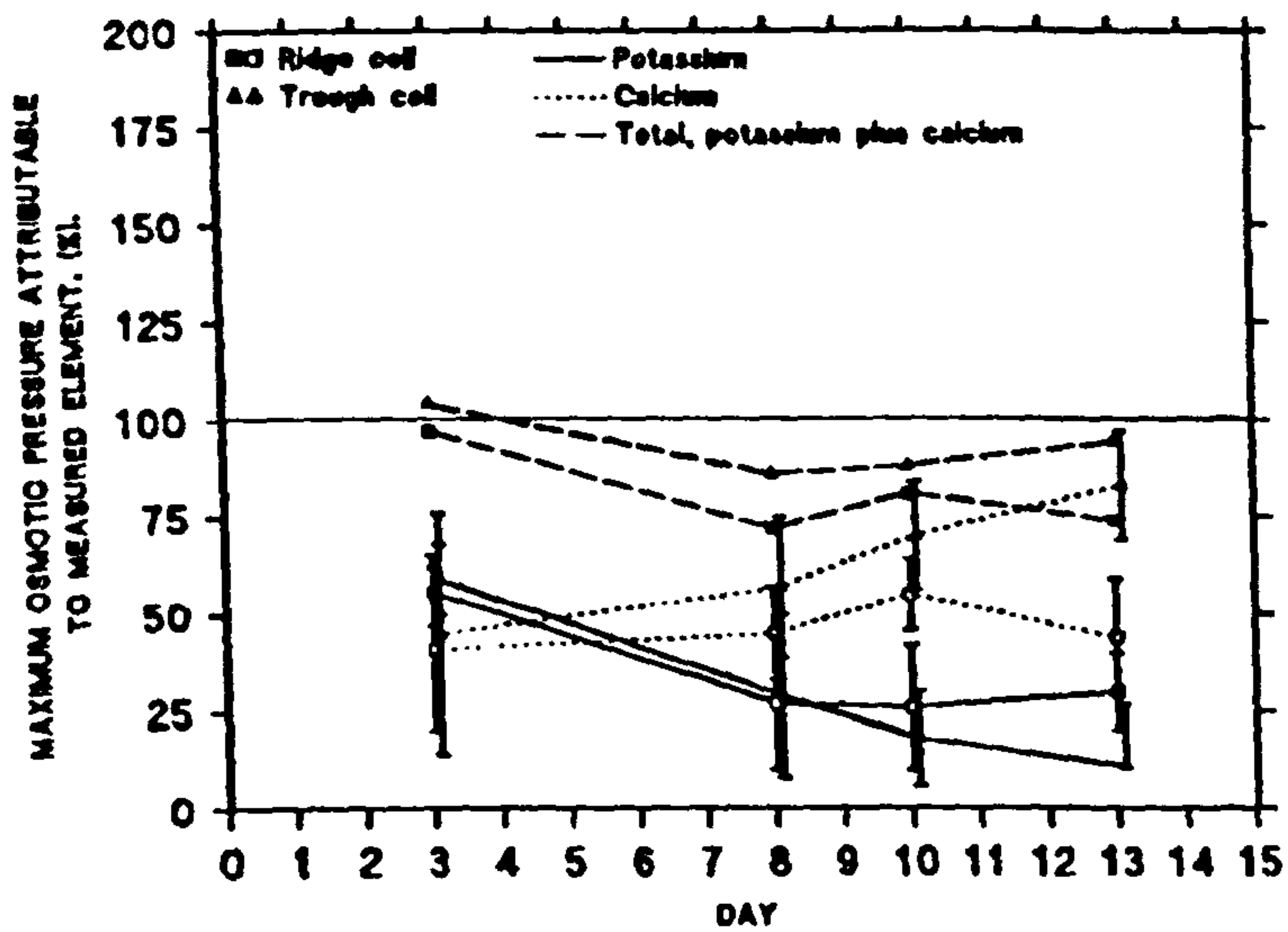


Fig 8.4 Maximum possible contributions of vacuolar potassium and calcium concentrations to the vacuolar osmotic pressure of the mesophyll. Barley plants were grown in 0.2 mM K. In a) and b) the plants were analysed using the pressure-probe approach. In c), the plants were analysed using the frozen-tissue approach whilst representative osmotic pressure values were obtained from different plants to those analysed. Datapoints show the means and SD of at least 3 cells per sampling date.

these potassium and calcium salts can account for virtually all the vacuolar osmotic pressure in the epidermal vacuole. Of particular significance is the fact that the proportion of osmotic pressure accountable for by potassium and calcium is fairly constant with time. This suggests that the value of osmotic pressure observed over the same time period may be the consequence of a strict relationship between vacuolar potassium and calcium concentrations. The nature of this relationship between potassium and calcium is further investigated in Chapter 9.



## Chapter 9. Vacuolar potassium and calcium

### 9.1 The inverse correlation between potassium and calcium concentrations

In Chapter 8 it was shown that potassium and calcium could account for virtually all of the vacuolar osmotic pressure of the epidermis if they were balanced by monovalent anions. Moreover, the vacuolar osmotic pressure did not change during the exchange of calcium for potassium hence it was postulated that the exchange of potassium for calcium was to keep osmotic pressure constant.

In fact, if both potassium and calcium ions were balanced by monovalent anions the ratio of exchange that would keep osmotic pressure constant would be 3:2. This ratio reflects the valencies of these cations because whereas potassium can only be balanced by one monovalent anion, calcium is balanced by two. Essentially, 3 potassium ions (+3 monovalent anions), are osmotically equivalent to 2 calcium ions (+4 monovalent anions). Thus if potassium is plotted vs calcium for individual cells, a gradient of 0.666 would support the hypothesis that calcium balanced by monovalent anions can replace potassium in its osmotic role.

Potassium concentrations were plotted against calcium concentrations for every cell in 5 separate time course experiments. Two plots were prepared for plants grown in 4.0 mM K (Fig 9.1) whilst 3 plots were prepared for plants grown in 0.2 mM K (Fig 9.2). One of the plots of each treatment was prepared from data obtained using the frozen-tissue approach.

The slope that described the relationship between potassium and calcium concentrations was estimated from a geometric mean regression analysis (Ricker, 1984). This analysis takes account of the fact that both sets of data (ie potassium and calcium concentrations) are independent variables. In addition 95% confidence limits of the slope were also calculated and are plotted on each graph as dotted lines. The actual value of these confidence limits are given in parentheses in each graph. When calculating the data, the errors associated with individual concentration measurements were ignored.

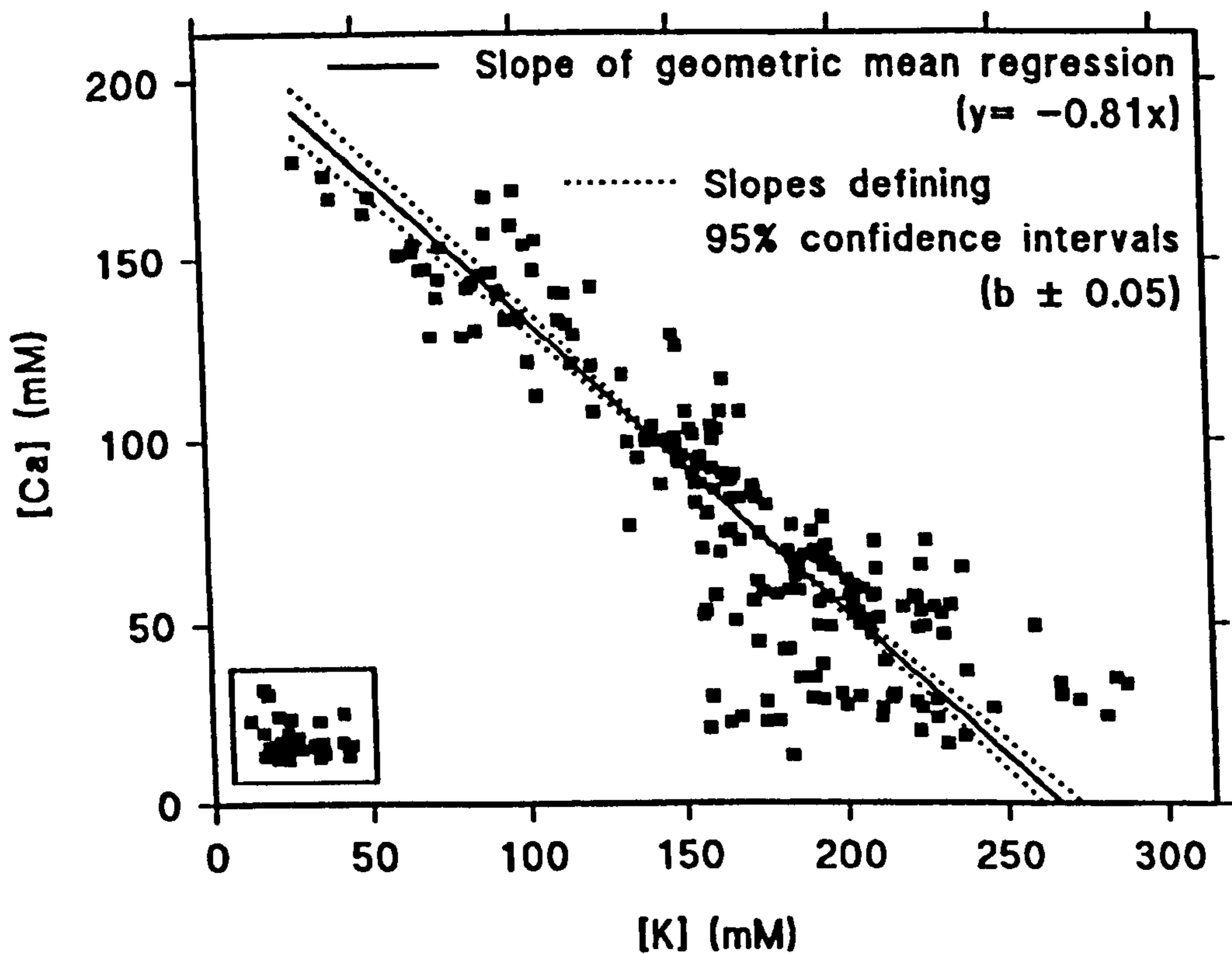
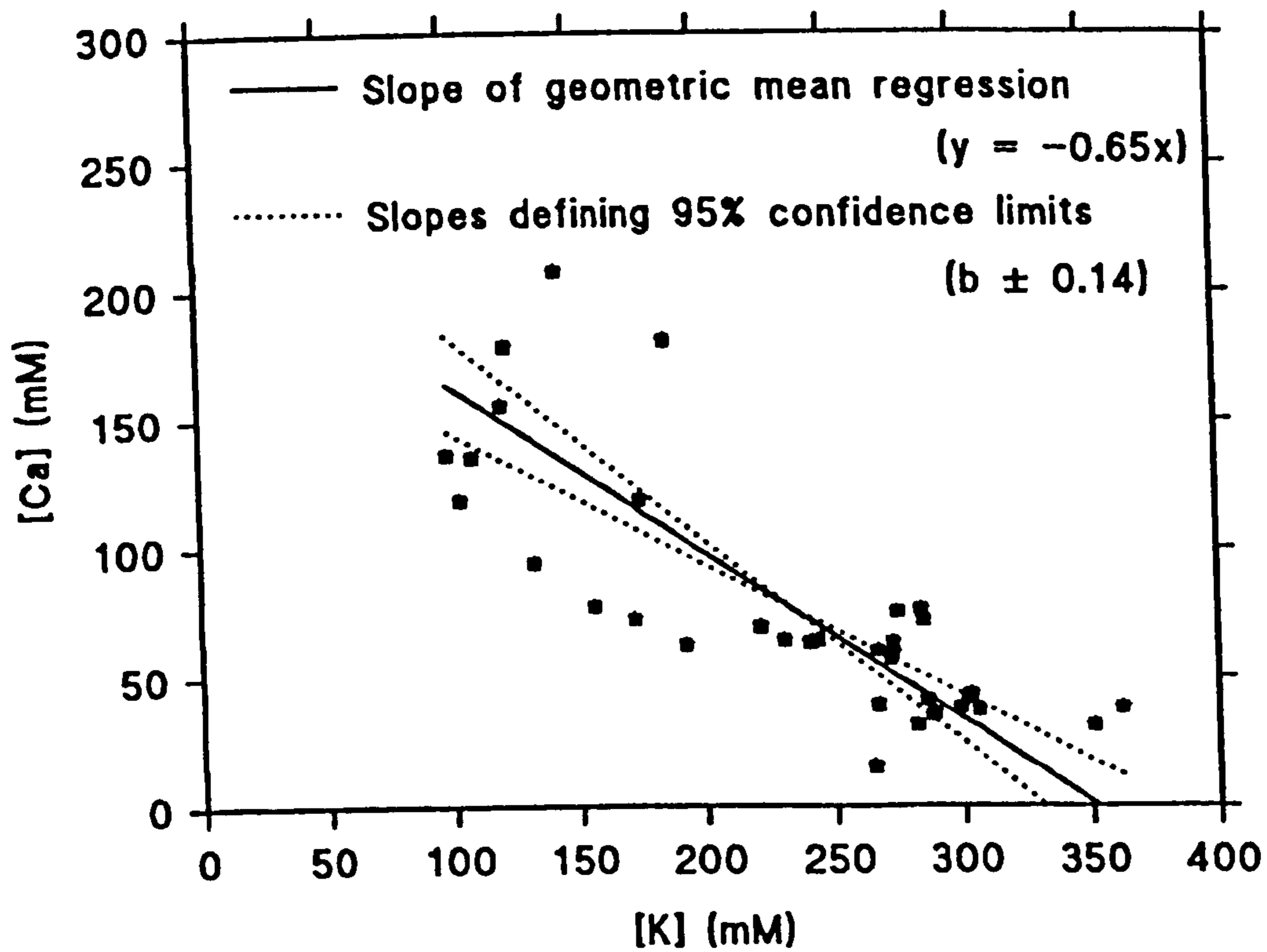


