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Osmotic Measurements on Stomatal Cells of *Commelina communis* L.

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Summary. Observations of aperture changes as sucrose is added to the solution bathing epidermal strips of *Commelina communis* L. allow calculation of the osmotic changes required to open or close the stomatal pore, for comparison with changes in potassium content. With "isolated" guard cells, in strips in which all cells other than guard cells have been killed, the internal osmotic changes required are 83 mosmol $kg^{-1}\,\mu m^{-1}$ below 10 μm aperture, 129 mosmol $kg^{-1}\,\mu m^{-1}$ in the range 10–15 μm, and 180 mosmol $kg^{-1}\,\mu m^{-1}$ above 15 μm. For opening against subsidiary cell turgor in addition to guard cell turgor, in intact strips with live subsidiary and epidermal cells, these figures should each be increased by about 33 mosmol $kg^{-1}\,\mu m^{-1}$. A change in subsidiary cell turgor is magnified in its effects on the water relations of the guard cell by a factor greater than 3.7 for equal changes in the water potential of the two cells, or greater than 4.7 at constant volume of the guard cell.

It is now generally accepted that the turgor changes in stomatal guard cells which are responsible for the opening and closing of the stomatal pore are the result of ion movements into and out of the guard cells, generally involving the accumulation and loss of potassium salts. There is evidence for accumulation of very high concentrations of potassium in open guard cells of many species, as summarized in recent reviews (Raschke, 1975, 1977; Meidner & Willmer, 1975; MacRobbie, 1977). It is, however, important to make a quantitative comparison of potassium changes and of osmotic changes required to open or close the stomatal pore, over the whole range of aperture. This paper is the first of three related to this aim, to determine the extent to which the measured potassium changes can account for the required osmotic changes. In this paper measurements of the osmotic changes are made, to be compared in the two later papers with measured changes in potassium content (MacRobbie & Lettau, 1980, *in preparation*).

The species chosen is *Commelina communis* L., on which a good deal of previous work has been done. In this species the stomatal complex consists of eight cells, a pair of guard cells surrounded by pairs of inner lateral subsidiary cells, outer lateral subsidiary cells, and terminal subsidiary cells; in addition unspecialized epidermal cells separate the complexes. Penny and Bowling (1974) used K-sensitive microelectrodes to measure potassium levels in the various cell types, in conditions in which the stomata were wide open or were closed, and these measurements are extended in the two following papers. *Commelina* has the further advantage for stomatal work of allowing the use of "isolated" guard cells, in epidermal strips in which all cells other than guard cells have been killed by a treatment at low pH (Squire & Mansfield, 1972). In such tissue tracer measurements of ion concentration changes in guard cells are possible, allowing a check on measurements with ion-sensitive electrodes. The second paper is concerned with potassium measurements in "isolated" guard cells, the third with potassium measurements in the intact epidermal strips, for comparison with the present osmotic measurements.

Osmotic measurements of this kind also allow assessment of the relative importance of the pressure in the guard cells, and that in the subsidiary cells, in determining the stomatal aperture, and provide further illustration of the "mechanical advantage" of the subsidiary cell, discussed by previous authors.

Materials and Methods

Preparation of Epidermal Strips

Plants of *Commelina communis* L. (originally from seed provided by Professor T.A. Mansfield, Lancaster University) were grown in a greenhouse with supplementary lighting for 15 hours/day, until they were about 6 weeks old. The two youngest fully expanded leaves were used. To ensure that all cells were fully turgid, leaves

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were kept on wet paper tissues in a closed Petri dish before epidermal strips were taken, in light to open stomates and in the dark to close them. Strips of lower epidermis were taken, floated briefly on distilled water, and cut to a size of about $5 \times 5 - 10$ mm.

Intact epidermal strips were observed at once, in an initial solution containing 75 mM KCl, 10 mM MOPS at pH 6.7 (3-(N-morpholino) propanesulphonic acid).