Fig 9.1 Relationship between vacuolar potassium and calcium concentrations in epidermal vacuoles of barley grown in 4.0 mM K. In a) the solutes were measured using the dried microdroplet approach. In b) the solutes were measured using the frozen-tissue approach. The fitted line was calculated from a geometric mean analysis.  $r^2$  for a) was 0.64 and for b) was 0.78.

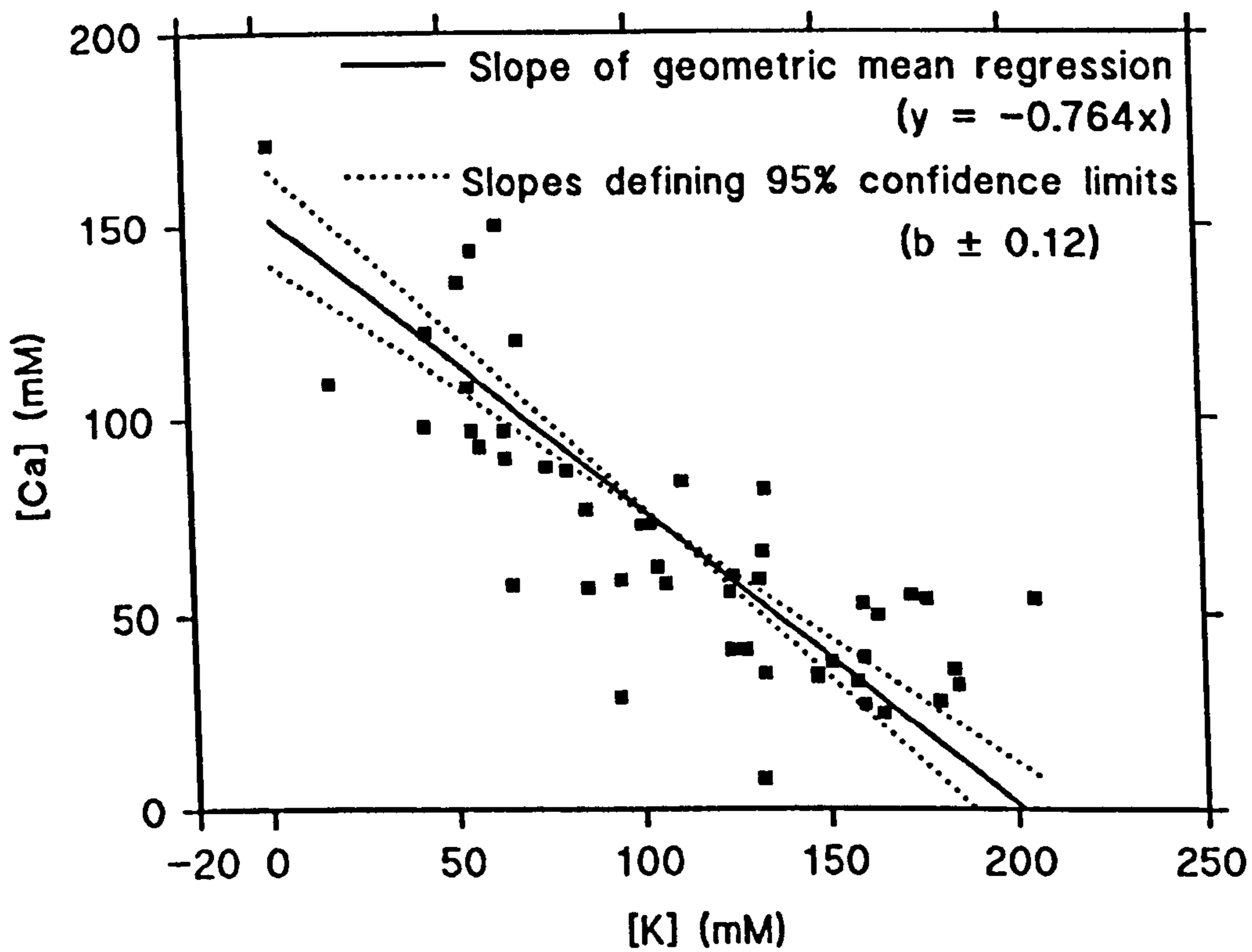
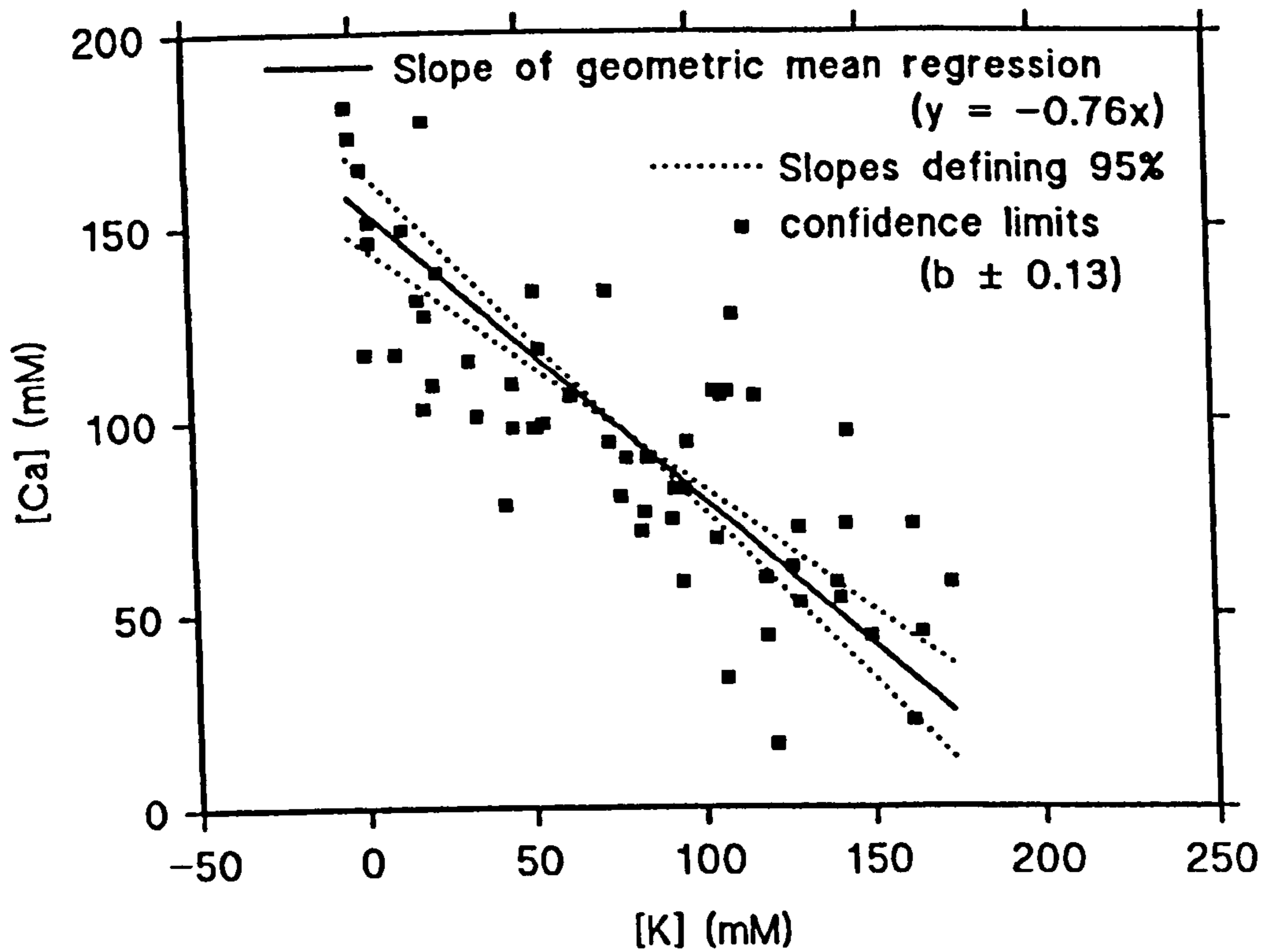


Fig 9.2 Relationship between vacuolar potassium and calcium concentrations in epidermal vacuoles of barley grown in 0.2 mM K. Graphs show the results of two completely independent experiments both measured using the dried microdroplet approach. The fitted line was calculated from a geometric mean analysis.  $r^2$  for a) was 0.58 and for b) was 0.68.

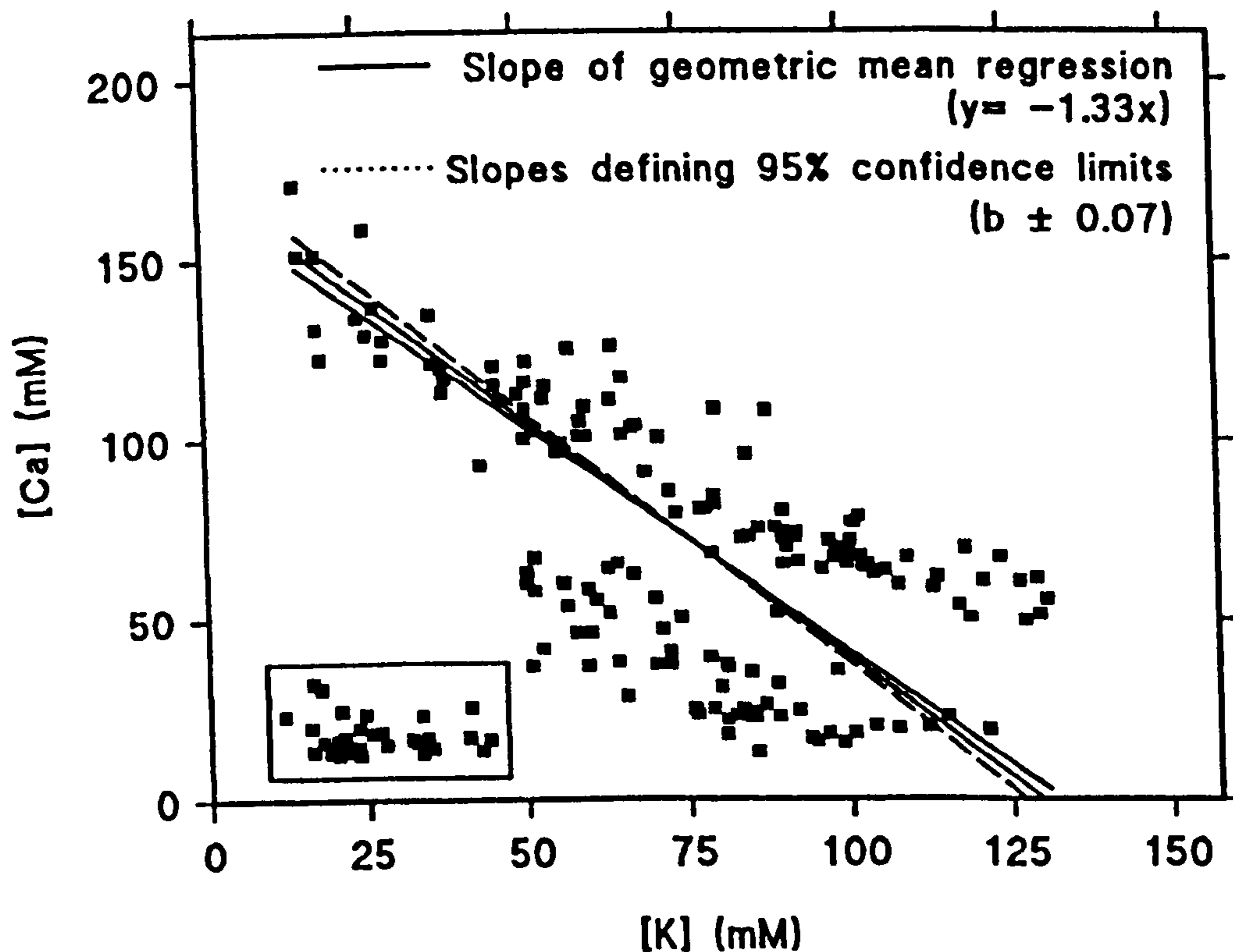


Fig 9.2c Relationship between vacuolar potassium and calcium concentrations in epidermal vacuoles of barley grown in 0.2 mM K. Graphs show the results of an experiment where concentrations of potassium and calcium were measured using the frozen tissue approach. The fitted line<sup>8</sup> was calculated from a geometric mean regression analysis.  $r^2$  for the data was 0.4.

## 9.2 Interpretation of the relationship between potassium and calcium

There is a clear relationship between the potassium and calcium concentrations in the barley epidermis vacuole. This relationship persists even when the plants were grown in 0.2 mM K.

The purpose of these plots was to test the hypothesis that a 2:3 substitution of calcium for potassium maintained osmotic pressure constant. In fact, the data obtained using the dried microdroplet approach (Figs 9.1 a, 9.2 b,c) were consistent with this

<sup>8</sup> This plot was constructed from two datasets obtained at different sampling dates. The data of these were pooled before the line was fitted. When the data from these apparently separate datasets were considered individually, identical slopes of 0.91 were obtained with confidence intervals of 0.15 (upper dataset) and 0.16 (lower dataset).

theory because the slopes of 0.666 lay within the 95% confidence limits of the fitted curves. The data obtained using the frozen tissue approach however was not consistent with this hypothesis since the slope describing the potassium / calcium exchange was steeper than 0.666. This steeper slope shows that *less than 3* potassium ions were exported for every two calcium ions accumulated. For instance, for Fig 9.1 b, the slope of 0.8 (or 4/5) would suggest that only 2.5 potassium ions were exported for every 2 calcium ions accumulated. If all the cations were balanced by monovalent anions this exchange would lead to an increase in osmotic pressure of 20%. Similarly, the relationships described in Figs 9.2 a,b would both lead to an increase in osmotic pressure of 12.5%, whilst that described in Fig 9.2c would lead to an increase in osmotic pressure of 35%.

The calcium/ potassium relationships observed can be interpreted in either of two ways, namely:

- 1) The relationship leads to a change in the osmotic pressure when the slope of the calcium/ potassium interchange is steeper than 0.666.
- 2) A proportion of the accumulated calcium may not be soluble, or not be balanced by monovalent anions.

#### 9.2.1 The K/Ca relationship in plants grown in 4.0 mM K

Direct measurement of the vacuolar osmotic pressure shows that it remains fairly constant throughout the life of the leaf (Fig 7.1). The relationship described in Fig 9.1a is consistent with the hypothesis that osmotic pressure is maintained constant because the slope describing the relationship is 0.65. The slope of 0.66 is well within the 95% confidence limits for the fitted slope.

The relation between potassium and calcium presented in Fig 9.1b is constructed from data obtained from plants grown in Hannover. Different growth conditions there may have accounted for the difference in the slope observed. The slope of 0.666 lies outside the 95% confidence limits for the fitted slope of 0.81

which is consistent with 2) above. A reexamination of the osmotic pressure data for the plants grown in Hannover, however, suggests that although osmotic pressure was generally fairly constant, it did increase with time in one particular batch of plants (Fig 7.1c). The observed increase of some 20% is that predicted from the potassium/calcium relationship, hence it is not possible to exclude 1) above as an explanation for the relationship. The results are difficult to interpret further because the osmotic pressure values were not obtained from those plants actually measured for their vacuolar concentrations. This difficulty in interpretation further emphasises the advantage of being able to simultaneously measure water and solute relations using the pressure-probe approach.

### 9.2.2 The K/Ca relationship in plants grown in 0.2 mM K

Plants grown in 0.2 mM K increase their vacuolar osmotic pressure throughout development (Fig 7.2). The proportion of osmotic pressure accounted for by potassium and calcium, however, stays the same (Fig 8.4). This constancy suggests that the potassium / calcium exchange leads to an increase in osmotic pressure in these plants. In fact, the slopes of the relationships shown in Fig 9.2 would lead to an increase in osmotic pressure if the cations were balanced by monovalent anions. The slopes described in Figs 9.2 a,b both suggest an increase in osmotic pressure of 12.5% whilst the relationship observed in Fig 9.2 c would suggest an increase in osmotic pressure of 35%. When these predicted increases in osmotic pressure were compared with the real measured osmotic pressure the values were very close. For instance osmotic pressure increased by approximately 15% in those plants illustrated in Fig 9.2 a,b (Fig 7.2 a) and by approximately 30 to 40% in those plants illustrated in Fig 9.2 c (Fig 7.2 b).

### 9.3 Summary

The relationship between the potassium and calcium concentrations is influenced by the potassium nutrition of the plants. When potassium is withheld its

vacuolar osmotic pressure of the epidermis is initially lower than in plants grown in 4.0 mM K, but thereafter increases. The extent of this increase is apparently related to the behaviour of the vacuolar potassium and calcium concentrations and it appears that there is a purposeful adjustment of the relationship in order to maintain a set osmotic pressure.

Osmotic pressure regulation by balance of the potassium and calcium concentrations is apparently not controlled by increasing the quantity of calcium taken up by the vacuole because its concentration does not generally increase under potassium-deficient conditions (compare Figs 5.1, 5.2, 6.3 and 6.4). This shows that osmotic pressure is increased by restricting the release of potassium from the vacuole and shows that potassium plays an important role in the regulation of vacuolar osmotic pressure.

## Chapter 10. General discussion

The first leaf of barley is a heterogenous structure that in our growth system has a life of approximately two to three weeks. During that period, the osmotic pressure of the epidermis remains fairly constant, and supports a turgor pressure of 1.3 MPa. Over the experimental period investigated from day 4 onwards, the cells within this leaf are fully expanded and growth has ceased. As the leaf develops, calcium accumulates in the epidermis where it is stored preferentially in cells furthest from the vascular tissue. This accumulation leads to developments of gradients of calcium with peaks ~~|maximised|~~ over the morphologically distinct trough cells. The import of calcium appears to be related to the export of potassium from the cell such that the osmotic pressure remains constant. When the potassium in the nutrient solution is reduced from 4.0 to 0.2 mM, vacuolar osmotic pressure is initially low. During leaf development, however, the osmotic pressure in these low K plants increases until by day 13, it is close to the osmotic pressure of the high K plants.

Calcium does not accumulate in the mesophyll cells (Fig 10.1 a) and phosphorus is not accumulated in the epidermal cells (Fig 10.1 b). These observations are consistent with other studies of heterogeneity in barley leaves (Leigh and Storey 1986, 1992, Dietz *et al.* 1992 a,b).

### 10.1 Are all cells identical within one tissue type?

Vacuolar heterogeneity between different tissue types may result from their different membrane transport properties (Dietz *et al.* 1992 a,b). To date, however, cell heterogeneity within single tissue types has not been reported, and the approach taken by Dietz *et al.* (1992a,b) is incapable of discriminating differences between individual cells.

Data obtained using the single cell microsampling approach has demonstrated conclusively that an apparently non-random heterogenous distribution of elements may occur within one tissue type. The time-course studies demonstrate that the pattern of this heterogeneity is dependent upon leaf development. It is possible that such heterogeneity develops with time in other species.