"Isolated" guard cells were prepared by floating the strips (cuticle up) at low pH for several hours, the treatment introduced by Squire and Mansfield (1972) to kill all cells in the epidermal strips other than the guard cells. In general, a treatment of 2 hr in 10 mM MES (2-(N-morpholino) ethanesulphonic acid) at pH 3.9 was used, in a large volume of solution, with several changes. The effectiveness of the killing treatment may be checked by the inability of the dead cells to take up neutral red (0.01 % solution), or their penetration by Evans Blue, but is also clearly seen in the microscopic appearance of cells under high power, and the presence or absence of crystals in the epidermal cells. Guard cells will survive for days at pH 3.9 and given KCl will open widely. In general, treatment for 2 hr at pH 3.9 did kill epidermal cells, but sometimes longer treatment was necessary, and on several batches of plants grown in very hot conditions many epidermal cells seemed able to survive prolonged treatment at low pH. The reason for this resistance is not known. It is possible that killing results from prolonged treatment in the absence of Ca^{2+}; if so, then in plants grown in conditions of high transpiration, with large numbers of calcium oxalate crystals in their epidermal cells, it is possible that enough Ca^{2+} is released from the first cells damaged by low pH to protect a significant fraction of the epidermal cells in the strips, even with several solution changes. For this reason it was essential to check the condition of all the strips used as "isolated" guard cells.

After the isolation pretreatment, strips were treated overnight or longer in a thermostatted cabinet at 30 °C in light, floating on 50–75 mM KCl, 10 mM MES, pH 3.9, to open the stomata.

Observation of Osmotic Changes

For observation of aperture changes produced by osmotic changes in the external bathing solution the epidermal strips were placed, cuticle side up, across two greased fragments of cover slip (6×25 mm), stuck to a glass slide with a channel 3 mm wide between them; a cover slip with greased edges was then placed on top, and the solution bathing the strip could be changed rapidly by drawing solution through the chamber thus formed. The strip was observed at magnification of $640\times$, using Nomarski optics, in a Zeiss Standard Universal microscope. Stomatal apertures were measured with a micrometer eyepiece (1 division = 1.54 μm, measured to 0.5 division), or were photographed and measured on enlarged prints.

It was essential to work as quickly as possible to minimize solute leakage from the cells. All solutions contained the same K concentration in any one experiment (50 or 75 mM, with 10 mM MES, pH 3.9, or MOPS, pH 6.7), but varying sucrose concentrations. Aperture changes were fast; e.g., on removal of 1079 mosmol kg^{-1} sucrose the aperture of one stomate increased from 10.8 to 13.9 μm within the first minute (and was very close to this within 25 sec), and changes were complete within 2 min. (Similar rapid equilibration of water across guard cell membranes was found by Meidner and Bannister (1979) in *Tradescantia virginiana*, and by Raschke (1979) in *Vicia faba*.) In each strip the apertures were measured in each of a group of 6–10 neighboring guard cells. After a solution change, with several flushes through, about 2 min were allowed for equilibration, and then the apertures were measured rapidly; exposure to each solution lasted for 7–10 min, and the whole series for 46–60 min or so. In nearly all cases the aperture recovered to its original value on removal of sucrose, and it is argued that loss of solute content was not a serious problem – probably because of the presence of high KCl in all solutions.

Osmotic pressures of sucrose solutions were taken from tables (Handbook of Chemistry and Physics, Weast, CRC Press) but solutions were also checked by use of a vapor pressure osmometer (Wescor).

Plasmolysis was observed easily in epidermal cells, outer lateral and terminary subsidiary cells. It was hard to see in inner lateral subsidiary cells, particularly when these were compressed by open guard cells. It could be seen in guard cells, but it was hard to estimate the stage of incipient plasmolysis, and was often not reached in open guard cells in the solutions used. Because of the need to observe apertures in a number of neighboring complexes as quickly as possible, careful searching for incipient plasmolysis in guard cells was not attempted in this study.

Measurements of Cell Volumes

Stomatal complexes were photographed, using Nomarski optics at $640\times$, and enlarged prints were made, on which areas were measured either by planimetry or by cutting out and weighing the paper. Depths were calculated from the fine focussing control on the microscope. Initially measurements were made on a series of strips, with varying stomatal apertures, mounted in liquid paraffin. In a later series individual guard cells were photographed at different apertures, after changes of external osmotic pressure, as part of the main series of measurements.

Treatment of Results

Plots of aperture against the osmotic concentration of sucrose added (π_o, mosmol kg^{-1}) were drawn. In intact strips, the initial changes as sucrose is added are a consequence of turgor changes in both guard cells and subsidiary cells. As subsidiary cell turgor is reduced the pore tends to open, but in due course the subsidiary cell turgor disappears, and the changes then reflect the changes in the guard cell alone (Fig. 1). The contribution of the subsidiary cell turgor to the water relations of the guard cell (P_{gs}) can be estimated as the value of π_o required to bring the pore back to the initial aperture, after release of subsidiary cell turgor. Slopes of the curves of aperture against π_o were estimated in the region of zero subsidiary cell turgor.