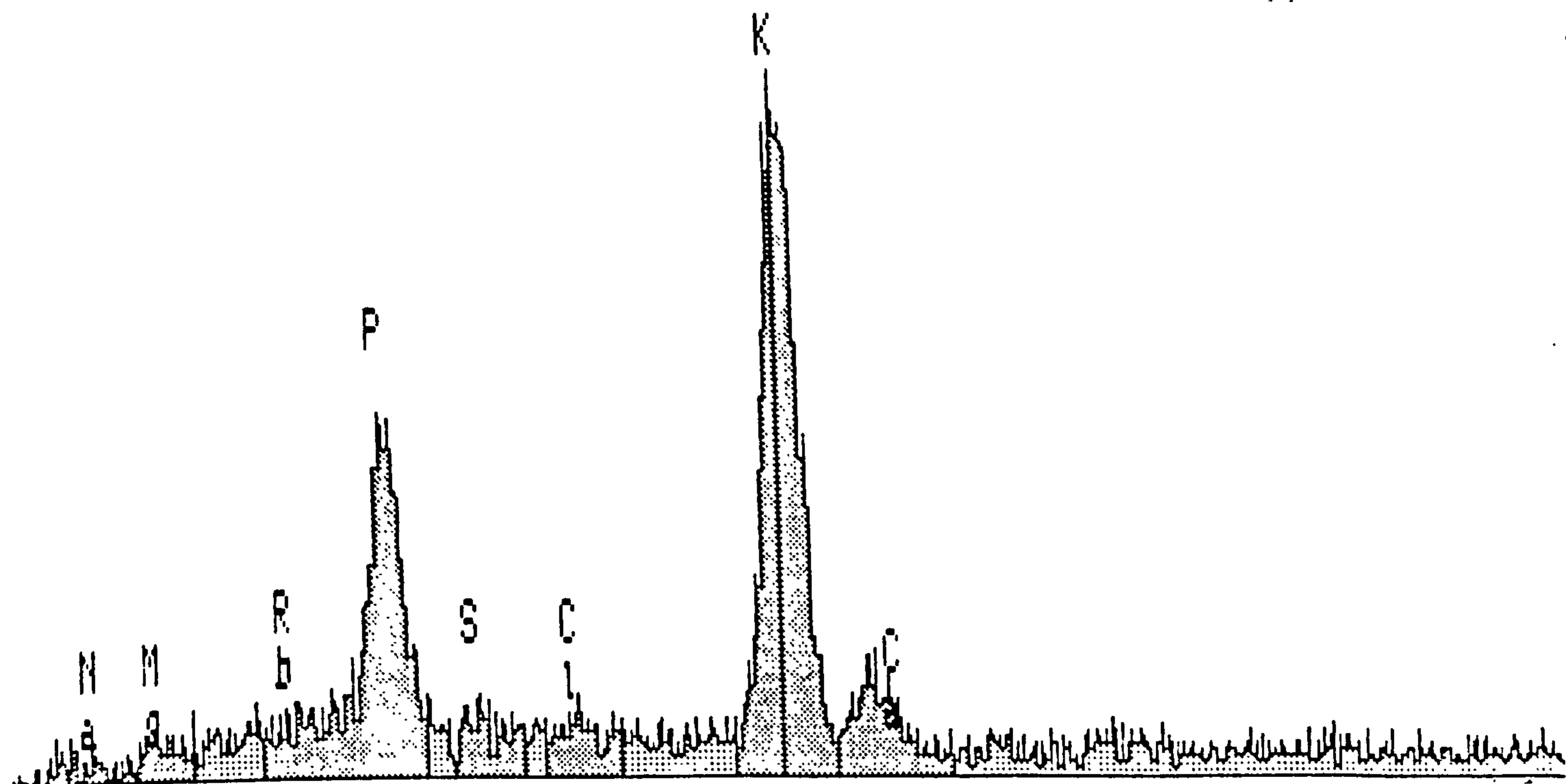


Fig 10.1a Qualitative X-ray microanalytical spectrum of dried mesophyll cell sap of barley at day 13. The spectrum shows that no calcium has accumulated (compare with Fig 10.1 b). The sap was extracted from the first cell encountered after entry into the leaf via a stoma.

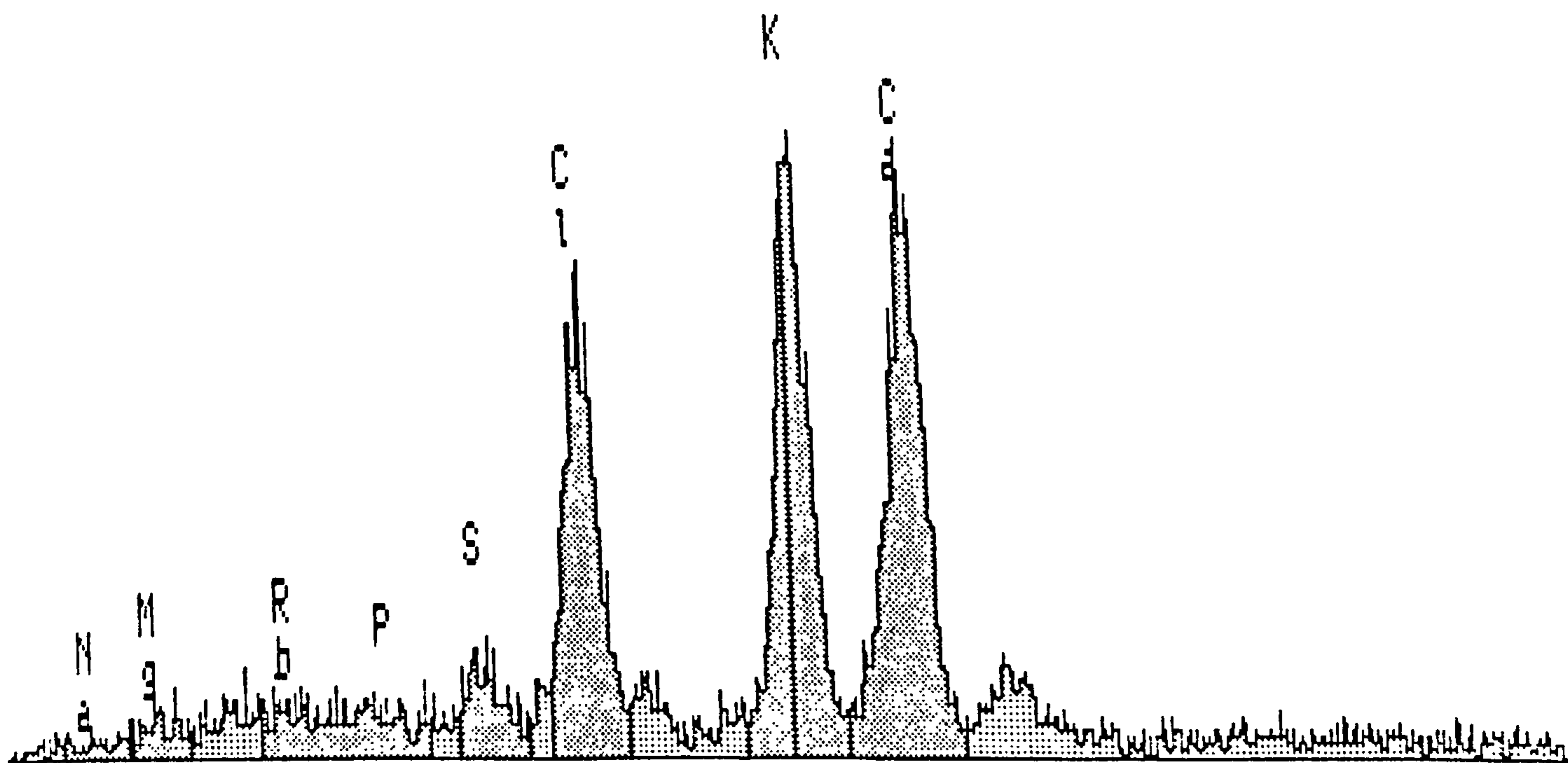


Fig 10.1b Qualitative X-ray microanalytical spectrum of dried sap extracted from an epidermal trough cell of barley at day 13. Note the lack of phosphorus. (compare with Fig 10.1 a).

A previous application of microdroplet analyses to investigate heterogeneity in the epidermis of wheat (Malone *et al.* 1991) did not find any statistical differences between ridge and trough cells. Their investigation, however was of young tissue which may have not developed such gradients. Recently, Richardson (1993) demonstrated differences in vacuolar concentrations between cells of the upper and lower epidermis of wheat 2<sup>nd</sup> leaves. The lower epidermis accumulated less calcium, but more potassium than the upper epidermis suggesting that there is a further level of heterogeneity in the leaf. These differences were not investigated in the work presented here.

The largest developmental swings in vacuolar ion concentrations have been observed in the epidermal trough cells lying furthest from the vascular tissue. It is suggested that by allowing these cells to accumulate calcium and senesce first, that a route remains whereby the orderly withdrawal of nutrients from the plant may occur. This withdrawal is consistent with a general observation of the senescing leaf in that necrosis is frequently observed to occur first in the regions of the trough cells closest to the tip and edge of the leaf. This apparent link between epidermal heterogeneity and development is further substantiated if the changes observed in the epidermis are viewed alongside changes occurring in the whole leaf (Figs 5.3, 5.4 a,b, 6.5 a,b). Such comparisons reveal that the changes in ion concentrations within the epidermis reflect a general redistribution of potassium within the leaf as development proceeds. This single observation is important since it emphasises the individuality of single cell vacuoles as independent determinants of tissue function as implied by Tomos and Wyn Jones (1988). It would also imply that conclusions drawn from whole tissue measurements may be misleading.

## 10.2 Turgor and osmotic pressure in the epidermis of barley

One of the major advantages of the microsampling approach over other quantitative methods is its capability of analysing several related physiological parameters (turgor, osmotic pressure and solute composition) in individual cells. The microsampling method has been used here to investigate the relationship between the development of solute heterogeneity and the water relations parameters of the

epidermis. In general, the data presented in this thesis are comparable with the findings of Leigh *et al.* (1986) in that soluble potassium and calcium constitute nearly all the osmotic pressure in barley and the osmotic pressure of whole leaf sap was similar in plants regardless of their potassium nutrition. However, the latter study could not account for cell water relations at the resolution of the single cell nor could it accurately measure single cell solute concentrations. Using the micropressure probe approach it has been demonstrated that virtually all the osmotic pressure in these cells can be accounted for by soluble potassium and calcium salts. The proportions of these salts in the vacuole change with time, maintaining osmotic pressure constant in plants grown in 4.0 mM K, but increasing osmotic pressure in those plants grown in 0.2 mM K that had an initially lower osmotic pressure. Absolute comparisons of the data obtained in this thesis with other published data are complicated by the fact that no parallel studies have addressed the question of cell heterogeneity.

#### 10.2.1 Osmoregulation or turgor regulation?

Osmotic and turgor pressure remain constant in potassium sufficient plants throughout most of the leaf life. In 'low K' plants both osmotic and turgor pressure increase towards the same value as in the 'high K' plants. This behaviour is consistent with some form of regulation (see Section 1.2.5), but poses the question as to what is the regulated parameter here?

In fact, due to their interdependency, it is difficult to confirm whether osmotic pressure or turgor pressure is the regulated parameter in the barley epidermis. In the 'high K' plants both turgor and osmotic pressure remained constant at approximately 1.3 MPa throughout the development of the leaf. In the 'low K' plants both osmotic and turgor pressure increased.

The concept of regulation implies that there are target values of pressure in the cell. The constancy of both osmotic and turgor pressure in the 'high K' plants suggests that this target value (or "set-point" (Cram 1976)) is approximately 1.3 MPa. The increase in pressure in the epidermis of 'low K' plants can be interpreted as an attempt to reach this target pressure of 1.3 MPa. Osmotic pressure (Fig 7.1 b,c)

certainly does not *decrease* to the extent implied by the observed decrease in vacuolar potassium (Figs 4.5, 6.4 a,b, Table 9.1) and did not increase indefinitely.

In the experimental systems investigated, two potential sources of osmotic stress were identifiable. The first was self-imposed. This was the export of potassium from the epidermis. The second was externally imposed by withholding potassium (i.e. the major osmotic cation in young leaves) from the plant. The export of potassium with development was associated with the appearance of calcium, but the relationship between these cations was dependent upon the potassium nutrition of the plant. When the osmotic pressure and turgor pressure of the epidermis had already achieved its target turgor/ osmotic pressure, the exchange of potassium and calcium led to no change in these parameters. When, however, the turgor/ osmotic pressure was lower than the target value of 1.3 MPa the exchange of potassium and calcium led to an increase in turgor/ osmotic pressure. Thus the cell apparently regulates using a combination of potassium and calcium in the vacuole. The evidence suggests, however, that the solute being regulated is potassium and not calcium. Potassium deficiency does not lead to an increase in calcium in the cells, but it does appear to lead to a net decrease in the quantity of potassium exported. Such a net decrease in potassium export could be effected either by releasing less potassium per calcium accumulated or by taking up potassium in a separate process.

Given that potassium is used by the cells in instances of potassium deficiency to regulate turgor, it is surprising to see vacuolar potassium decline at all. For instance if potassium were not exported at all, the target osmotic pressure would be achieved earlier. Export of potassium concomitant with the arrival of other solutes may be a developmental feature of barley epidermal cells that would normally allow the cell to regulate turgor or osmotic pressure under typical growth conditions. It is possible that the sink for this exported potassium requires potassium, hence the behaviour observed may result from the competition for potassium by both the regulation system and this sink. The fact that calcium does not generally accumulate in the epidermis under potassium-deficient conditions suggests that it cannot perform the role of an osmotic adjuster. However, the observation that some cells entirely emptied of their potassium (Table 8.2) suggests that epidermal cells do possess the capability of regulating their osmotic pressure solely by calcium. It is possible that the

reason why cells did not generally accumulate more calcium under the potassium-deficient conditions used was that the supply of calcium was limited. Leigh *et al.* (1986) showed that soluble calcium was accumulated in shoots of barley deprived of potassium although the concentration available in the soil was not given.

Irrespective of the subject of regulation, it was apparent that the time course of turgor pressure increase reflected the trend in osmotic pressure. In the potassium-deficient treatment, turgor increased at a slightly faster rate than vacuolar osmotic pressure right up to the last measurement taken at day 13 (Fig 7.3 b). In the control, turgor was constant as was the osmotic pressure (Fig 7.3 a). The policy of attempting to randomly sample cells from one small region of the plant led occasionally to cells being sampled adjacent to necrotic regions (see Plate 7.1). Such cells often had low turgor pressures at day 13 (Fig 7.3 a). It was postulated that low turgors may have resulted from local decreases in wall water potential caused by flooding of the wall space with solutes derived from dead cells. This flooding would account for the large degree of turgor variation observed in the cells and could possibly explain the tendency for necrosis to occur in patches. A constancy of osmotic pressure in cells adjacent to these dying patches rather than a constancy of turgor pressure is suggestive of osmoregulation rather than turgor regulation but does not confirm it. If turgor pressure were being regulated, osmotic pressure within the vacuole would increase. In this instance though, osmotic pressure was remarkably constant. Caution should be exercised in interpreting these observations. Turgor could be regulated, but the turgor control mechanism may be abandoned or derestricted as senescence advances, and the need to sustain high turgors diminishes.

Thus the barley epidermis apparently turgor/ osmo-regulates despite an ongoing developmental process which leads to the loss of potassium from the cell concomitant with the accumulation of calcium. It is suggested that control of vacuolar potassium is used to regulate osmotic or turgor pressure.

### 10.3 Water potential and potassium-deficiency

The time course of turgor and osmotic pressure change in the potassium-deficient treatment showed that the increase in osmotic pressure measured in the epidermis was not proportionately related to an increase in turgor. Indeed, during early leaf development the osmotic pressure is greater than the turgor pressure indicating a reduced wall water potential. This reduction may have been a consequence of increased transpiration (Brag 1972), or the accumulation of wall solutes. Although the basis of this reduced water potential is still unclear, the wall solute explanation is favoured because the stomata appeared closed in both treatments. The increase in turgor pressure at a rate faster than the increase in osmotic pressure suggests that these solutes are eventually taken up by the cell from the apoplast. ✓

### 10.4 Do the results compare with other studies of vacuolar composition?

In a previous investigation of osmotic pressure maintenance in barley shoots, Leigh *et al.* (1986) showed that osmotically-active calcium and magnesium were accumulated in response to potassium-deficiency. In the study reported here, calcium did not differ significantly between treatments. This difference may reflect the different growth conditions used and the choice of tissue analysed. The light intensity under which our plants were grown ( $130\mu\text{E m}^{-2}\text{s}^{-1}$ ) was approximately one third that of the earlier study of Leigh *et al.* (1986) and may have reduced both photosynthesis and transpiration. Significantly, the data of Leigh *et al.* (1986) was obtained from whole shoots grown in soil whereas this investigation was of primary leaves grown hydroponically. Measurement of whole shoots inevitably includes different tissue and organ types which may have an intrinsically different response to the first leaf. If it is accepted that the explanation of the differences lies in the choice of the plant tissue analysed, it would suggest that the first leaf differs fundamentally from the younger leaves because of its inability to osmotically adjust with calcium whilst the older leaves can. Interestingly, Leigh *et al.* (1986) did find that in the first leaf of barley that low potassium concentrations in the vacuole could be associated with high

concentrations of calcium. It is difficult to further discuss these differences in solute distribution without further quantitative information on these leaves.

Magnesium was not accumulated in the epidermis even though it was present in the nutrient solution. Lack of uptake of magnesium might suggest that either the epidermal vacuole is unable to accumulate magnesium to osmotically significant concentrations, or that the epidermal vacuole does not have an uptake capability for this ion. An alternative explanation is that magnesium in the transpiration stream is sequestered by other cells for the purpose of osmoregulation. Leigh *et al.* (1986) demonstrated that the leaf concentration of magnesium quadrupled when the availability of potassium and sodium was restricted. Unfortunately, whole tissue measurements of magnesium were not made, but if vacuolar accumulation did occur then its distribution must be restricted to tissues other than those of the epidermis because it did not accumulate in these cells (Fig 10.1b). Typical concentrations of magnesium in barley leaves are from 5-10 mM (Leigh *et al.*, 1986; Dietz *et al.*, 1992b).

The absence of a magnesium peak in the X-ray spectrum of extracted mesophyll sap suggests that magnesium is not accumulated in the mesophyll vacuole (Fig 10.1a). However, other workers have found significant quantities of magnesium in the cytoplasm and vacuole of mesophyll cells and in bundle sheath cells (Dietz *et al.* 1992b, Williams *et al.* 1993). Its accumulation in both the vacuole and cytoplasm of the mesophyll is apparently dependent upon the calcium nutrition of the cells. The behaviour of these ions in the cytoplasm is unlikely to balance osmotic pressure. However, the increase of soluble magnesium under potassium-deficient conditions suggests that magnesium may play a role as an osmoticum in certain instances although its compartmentation under these conditions needs further investigation (Leigh *et al.* 1986).

The epidermal vacuole can nevertheless accumulate other ions than those observed in this work. For instance sodium can be accumulated by both epidermal cells and mesophyll cells of barley (Flowers and Läuchli, 1983; Leigh and Storey, 1993; Fricke pers comm). The accumulation of sodium is used to balance leaf osmotic pressure when the availability of potassium is restricted (Leigh *et al.* 1986). Similarly, the epidermis can also accumulate a variety of different anions. Data of Richardson

*et al.* (1992) and of Dietz *et al.* (1992) suggest that the anionic balance may alter depending upon the nutrient supply. The data of Richardson *et al.* (1992) is particularly interesting since it was obtained from wheat plants analysed under identical conditions. Direct measurement confirmed that epidermal cells of wheat osmo/ turgor adjusted using a variety of anions (phosphate, sulphate, nitrate and chloride) dependent upon nutrient supply. These were accumulated whenever nitrate, the favoured anion, was restricted and potassium was present to excess.

### 10.5 Why regulation at all?

One possible significance of turgor to epidermal cells of barley is suggested from observation of the plants' habit after 13 days (see Plate 2.1). Under potassium-deficiency, the youngest leaves had a tendency to bend and collapse especially when the plants were moved. This observation would suggest that lack of turgor in the epidermis may lead to susceptibility to damage which could be particularly significant for plants grown outdoors.

Turgor is an important determinant of leaf structure (see Section 1.3). This being so it might be argued that control of turgor is important at an early stage of leaf development. In general, however, the primary leaves of both treatments were similar in appearance, even though their turgor pressures were different. Similarly, although preliminary observations suggest that the low potassium treatment was more susceptible to collapse, their habit was similar to the controls. This suggests that a high turgor is not crucial to development, but merely makes the plant more robust. Both treatments had leaves which were fully unrolled. If leaf unrolling is a turgor dependent phenomenon it can obviously occur at turgor pressures much lower than those maximally attained in the control plants. It is possible that stomata work most efficiently when contained within an epidermis of a certain turgor (Brag 1972, Meidner 1990).



## 10.6 Why is potassium exported from epidermal cells?

The retranslocation of potassium from leaves increases with age, even when potassium is not limiting (Greenway and Pitman 1965, Jeschke and Wolf 1985, Bogemans *et al.* 1990). It is postulated that the time dependent redistribution of potassium within the leaf (Figs 5.3, 5.4 a, 6.5 a,b, 10.1 a,b) reflects the fact that the epidermis acts as the source of such retranslocation.

More potassium was exported from epidermal cells of plants grown in 4.0 mM K than from plants grown in 0.2 mM K. This observation suggests that demand for potassium by other parts of the plant is not the primary event determining the rate and extent of potassium export. In contrast to the potassium, the calcium was observed to increase in the leaf at a constant rate regardless of treatment (Fig 6.3). If the calcium arrives at a constant rate regardless of the plant nutrient status this suggests that the import of calcium may determine the efflux of potassium from the cell. If osmotic pressure is maintained by the potassium/ calcium exchange, this suggests that the import of calcium may drive the export of potassium.

In fact, the potassium/ calcium interchange may have a parallel. In Section 1.4, retranslocation of potassium was discussed and was shown to increase when barley plants were fed sodium even when potassium was present to excess (e.g. Ward, 1958; Wolf *et al.* 1990,1991). In the light of the data obtained in the present study, this would be consistent with sequestration of sodium, and subsequent osmo/ turgor regulation by potassium. Indeed, sodium is similar to calcium in that once accumulated, it tends to remain in the leaf. This would imply that osmotic pressure in these leaves would continuously increase unless measures were taken to stop it. ✓ *qerr'*

## 10.7 A hypothesis for the role of the epidermis in barley

The potassium/ calcium relationship observed in the epidermal vacuole regulates either turgor or osmotic pressure. This relationship suggests that the epidermis is not a mere repository for undesirable solutes, but is under some control. This conclusion is further supported by other workers who have found that the various

combinations of vacuolar solutes results in a fairly constant osmotic pressure (see Section 10.5).

The hypothesis is proposed that in the epidermis, calcium in a soluble form replaces the potassium that is retranslocated from the leaf. Judging by its apparent absence from the mesophyll, and the very high contents of calcium within the epidermal vacuole, it is proposed that this is an important function of the epidermis, since it:

- 1) Would provide a compartment apparently free of phosphate that might otherwise be precipitated and made unavailable if calcium were stored in the mesophyll.
- 2) Would provide a compartment where calcium may be stored in a fully soluble state where it could function as an osmoticum, and in doing so would sequester a potentially toxic cation away from cells that may require only low concentrations.
- 3) As accumulation of calcium progresses, it would allow release potassium for export to other developing parts of the plant whilst keeping the vacuolar osmotic pressure unchanged.