In "isolated" guard cells, in the absence of any subsidiary cell turgor, the aperture falls from the start, as sucrose is added, and slopes may be estimated from the start of the experiment.

From such plots of aperture against π_o, estimates of the dependence of the aperture on the internal solute content were made in two ways, either (a) from the relation between aperture and π_o in a single stomate, or (b) by comparison of the different external osmotic changes necessary to produce a given aperture in a number of stomates of different initial apertures. The second method was used only for isolated guard cells, in which the range of initial apertures in a single strip was wider than that in an intact strip, and in which the initial aperture did not also depend on the subsidiary cell turgor.

(a) For a single guard cell we may write $\Psi_o = \Psi_{cell}$ in any given solution, where Ψ is water potential. i.e.,

$$-\pi_o = P - \pi = P - Q/V \tag{1}$$

where P is the cell turgor pressure, π the cell osmotic pressure, Q the cell solute content, and V the cell volume.

Changes in the cell water content may be produced by the water flows induced either by a change in π_o, as in the experiment, or by a change in solute content, by potassium accumulation for example. In either case we may write:

$$d\Psi = dP - \frac{1}{V}dQ + \frac{Q}{V^2}dV. \quad (2)$$

Under experimental conditions Q is taken to be constant, and

$$-d\pi_o = d\Psi = dP + \frac{Q}{V^2}dV. \quad (3)$$

Pressure changes and volume changes are related by the elastic coefficient of the cell wall, ε. For small changes in volume we may write:

$$dP = \frac{dV}{V} \cdot \varepsilon \quad (4)$$

although ε may change with volume over an extended range. In the intact strip both P and ε for the guard cell will depend on the elastic properties of both the guard cell wall and the surrounding subsidiary cells. At any volume the total pressure in the guard cell will have two components, P_g depending only on guard cell and its resistance to expansion, and P_{gs}, the effective pressure of the subsidiary cells as seen from the guard cells (the tissue pressure); Similarly ε will have two components, one expressing the resistance of the guard cell wall to expansion, and another (ε_{gs}) expressing the resistance of the subsidiary cell to the guard cell expansion. As will be shown in Results, in the intact strip the second component is more important. Once the subsidiary cell turgor is released then only the components of P and ε determined by the guard cell wall need be considered.

If we insert Eq. (4) into Eq. (3) then we have

$$d\Psi = (\varepsilon + \pi)\frac{dV}{V}. \quad (5)$$

Thus if we determine the slope $\frac{d\Psi}{dA}$, we are measuring the quantity $\frac{(\varepsilon+\pi)}{V}\frac{dV}{dA}$, and if we know the relation between volume and aperture we may determine $(\varepsilon + \pi)$.

However, we may also consider an alternative condition, of a volume change produced by a change in solute content, at constant water potential. Then in Eq. (2) we may put $d\Psi = 0$, and we have

$$\frac{dQ}{V} = dP + \frac{Q}{V^2}dV. \quad (6)$$

Comparing Eqs. (3) and (6), it is clear that the slope $\frac{d\pi_o}{dA}$, determined from the dependence of aperture on external π_o, may also be used as an estimate of the slope $\frac{1}{V}\frac{dQ}{dA}$, the dependence of aperture on solute content, at constant water potential. Since volume changes are small, the slope $\frac{1}{V}\frac{dQ}{dA}$ will be close to $\frac{d\pi}{dA}$, and will be an estimate of the change in internal osmotic pressure required to open and close the pore, at constant water potential.

Consider a group of isolated guard cells having different solute contents, and therefore different initial apertures A. Each may be brought to a given aperture x but requires a different external osmotic pressure (π_o^x) to do so. At aperture x, all the guard cells have the same volume V_x, and therefore the same pressure P_x, but different solute contents.

For each we may write

$$-\pi_o^x = P_x - Q/V_x. \quad (7)$$

If we consider the dependence of π_o^x on the initial aperture then we are, in effect, considering the relation between solute content and initial aperture. We may write:

$$\frac{d\pi_o^x}{dA_i} = \frac{1}{V_x}\frac{dQ}{dA_i}. \quad (8)$$

Graphs of A against π_o were drawn for all the cells of each group, and values of π_o^x for each cell were read off these graphs, for various values of x. The slope of the graph of π_o^x against the initial aperture A_i is then an estimate of $\frac{1}{V_x}\frac{dQ}{dA_i}$, and provides an alternative way of determining the relation between aperture and solute content.

This method could also be used for a group of intact guard cells provided π_o^x could be plotted against an estimate of the aperture which would be reached at the given initial solute content in the absence of subsidiary cell turgor. This could be estimated by extrapolating the lines obtained for aperture against π_o once the subsidiary cell turgor has been released, back to zero added sucrose. However, in practice, since the range of initial apertures in an intact strip was not very wide the method was not used, and results are quoted for intact strips only as slopes $\frac{d\Psi}{dA}$.

Results

Changes of Aperture with Increasing π_o

Figure 1 shows a typical set of curves for the change in stomatal aperture as sucrose is added to the solution bathing an intact strip. After an initial portion where the release of subsidiary cell turgor masks the closing effects of reducing guard cell turgor, the aperture falls as external sucrose is increased, and slopes may be calculated from the subsequent parts of such curves. The changes in water potential required to change the aperture are shown in Table 1. The slopes change with aperture, as might be expected, and therefore the results are quoted for three aperture ranges. The slopes may be used as estimates of $\frac{1}{V}\frac{dQ}{dA}$, the increase in internal osmotic concentration required to open the pore, and will be compared with changes of potassium concentration measured by other methods. (*See* MacRobbie & Lettau, 1980, and *in preparation.*)

The effect of adding sucrose to the solution bathing "isolated" guard cells is shown in Fig. 2. In the absence of subsidiary cell turgor the aperture falls from the start and slopes may be estimated over the whole range of sucrose solutions. The figures for "isolated" guard cells are also collected in Table 1 and agree reasonably well with those obtained from intact strips. Table 2 collects estimates of the guard cell properties which may be obtained from such results. In pressure units the average values of the

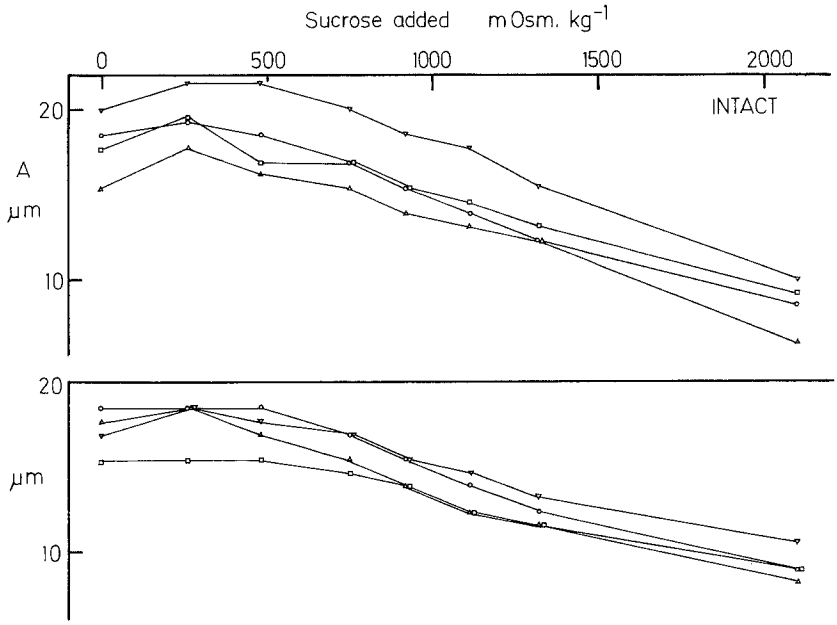

Fig. 1. Effect on aperture of adding sucrose to the solution bathing an intact epidermal strip; all solutions contain 75 mM KCl, 10 mM MOPS at pH 6.7. Each of the eight curves represents a single stomatal complex in the same strip

Table 1. Changes in guard cell water potential required to change the aperture: $\frac{d\Psi}{dA}$ / mosmol kg^{-1} μm^{-1}

	$\frac{d\Psi}{dA}$ / mosmol kg^{-1} μm^{-1}		
	Above 15 μm	10–15 μm	Below 10 μm
Intact	162 ± 17 (4)[a]	151 + 15 (8)[a]	52 ± 7 (5)
		129 ± 7 (6)	53 ± 4 (7)
		125 ± 4 (6)	102 ± 8 (9)
			100 ± 8 (5)
			138 ± 7 (4)
			133 (3)
Means	162 ± 17 (4)	137 ± 7 (20)	91 ± 6 (33)
Isolated	188 + 21 (9)[b]	139 ± 9 (10)[b]	108 ± 7 (6)[b]
		109 ± 15 (7)[c]	58 ± 4 (7)[c]
		104 ± 16 (5)[d]	85 ± 16 (7)[d]
			50 ± 4 (7)[e]
Means	188 ± 21 (9)	121 ± 8 (22)	74 ± 6 (27)

[a] Figures are given for the means of stomata measured in individual strips, and for combined means. Superscripts indicate measurements in different aperture ranges on the same strips, or identify strips for comparison with other figures. All "intact" measurements were made on fresh strips, at pH 6.7. "Isolated" treatments:

[b] Measurements at pH 3.9 after 44 hr pretreatment at pH 3.9.