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Section 10.8 addresses the energetics associated with the accumulation of high concentrations of calcium ions and the export of vacuolar potassium.

## 10.8 The implications if calcium as a free ion accumulates to osmotically significant concentrations

The accumulation of calcium to high levels in the vacuole implies that a large electrochemical gradient for calcium would be generated across the tonoplast since it is now well established that the cytoplasmic free calcium concentration is maintained at a very low level (in the order of  $0.1 \mu\text{M}$  (e.g. Allan and Trewavas (1987) Evans *et al.* (1991) and references therein)). Assuming that the calcium accumulated in the vacuole is fully soluble, then accumulation of 150 mM calcium would create a concentration gradient of some 1.5 million across the tonoplast. Generally, estimates of the vacuolar free calcium concentration are much lower than this (e.g. 1 mM in *Nitellopsis* (Miller and Sanders 1987), 1-2 mM in both *Zea* and *Riccia* (Felle 1988), 12 mM in *Nitella*, (Spanswick and Williams 1965)).

The tonoplast lacks a calcium dependent ATPase and  $\text{Ca}^{2+}$  transport is achieved via the  $\text{H}^+/\text{Ca}^{2+}$  antiport (Evans *et al.* 1991). The thermodynamic feasibility of calcium accumulation via the proton/ calcium antiport depends on the stoichiometric ratio ( $\text{H}^+:\text{Ca}^{2+}=n$ ) for the transport reaction, according to the following relationship described by Blackford *et al.* (1990):

$$\Delta G = (2 - n) \Delta \psi + 59 [n(\text{pH}_v - \text{pH}_c) + (\text{pCa}_c - \text{pCa}_v)] \quad (\text{Eqn 10.1})$$

Where:

$\Delta \psi$  is the membrane potential

$\Delta G$  is the free energy liberated (negative value) or utilised (positive value) by the transport system in mV.

$\text{pH}_v, \text{pH}_c$  is the pH of the vacuole and cytoplasm respectively, and;

$\text{pCa}_v, \text{pCa}_c$  is  $-\log_{10}[\text{Ca}^{2+}]$  in the vacuole and cytoplasm respectively.

By substituting physiological parameters into eqn 10.1<sup>9</sup>, Blackford *et al.* (1990) have investigated the feasibility of the calcium transport system as a means of accumulating calcium in the vacuole. (The physiological parameters were:  $\Delta\psi = 20$  mV,  $\text{pH}_v = 5.5$ ,  $\text{pH}_c = 7.5$ ,  $\text{pCa}_c = 7.0$ ,  $\text{pCa}_v = 3.0$ ). It was demonstrated that a ratio of 1 proton per calcium ion was capable only of sustaining transport of calcium from the vacuole to cytosol. When  $n=3$ , transport would proceed spontaneously driven by a  $\Delta G$  of -140 mV against a gradient of 1 mM calcium. Using the same physiological parameters as Blackford *et al.* (1990) Transport of calcium against a 150 mM ( $\text{pCa}_v = 0.82$ ) calcium gradient can be calculated to occur when  $n=3$  driven by a  $\Delta G$  of -9.38 mV, and when  $n=4$  by a  $\Delta G$  of -150 mV.

Assuming that  $n$  was an integer, Blackford *et al.* (1990) suggest that its value be at least  $n=3$  since a value of  $n=2$  would poise the transport system at equilibrium for normal *in vivo* ionic gradients. This would make the accumulation of free calcium to high concentrations at least a tenable hypothesis, based on average physiological parameters.

### 10.9 The implications of the very low potassium concentrations occasionally measured in the vacuole

The vacuolar accumulation of potassium is energy-dependent although the mechanism by which this occurs is unclear (Martinoia 1992). The activity of transporters acting in the reverse direction that would be necessary for complete export of potassium from the vacuole (i.e. in a reversal of the usual electrochemical gradient of K) is to date unreported. ✓

Leigh and Wyn Jones (1984) suggested that the vacuolar concentration of potassium in cells is maintained above a minimum level of approximately 10-20 mM

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<sup>9</sup> The physiological parameters substituted into eqn 10.1 have not actually been confirmed in barley epidermal tissue and may not be true for it. It is possible that a tissue responsible for calcium accumulation has different values of these parameters.

and does not decline to zero. This hypothesis is supported by observations of Memon *et al.* (1985), who demonstrated that retention of vacuolar potassium in potassium-deficient barley occurred in a variety able to accumulate high vacuolar concentrations of this ion. Failure of cytoplasmic homeostasis for potassium when vacuolar concentrations are low suggests that the vacuole must maintain a minimum level. Indeed, in barley roots grown without potassium the root tissue contained a concentration of 10-20  $\mu\text{mol}$  of potassium per gram fresh weight (Pitman *et al.* 1981). X-ray microanalysis suggested that the vacuole still had approximately one third of the cytoplasmic concentrations of potassium.

In the current investigation, vacuolar potassium in several epidermal trough cells of the low potassium treatment had declined to zero towards the end of leaf development (Table 8.2) showing that certain epidermal cells possess the capacity to entirely empty of their potassium. The competence to do this is consistent with a previous postulate that the epidermis acts as a source of retranslocated potassium. ✓

### 10.9.1 The minimum level of vacuolar potassium sustainable by a potassium proton antiport

The export of potassium from the vacuole at the assumed tonoplast membrane potential of +20 mV against a cytoplasmic potassium concentration of 100 mM (see Section 10.8) can occur without the input of energy if the vacuolar potassium concentration is above a particular level. This level can be calculated using the Nernst equation (Eqn 10.2) and it describes the equilibrium concentration of potassium in the vacuole. Export of potassium to below this equilibrium concentration requires an input of energy (or a change in the physiological parameters of the cell).

$$E_x = RT/F \ln [K_o]/[K_i] \quad \text{Eqn 10.2}$$

where  $E_x$  is the equilibrium membrane potential (mV)

R is the gas constant (0.0083 kJ mole<sup>-1</sup> K<sup>-1</sup>)

T is the absolute temperature (298 K at 25°C)

F is the Faraday constant (0.0965 kJ mole<sup>-1</sup> mV<sup>-1</sup>)

$K_o$  is the cytoplasmic potassium concentration (100 mM)

$K_i$  is the vacuolar potassium concentration

thus:

$$20 = 25.63 \ln [100]/[K_i]$$

$$2.178 = [100]/[K_i]$$

$$K_i = 45 \text{ mM}$$

At equilibrium, the cell would only be able to passively export potassium down to a concentration of 45 mM under the conditions assumed. Complete export of potassium from the vacuole under potassium-deficient conditions requires an energy input. This observation can be interpreted in 2 ways. Firstly, the parameters

substituted into Eqn 10.2 may be incorrect for barley epidermal cells. Secondly there may be an outwardly directed active potassium transport mechanism in the tonoplast of barley epidermal cells.

Tissue heterogeneity shows that cells differ in their vacuolar content. However, the dogma is that cytoplasmic potassium homeostasis is universal and potassium is maintained at about 100mM across all tissues (Tomos and Wyn Jones, 1988). It is possible that the parameters substituted into Eqn 10.2 are not true for barley epidermal tissue or become untrue for barley tissue once the vacuolar potassium concentration declines to 45 mM. The results cannot simply be explained by cell death because the cells without potassium were still turgid and still able to regulate their turgor/ osmotic pressure. ✓

#### 10.10 A model for the transport of solutes within the leaf

Considering the data collectively, it is possible to speculate as to the behaviour of solutes during leaf development and senescence. One day before full leaf expansion potassium is at a higher concentration in the epidermis than is found in the whole tissue (Figs 5.4, 6.6). This may indicate that potassium is an important solute for leaf support early in the life of the leaf. Neither the mesophyll nor epidermis appear to have prioritised receipt of potassium at this stage since in potassium-deficient plants the distribution trends are comparable. After the potassium has accumulated in both tissue types the epidermis turgor or osmoregulates. Thereafter, it must be able to tolerate other solutes arriving in the leaf. Calcium is of particular significance to the plant because it is phloem immobile and is therefore retained within the leaf (Clarkson, 1984). In the system studied, calcium is accumulated steadily (and comparably) in both control and potassium-deficient plants suggesting that it does not perform a primary role as an osmotic adjuster in turgor regulation. Soluble calcium accumulates preferentially in the epidermis. Its sequestration here may be related to its toxicity to other cells, or because it would precipitate if mixed with the vacuolar contents in these cells (refer to Table 8.3, Fig 10.1a). ✓ *ajord*

Uncompensated accumulation of calcium in the epidermis would increase the cell osmotic pressure. In order to regulate either turgor or osmotic pressure, potassium is released from the cell. This release is retarded if cell osmotic/ turgor pressure has not reached its target level showing that potassium is used to turgor/ osmoregulate in epidermal tissue.

Regardless of the osmotic pressure status of the epidermal cell, potassium is still exported from epidermal cells. This apparent contradiction may reflect a process of turgor/ osmotic pressure regulation by potassium that works concomitantly with a developmental process that normally exports potassium.

The hypothesis proposed would suggest that the export of potassium from the epidermis is normally under the direct influence of the cell turgor or osmotic pressure. This osmotic/ turgor pressure will change when other solutes such as sodium or calcium arrive in the leaf, and this change is balanced by loss of potassium. The potassium that is exported is therefore made available once more to the rest of the plant. If this is so, it would suggest that the epidermis acts as a potassium storage tissue in the leaf as well as a structural tissue. It is concluded that the epidermis may be the tissue-type responsible for supplying the retranslocation pathway with potassium. To achieve this, the epidermis appears to possess transport properties distinct from the mesophyll. If tissue heterogeneity is now reconsidered in terms of the possible sequestration of potentially toxic elements then a further reason for leaf heterogeneity becomes apparent. Solutes that are deleterious to certain tissues are clearly best segregated from them in more tolerant tissues that have the ability to accommodate them. Restriction of calcium to the epidermis (Leigh *et al.* 1986) may represent an example of this. It may be a means of segregating ions that would otherwise precipitate since calcium partitioning away from phosphate was also observed in *Vicia faba* (Outlaw *et al.* 1984), although in this case, the mesophyll contained the calcium and the epidermis the phosphate. This reverse distribution suggests that strict compartmentation between different tissue types is more than a mechanism that ensures supply of specific cell types with particular ions.



## **10.11 Conclusion: The role of potassium as an osmoticum**

At the end of Chapter 1 a question was proposed:

**"What is the role of potassium as an osmoticum in individual leaf epidermal cells of barley developing under potassium sufficient and deficient growth conditions?"**

In summary, it has been demonstrated that potassium (and its counter ion) is the most important solute accumulated as an osmoticum in barley leaf epidermal cells. and is therefore important for generating the turgor in these cells. This is true for plants grown in both 4.0 mM and 0.2 mM potassium. In plants grown in 0.2 mM potassium, however, the absolute value of osmotic pressure is initially lower. This is a direct consequence of potassium nutrition, and shows that its initial role as an osmoticum cannot be substituted for (at least in the absence of sodium).

As well as being the primary osmoticum accumulated, potassium plays a second important role as a regulator of turgor. Its export from the vacuole normally balances the change in osmotic pressure that would otherwise occur if the import of soluble calcium were not compensated for. It may play a similar role in regulating turgor or osmotic pressure when sodium is accumulated.