[c] Measurements at pH 6.7 after 20 hr pretreatment at pH 3.9.

[d] Measurements at pH 6.7 after pretreatment of 7 hr at pH 3.9 followed by 41 hr at pH 6.7.

[e] Measurements at pH 6.7 after pretreatment of 7 hr at pH 3.9 followed by 20 hr at pH 6.7.

slopes $\frac{d\Psi}{dA}$ are 0.44 MPa μm^{-1} above 15 μm, 0.31 MPa μm^{-1} in the range 10–15 μm, and 0.20 MPa μm^{-1} below 10 μm (1 MPa = 10 bar).

Table 2. Properties of "isolated" guard cells

Aperture	6–9 μm	11–14 μm	16–20 μm
$\frac{d\Psi}{dA}$ / MPa μm^{-1}	0.20	0.31	0.44
$\frac{dA}{d\Psi}$ / μm MPa^{-1}			
(constant Q)	5.0	3.2	2.3
$(\varepsilon+\pi)$/MPa	7.5	12	16
approx. π/MPa	0.8	$1.5<\pi<2.2$	$3.8<\pi<5.7$
approx. ε/MPa	6.7	10	11
approx. $\varepsilon/\varepsilon+\pi$	0.89	0.83	0.69
approx. $\frac{dP_g}{dA}$ / MPa μm^{-1}	0.18	0.26	0.30
approx. $\frac{dA}{dP_g}$ / μm Mpa^{-1}	5.6	3.8	3.3

$(\varepsilon+\pi)$ assumes a fractional volume change of 0.027 μm^{-1}.

$\frac{dA}{dP_g}$ is the coefficient b_g in the nomenclature of Cooke et al. (1976).

Such slopes are also estimates of the quantity $(\varepsilon+\pi)\cdot\frac{1}{V}\cdot\frac{dV}{dA}$. If $\frac{1}{V}\frac{dV}{dA}$, the fractional change in volume per unit change in aperture, is known, then we may calculate $(\varepsilon+\pi)$. The fractional change in volume will be the sum of the fractional changes in area and in depth, all for a change of 1 μm in aperture. Fractional changes in area were estimated from photographs of "isolated" guard cells as the aperture changed, and the mean value was 0.019 ± 0.002 (8) μm^{-1}. The fractional change in depth with aperture was much smaller and was difficult to measure accurately; at the position of overlap with the inner lateral subsidiary

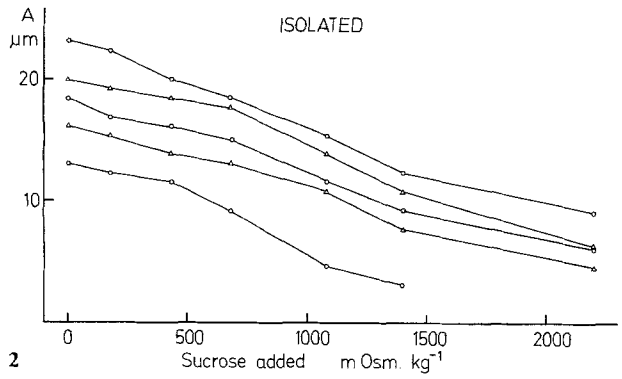

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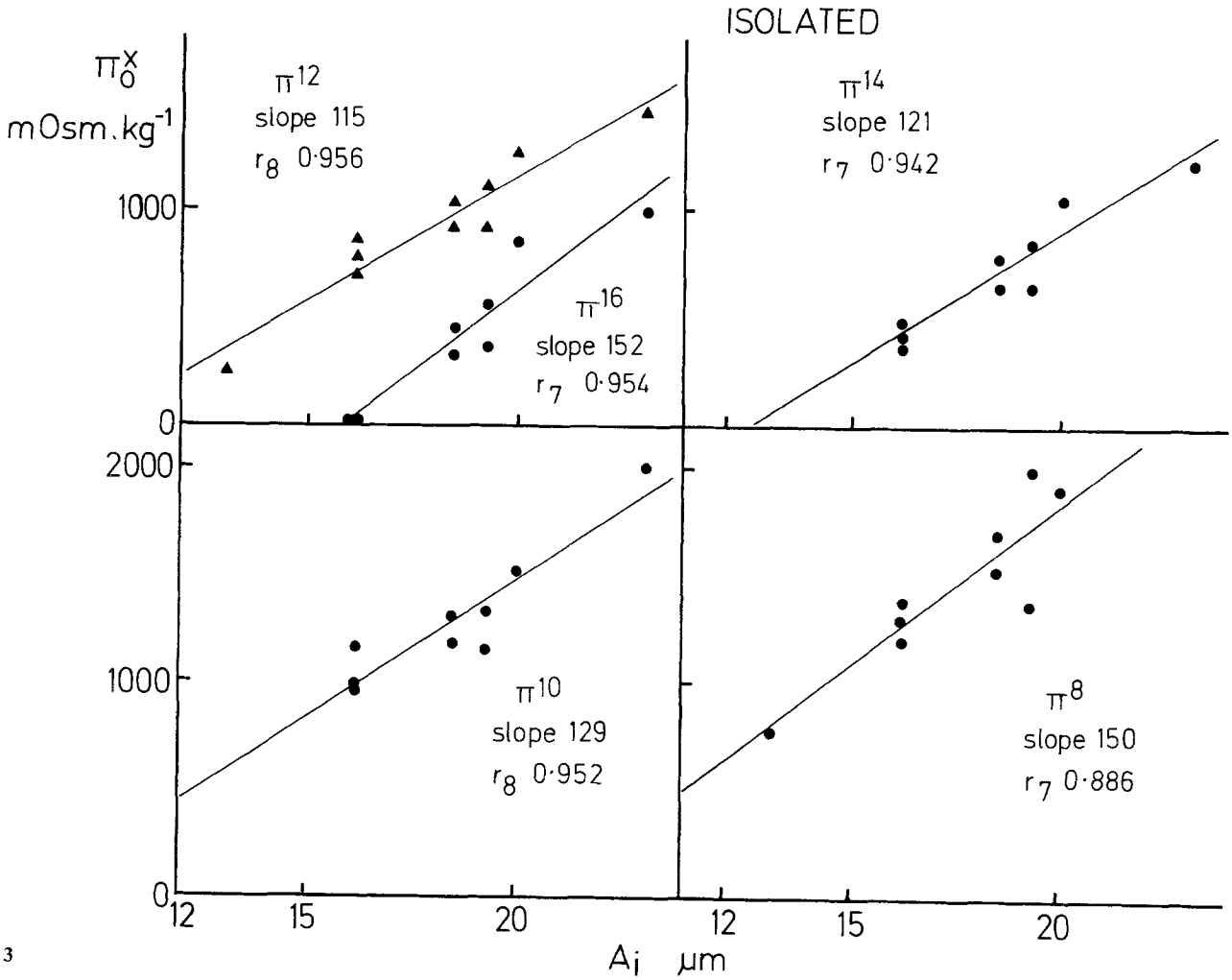
Fig. 2. Effect on aperture of adding sucrose to the solution bathing an "isolated" epidermal strip; all solutions contain 75 mM KCl, 10 mM MES at pH 3.9. Each curve represents a single stomatal complex in the same strip

Fig. 3. External concentrations of sucrose (π_o^x) required to produce given apertures (8–16 μm) in complexes with different initial aperture (A); thus π^{12} is the osmotic pressure of sucrose required to produce an aperture of 12 μm, in 10 complexes of initial apertures 13–23 μm. All solutions contain 75 mM KCl, 10 mM MES at pH 3.9. (Tissue *b* in Table 1)



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cell, the fractional change in depth was about $0.0075\,\mu m^{-1}$. Thus the fractional volume change is about $0.027\,\mu m^{-1}$, allowing the estimate of $(\varepsilon+\pi)$ in Table 2. To the extent that fractional volume changes are underestimated because of the wall thickness, these figures will be overestimates. Because of the difficulty of detecting incipient plasmolysis and the need to work quickly, accurate estimates of π were not made in this study. Approximate estimates of π can be obtained from the osmotic pressures of the highest solution tried in which the guard cells were not plasmolysed and the lowest solution in which