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## 10.12 The methodology

### 10.12.1 The analysis of dried microdroplets

The single cell approach to analysis was developed to overcome limitations in traditional techniques (see Section 1.6). The dried microdroplet method adopted for most of the work was an improvement of an existing technique (Malone *et al.* 1992) because it incorporated an internal standard (Hyatt and Marshall, 1985) which simplified the preparation procedure and enabled quantification of droplets that freeze-dried unevenly. Since its instigation, the approach has become routine and has been extended to analysis of sodium in maize root vacuoles (Pritchard, *pers comm*), of fluid extracted from the rat blastocoel (Brison *et al.*, 1992), of mesophyll and bundle-sheath cell vacuoles (Fricke *pers comm*) and of cells in the pulvinus of *Phaseolus* (Irving, *pers comm*). Phosphorus and sulphur concentrations have similarly been successfully measured in epidermal vacuole extracts of wheat grown under various nutrient regimes (Richardson, 1993). Some caution should still be exercised in interrelating water and solute relations by this approach because of the inability of X-ray microanalysis to distinguish between different chemical states of elements.

The quantitative measurements of the ion concentrations in epidermal vacuoles obtained using the frozen-hydrated material were comparable with those obtained using the dried microdroplet approach, but could give no measure of the water relations of the cell. This was a particular disadvantage in the present study because the hypotheses tested were of the role of vacuolar salts as osmotica. A great advantage of the pressure-probe/ extracted sap approach used in this thesis has been highlighted by Tomos and Wyn Jones (1988). They argue that the interdependence of solute and water relations provide a check on the validity of the data obtained. They wrote: "proposed solute concentrations for each compartment must be consistent with the measured osmotic potentials of the compartments", and "the difference between the proposed protoplast osmotic potential and wall water potential must be consistent with measured turgor pressures". Certainly the solute and water relations parameters measured in this report are in agreement. Since the approach used offers the possibility of taking multiple equal volume samples from the same extracted sap, the

technique also offers the advantage of allowing for some estimate of the degree of experimental variation, as distinct from cell to cell variation.

#### ***10.12.1.1 Is the sap extracted from the cell representative of in vivo concentrations?***

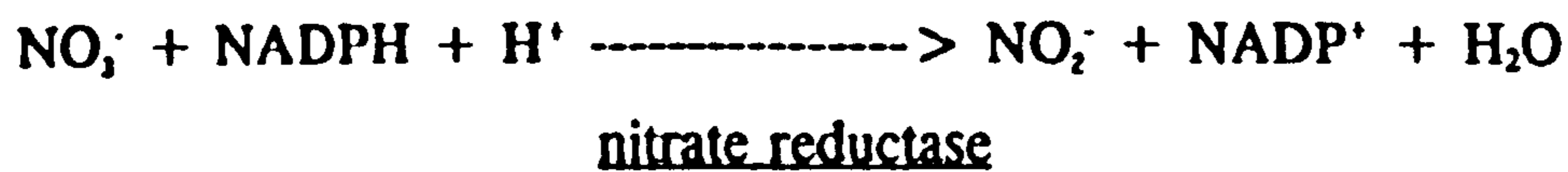
The putative benefit of the microsampling approach is that the contents of single vacuoles can be accurately measured. Whilst the analytical techniques as applied to the extracted droplets are reliable it is necessary to show that the samples are vacuolar and that they are representative of *in vivo* concentrations.

The cytoplasm of barley epidermal cells constitutes only a small fraction (1%) of the cell volume (Plate 2.2 a, also Winter *et al.* 1993) These epidermal cells yielded aqueous samples different from those obtained from mesophyll cells where the vacuole: cytoplasm ratio was much lower, and the samples were more viscous. It is concluded that the vast majority of sap extracted from the epidermis was vacuolar. In cells that are of low turgor or less vacuolate such as the mesophyll this assumption may be less valid. A final observation suggesting that cytoplasmic contamination is negligible draws on the inferences of Leigh *et al.* (1986). It was suggested that the cytoplasm of plant cells contains 100-200 mM potassium, even under potassium-deficient conditions. If cytoplasmic contamination were severe then zero vacuolar potassium (Table 8.2) would not be observed. (Since the completion of the work described here Fricke *et al.* (1994) have looked at the levels of malate dehydrogenase in samples as an indicator of cytoplasmic contamination and have shown that whilst epidermal samples do seem to be free of contamination, mesophyll cells are significantly contaminated.)

#### **10.12.2 Organic constituents of the vacuolar sap**

Concurrent with the development of the X-ray microanalytical technique in our laboratory, other techniques were being developed that enabled measurements of several metabolites in the vacuolar samples. These techniques utilised a microscope fluorometer to assay quantities of NADPH and NADH that varied according to the

stoichiometry of various enzyme-linked reactions. Data have been included in this thesis (Table 8.1) that utilised the enzyme nitrate reductase to assay the vacuolar nitrate concentrations according to the following reaction scheme:



Using a similar approach, organic compounds have been measured in vacuolar sap extracted from leaves of control plants. Preliminary measurements made by Drs Williams and Koyro (pers comm) estimated total sugars (Glucose + fructose + sucrose) to be at maximum 10 mM. It must be emphasised that the data obtained were of a preliminary nature, since the assays were being developed concomitant with the measurements. Since this date, however, Fricke *et al.* 1994 a,b) has reestimated the organic solute concentrations in vacuolar epidermal and mesophyll extracts of the third leaf of barley (cv Klaxon). In a series of measurements from 13 plants, total sugars in the epidermis were found to be  $1.6 \pm 1.4$  mM and in the mesophyll  $36 \pm 18.7$  mM (mean  $\pm$  SD). Amino acids were low in the epidermis ( $0.2 \pm 0.2$  mM) and higher in the mesophyll ( $11.2 \pm 2$  mM), and malate concentrations in the epidermis were very low (0-1 mM). Of the solutes quantified so far, the inorganic component constitutes virtually all the osmotic pressure in the epidermis. In the mesophyll, potassium and nitrate similarly constitute the majority of the osmotic pressure although other organic solutes are present at much greater concentrations than in the epidermis, and therefore constitute a greater proportion of the osmotic pressure.

The ability to measure organic solutes as well as inorganic solutes in extracted sap further reinforces the value of the extracted sap technique for investigation of the water and solute relations of single cells.

### 10.12.3 The analysis of frozen tissue

The mesophyll and other cells present particular technical difficulties for the microsampling technique. In brief, the microsampling technique is of limited use when cells are small, or are of low turgor and highly cytoplasmic. Analysis is made even more difficult if the analysed cells are situated deep within a tissue. To obtain data for mesophyll cells that could be compared with the epidermal data a second approach was followed. This was the X-ray analysis of frozen-sectioned material (Koyro and Stelzer, 1988). In addition, using this technique, an attempt was made to measure cytoplasmic ion concentrations.

Measurements of vacuolar potassium and calcium concentrations of the barley epidermis using both the extracted sap approach and the frozen-tissue approach were close, however, sap solute concentrations measured with the latter technique were generally lower. This difference could be a consequence of the differing growth conditions or of the technique itself because underestimation of solute concentrations would occur if water from the tissue sublimed to a lesser extent than from the standards. This differential etching could have occurred in the analytical system used because whereas the standard solutions were laid directly on top of the copper stub (Plate 2.3), the tissue was mounted within a hole drilled into this stub. Thermal contact between the tissue and the stub was improved by mounting the tissue in a weak detergent solution. However, ice is a poor thermal conductor of heat relative to copper, and the thermal properties of the cell wall are unknown. This arrangement therefore could have led to a faster rate of etching of the standards leading to an underestimation of the vacuolar concentrations.

It is noteworthy that whilst actual concentrations in the vacuole may be slightly underestimated using the frozen-tissue technique, the relationships observed between vacuolar solutes in a single cell should not be affected. Therefore, the relationships between potassium and calcium concentrations are real. The observation that there is relatively little scatter in the data (Figs 9.1b, 9.2c) further suggests that the degree of underetching is consistent. This being so, it could be corrected for if solute concentrations were 'corrected' by comparison of the data with results obtained using the dried microdroplet approach.

Because etching of mesophyll cells could be slower even than in epidermal cells (because of the point contacts between cells and the insulating vacuum in the former 'air spaces') the actual vacuolar concentrations in these cells could be further underestimated. However, the relative concentrations between vacuolar concentrations between epidermal and mesophyll cells should not be affected. This would suggest that the constancy of potassium concentration during a decline in epidermal potassium concentrations (Fig 5.2) is real and would suggest that the redistribution of potassium generally observed is not into the mesophyll vacuoles.

In summary, the extracted sap technique has been shown to be an accurate way of measuring inorganic solutes in dried epidermal sap (Section 3.1.5). It might also be concluded that the frozen-tissue approach is also a valid quantitative technique for measuring other tissues because the results obtained for the epidermal cells using this technique were similar. However, such a general conclusion should be drawn with some caution because of uncertainties in the relative etching rates of standards and samples. To date, it has not proved possible to test the frozen-tissue approach with an internal standard nor to obtain simultaneous water relations measurements of the measured individual cells.

#### *10.12.3.1 Quantitative measurement of the cytoplasm*

A putative advantage of the frozen-hydrated approach of X-ray microanalysis is its ability to measure very small compartments. It was therefore attempted to gain accurate quantitative measurements of the cytoplasm. Throughout the measurement of all cells, fractured surfaces were examined to establish whether cytoplasm could be discerned. In the mesophyll, the cytoplasm was often visible, but was packed with unfractured chloroplasts. This led to an uneven geometry and made selection of an area of pure cytoplasm impossible. Similarly, in the epidermal cells, the cytoplasm was rarely seen, and even when visible was closely appressed to the cell wall. Uneven topography can lead to the interception of the X-ray signal. This problem is confounded by the uncertainties of the spatial resolution of the beam (Morgan 1985) and of ion movement during preparation (Willison *et al.* 1984, Jeffree *et al.* 1987). Furthermore, small cytoplasmic volumes requires that the beam density per unit

volume excited need be higher to achieve adequate counts. Local heating may lead to mass loss (Morgan 1985) that would lead to errors of interpretation.

It is concluded that quantitative cytoplasmic measurement in barley leaves is not reliable using the technique devised for vacuolar measurement.