Table 3. Dependence of π_o^x on initial aperture

		$\frac{d\pi_o^x}{dA_i}(r, \mathrm{df})$ (mosmol kg^{-1} μm^{-1})			
Strip		I	II	III	IV
Label in Table 1		(*b*)	(*c*)	(*d*)	(*e*)
	A_i (μm)	13–23	7–17	6–15	6–10
Mean	$\bar{A}_i$	18.0±0.9 (10)	13.3±1.1 (9)	11.6±1.1 (8)	7.7±0.5 (7)
	16	152 (0.954,7)			
	14	121 (0.942,7)	63 (0.63,4)		
	12	115 (0.965,8)	63 (0.40,4)	55 (0.967,3)	
x (μm)	10	129 (0.952,8)	72 (0.82,3)		
	8	150 (0.886,7)	81.5 (0.956,4)	108 (0.933,4)	
	2		73.8 (0.981,4)	110 (0.887,6)	34 (0.918,5)
	4		68.4 (0.991,3)		

$\frac{d\pi_o^x}{sA_i}$ is equal to $\frac{1}{V_x}\frac{dQ}{dA_i}$.

they were, but no attempt was made to use narrow ranges of osmotic pressure and detect incipient plasmolysis. Such approximate estimates of π may then be used to calculate ε and the ratio $(\varepsilon+\pi)/\varepsilon$. This ratio may then be used to calculate $\frac{dA}{dP_g}$, the coefficient b_g in the nomenclature of Cooke et al. (1976), since $dP_g = (\varepsilon/\varepsilon+\pi)\cdot d\Psi$.

The dependence of aperture of "isolated" guard cells on internal solute concentration was also estimated by the second method already discussed; thus the osmotic concentrations of sucrose (π_o^x) required to produce a given aperture (x) in the different stomates measured in one strip were compared by plotting π_o^x against the initial aperture. Figures were read off graphs such as those in Fig. 2, and Fig. 3 shows one set of such results. In Fig. 3 are plotted the osmotic concentrations of sucrose required to produce apertures of 16, 14, 12, 10 and 8 μm in up to 10 stomates in one strip, against their initial apertures which were in the range 13–23 μm. π_o^x and A_i were linearly related, and the slope $\frac{d\pi_o^x}{dA_i}$ seemed to vary little with x. Since this slope is equal to $\frac{1}{V_x}\frac{dQ}{dA_i}$, this implies that volume changes with aperture were small over this range. The results of four such comparisons in different strips are collected in Table 3, which shows values for $\frac{d\pi_o^x}{dA_i}$ for different values of x, the correlation coefficients for the regression of π_o^x on A_i and the number of degrees of freedom on which these coefficients are based. The figures in each column may be compared with the slopes for the same strips in Table 1, measured as $\frac{d\pi_o}{dA}$ as A changes. The results imply that the initial aperture of "isolated" guard cells is linearly related to their solute content. However, this second method of estimating $\frac{d\pi}{dA}$ is likely to be less accurate than the first. In the first, the uncertainties in measuring apertures against the eyepiece micrometer should be evened out in estimating the slope for each stomate; in the second, a line has only one point from each pore and the fluctuations are less effectively ironed out. The values in Table 1 should therefore provide better estimates of $\frac{1}{V}\frac{dQ}{dA}$ than those of Table 3.

Effects of Subsidiary Cell Turgor

The effects of subsidiary cell turgor may also be estimated from curves of the form shown in Fig. 1. Figure 4 is a diagram showing how this was done. The final portions of such curves refer to a state in which subsidiary cell turgor has disappeared (and in which plasmolysis is visible in subsidiary and epidermal cells); if this linear portion is projected back to zero added sucrose we have an estimate of $A^0_{\max}$, the aperture appropriate to the given solute content in the absence of any opposing subsidiary cell turgor. The osmotic pressure of the sucrose added to bring the cells again to the initial aperture, after release of subsidiary cell turgor, is a measure of the contribution of subsidiary cell turgor to the water relations of the guard cell. This has been called P_{gs}, and is not