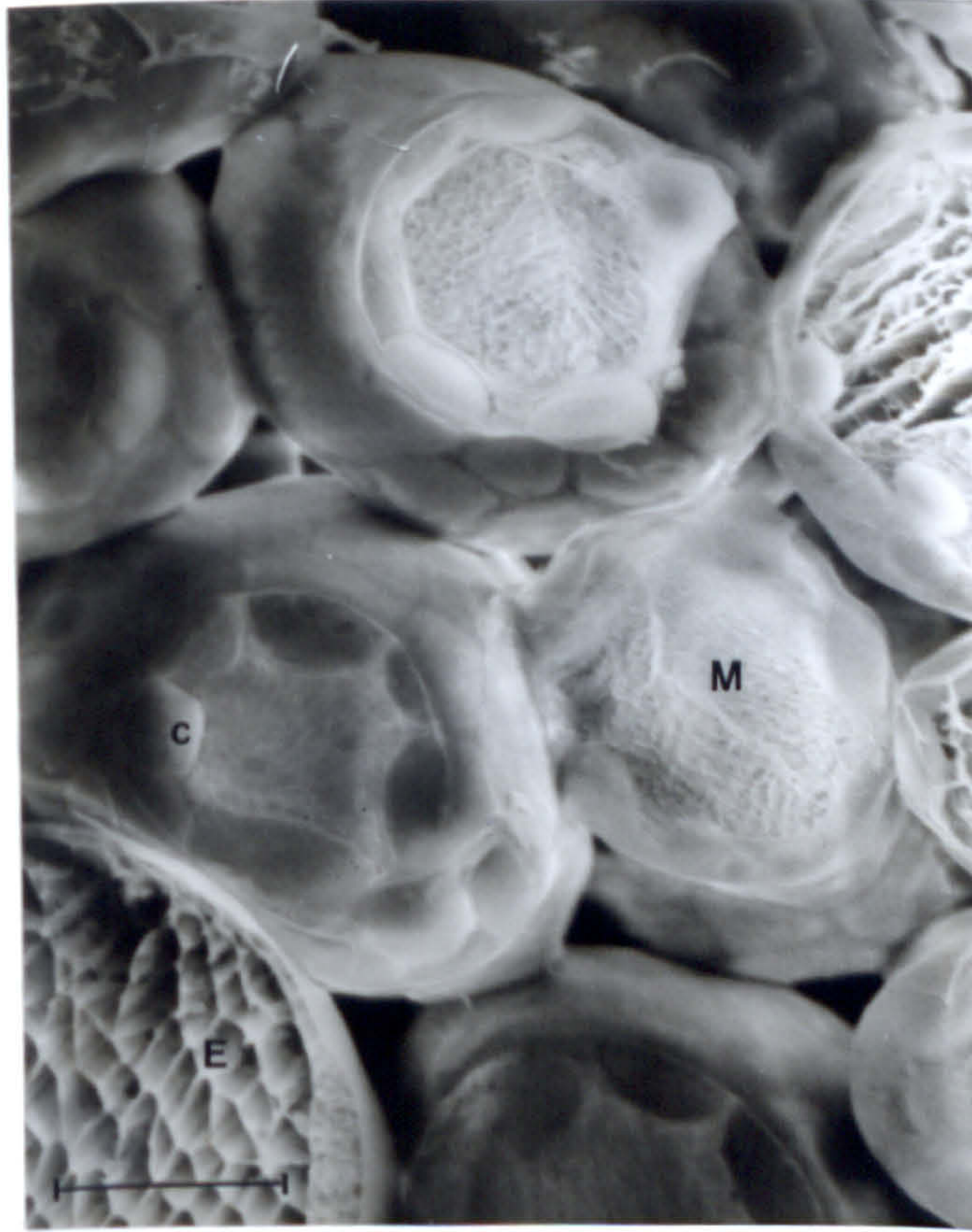


Plate 10.1 Scanning electron micrograph of a (heavily etched) frozen specimen of leaf tissue of barley illustrating the different etching characteristics of the vacuolar contents of the mesophyll (M) and epidermis (E) as caused by the preparatory procedure. The vacuolar contents of the mesophyll have a much finer lattice of crystals. Also visible are the tightly packed unfractured chloroplasts (c) around the periphery of the cell. Such an arrangement prohibited accurate assessments of cytoplasmic ion concentrations. The scale bar shows  $10\mu\text{m}$ .

### 10.13 A summary and comparison of the dried microdroplet and frozen-tissue approaches for quantitative X-ray microanalysis

It is concluded that the micropressure probe approach is the method of choice if solute relations are to be related to water relations in individual cells. Not only does this method allow for measurements of turgor pressure, osmotic pressure and elemental concentrations in single cells, but if necessary, this data can be supplemented with other measurements. Specifically, organic solutes can be measured by microfluorimetry (see Section 1.2.5.3). In addition, the veracity of the analytical techniques can often be checked by adding 'spikes' or internal standards of known concentration. The ability to divide extracted sap into subsamples means that errors of measurement can be quantified, and measurement variation be made distinct from cellular variation. Measurements of the cell osmotic pressure provides a rough check that the results obtained are tenable.

The major disadvantages of the pressure probe technique are:

- 1) It is currently only applicable to larger cells.
- 2) There is a risk of cytoplasmic contamination at the time of sample extraction.
- 3) There is a risk of sample dilution at the instant of extraction and of evaporation (especially of the smaller samples) after extraction. (Aside from minimising dilution, the practise of instant sampling has an added advantage in that the sample is taken before the plant has time to respond to other physiological perturbations imposed by having a single cell burst).
- 4) Cell sampling is dependent upon cell size and cell turgor, hence smaller less turgid cells give smaller samples (presumably with increased risk of cytoplasmic contamination).
- 5) The technique is better suited to the analysis of surface cells such as the epidermis. Uncertainties can arise as to the identification of cells sampled from within the plant tissue.



6) The technique is exceedingly time consuming, and requires much preparation and skilled manipulation under the light microscope. Even so, only a few cells can be analysed at a sitting.

If the disadvantages are inconsequential, then the pressure probe method is the method of choice for quantification since the method can be tested. The advantages of quantification of frozen hydrated material are:

- 1) Many separate cell types can be measured in a single plant even cells situated deep within the leaf can be measured.
- 2) Samples can be taken rapidly at a distinct point of time and do not necessitate tissue being subjected to an intense light source.
- 3) Analyses are performed on cooled specimens which may minimise selective sublimation of elements.
- 4) The technique can provide useful information regarding relative concentrations of elements in single vacuoles.

The disadvantages have been discussed, but essentially:

- 1) The technique relies upon certain assumptions that cannot be tested e.g. the cells themselves and standards may etch at different rates thus comparisons may be meaningless.
- 2) The tissue preparation itself may introduce artefacts stemming from tissue flooding and ice crystal formation.
- 3) The results cannot be expressed on the basis of measured osmotic or turgor pressures for individual cells.
- 4) Measurements made of inner-leaf tissues may be less reliable than of other tissues because they are more susceptible to errors of preparation (see 1) and 2)).

Although the results obtained with the freeze-etch technique were consistent those obtained by the extracted sap technique, it is the inability to test the data which makes it questionable. Clearly 'questionable' quantitative techniques by definition can not be quantitative.

#### 10.14 The experimental system

##### 10.14.1 Are the differences observed between treatments a nitrate or potassium effect?

The choice of barley as a plant material has been considered (see introduction). Epstein nutrient solution allowed good growth of the control plants whilst the potassium-deficient treatment was achieved simply by reducing the quantity of potassium nitrate added to the nutrient solution. Since the decrease in nitrate (from 12 to 8.2 mM) was not compensated for, it might be argued that the observed response was one of nitrate deficiency. In fact, even a concentration of 8.0 mM nitrate in the potassium-deficient nutrient solution is likely to constitute a "luxury" supply (Oertli, Richardson, *pers comm*). Furthermore there is evidence that replacing the cation with, for instance, calcium could itself influence the vacuolar concentrations (Atkinson 1991, Dietz *et al.* 1992b). Other evidence that suggests that the decline in turgor was due to potassium deprivation and was not a nitrate effect has been provided by Richardson (1993). It was demonstrated that turgor maintenance is independent of the anion accumulated, and that nitrate can be replaced in its osmotic role by for instance phosphate, sulphate or chloride. These were all present in the nutrient solution used. Analysis of nutrient solution potassium concentrations revealed that they did not decline below 2 mM in the control treatment (data not shown) hence control plants of the high potassium treatment were assumed to be growing optimally. ✓

### 10.14.2 Variable growth conditions and the comparability of experiments performed on different occasions

The variable growth conditions might account for the variation observed between repeats of the various time-course analyses. The light intensity was fairly low, and possibly limiting, and could possibly have varied as adjacent batches of plants were grown and removed from the growth room. Furthermore no strict routine was followed in removing set numbers of plants from the growth solution at set points in time. This, together with the variable temperature and humidity of the growth room may have influenced the development of the plants, and therefore made direct comparisons of different experiments difficult. The time course of senescence did not change markedly (as judged by the date at which necrotic patches became evident, day 13, in both control and potassium-deficient plants) whilst the pattern of solute movement over this period did. This was demonstrated by the variable response of vacuolar osmotic pressure.

### 10.15 Presentation of the data

#### 10.15.1 How do single cell concentrations compare with whole tissue concentrations?

The variability of growth conditions, the uncertainty of vacuolar dilution at the instant of extraction and plant to plant variation could obscure real physiological responses. Care has therefore been taken to minimise errors wherever possible by expressing solute and osmotic pressure values relative to the single cell and plant from which they were obtained. Due to growth condition variation, establishing the precise time course of leaf development was deemed less important than identifying the trends of ion movement over this period. Therefore the data have been presented as separate time courses. Typical errors of single cell concentration measurements can be seen in Table 8.2. The occasionally large standard deviation values observed when results were pooled arise therefore both from the additive errors of measurement and from plant to plant variation. These errors are especially apparent at day 13 when the plants

display the most epidermal heterogeneity, and more variability between individual plants.

Clearly, any obvious trend observed after real variation is averaged are likely to be representative of the actual physiological response of the plant. Indeed, plots of single cell concentrations against their mother plant concentration values demonstrate even more conclusively that the epidermal fluxes are far larger (and the probable cause) of the smaller leaf fluxes (data not shown).

### 10.16 Overall conclusion and future work

The data presented in this thesis emphasise the value of investigating solute and water relations at their component resolution. It shows that the microsampling approach to measurement is reliable, and also shows that adjacent cells may behave dissimilarly. By studying single cells it should be possible understand the behaviour and function of individual leaf tissues. This in turn will lead to a greater understanding of whole leaf behaviour. This reasoning can also be applied to other plant organs such as the root because cell heterogeneity in the root is also an important determinant of root function.

The data presented in this thesis has already led to some new hypotheses. For instance, the epidermis has been proposed as the source of potassium in potassium retranslocation. Similarly, vacuolar sodium is suggested to behave similarly to vacuolar calcium in its role as an osmoticum. These hypotheses need support from further experiment, however, future work could follow several different directions. The hypotheses discussed in this thesis beg further questions for instance:

- 1) How are the transport mechanisms controlled?
- 2) What is responsible for the pattern of heterogeneity observed?
- 3) Does the import of e.g. sodium or calcium dictate the rate of senescence of a particular leaf, or is the senescence of a leaf responsible for this mechanism?
- 4) How is the developmental senescence of the epidermis matched to the senescence of other cell types?

Any future work is likely to be performed under more controlled plant growth conditions, with light intensities typical of those normally experienced by the barley plants. Initially, calcium solubility could be confirmed by using microelectrodes to measure the calcium activity in either extracted sap samples or *in vivo*. An attempt will begin shortly to investigate cellular pathways by use of cell surgery to create lesions across proposed transport paths. For instance, if the route of calcium transport occurs to some extent within the epidermis itself, isolation of epidermal cells into 'islands' might be expected to retard the rate of senescence if this were due to the arrival of calcium. If such surgery leads to no difference in the rate of calcium import, then a mesophyll symplast or apoplastic route of calcium import would be implied. Other investigations include establishment of the presence or absence of plasmodesmata, and of measurements of turgor and wall water potentials of the mesophyll. This would establish the likelihood of apoplastic continuity between the epidermis and mesophyll. As an adjunct to this, tracer solutes or dyes could be released via the stomatal pores into the apoplast surrounding the mesophyll, so that subsequent transport could be investigated. These treatments could include mannitol to further investigate the role of turgor in these processes.

To date, the effect of sodium in the system has not been investigated. This may give some insight as to how the solutes interact and influence both osmotic pressure and senescence and plant growth. It is possible that sodium is tolerated as a cation because barley appears somewhat salt tolerant, and has been shown to accumulate in leaf tissue (Fricke pers comm, Leigh *et al.* 1986).

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