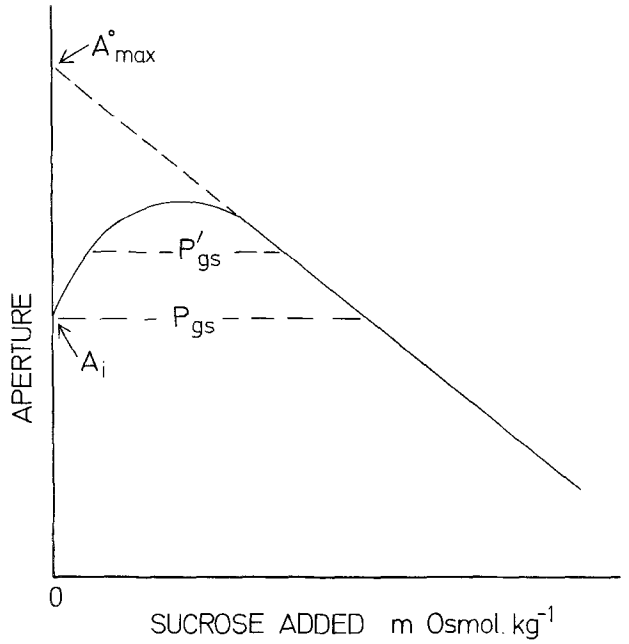

Fig. 4. Diagram showing method of estimating P_{gs}, the contribution of subsidiary cell turgor to the water relations of the guard cell. P_{gs} is the osmotic pressure of the sucrose required to bring the guard cells back to the initial aperture after release of subsidiary cell turgor.

A^{o}_{max} is an estimate of the aperture appropriate to the given solute content in the absence of any opposing subsidiary cell turgor. P'_{gs} shows the contribution of a lower value of subsidiary cell turgor to the water relations of the guard cell, as the subsidiary cell shrinks as sucrose is added; P'_{gs} is estimated as the difference in the osmotic pressures of the two sucrose solutions in which the aperture takes a given value, one with and one without a contribution from subsidiary cell turgor

Table 4. Effects of subsidiary cell turgor

Strip	Initial aperture A_i (μm)	Estimated A^{0}_{max} (μm)	P_{gs} (mosmol kg^{-1})
1	17.5±0.6(8)	21.1±0.7(8)	571±55(8)
2	16.5±0.4(6)	20.5±0.5(6)	399±29(6)
3	14.1±0.5(6)	15.9±0.6(6)	248±26(6)
4	12.3(2)	14.0(2)	253(2)
5	10.8±0 (4)	13.8±0.5(4)	280±30(4)
6	6.4±1.0(4)	7.7±1.2(4)	179±29(4)
7	6.4±0.5(8)	13.0±0.6(8)	657±40(8)
8	5.6±0.3(7)	6.7±0.3(7)	46±18(7)

equal to the turgor pressure of the subsidiary cell (P_s), but to the effective pressure exerted on the guard cell by the subsidiary cell; since the two cells overlap on only a part of their areas the two quantities will not be equal. Changes in P_{gs} as P_s declines may be estimated from the difference in the osmotic pressures of the two sucrose solutions in which the aperture takes a given value, one with and one without a contribution from subsidiary cell turgor (for example P'_{gs} in Fig. 4).

In practice it was not easy to estimate many values for P'_{gs} for a given cell; often the curve was very flat and sometimes showed small fluctuations in aperture in this region. Table 4 shows mean values of initial aperture, of estimated A^{o}_{max} in the absence of subsidiary cell turgor, and of estimated P_{gs}, for the groups measured in eight strips. For seven of the eight strips, the relation is as would be expected, that the effect of subsidiary cell turgor increases with aperture, as the bulge of the guard cell into the inner lateral subsidiary cell increases. (Areas of the inner lateral subsidiary cell were estimated at three levels of focus, at the two surface junctions with the guard cell, and at the median plane. The ratio of these areas was correlated with aperture; at low apertures the wall between guard cell and inner lateral subsidiary cell was nearly straight, but at wide opening the area in the median plane (a_m) was only about 0.5–0.6 of that in the surface plane (a_s). The relation was $a_m/a_s = 1.006-0.026A$ ($r=0.892$, 7df). Thus the guard cell clearly bulges into, and compresses, the inner lateral subsidiary cell as the pore opens.)

But in strip 7 in Table 4 a low initial aperture is associated with a very large change in aperture on release of subsidiary cell turgor and a high value of P_{gs}. For all cells except those in strip 7, the relations between A_i, P_{gs} and A^{o}_{max} can be expressed as:

$$P_{gs} = -110 + 33.2A_i \qquad r=0.811, 35\text{ df.}$$

$$A^{o}_{max} = -0.01 + 1.20A_i \qquad r=0.987, 35\text{ df.}$$

On this basis we would predict that strip 7, with a mean initial aperture of 6.4 μm should have $P_{gs} = 102$ mosmol kg^{-1} and $A^{o}_{max} = 7.7$ μm, instead of the 657±40(8) mosmol kg^{-1} and 13.0±0.6 μm, as observed. Thus in strip 7 the subsidiary cell turgor is abnormally high. It would seem therefore that transient increases in subsidiary cell solute content may occur in some conditions, perhaps in the early stages of changing aperture, and that this might repay further study.

In strip 7, where the pore opened widely as subsidiary cell turgor was reduced, it was possible to estimate values of P'_{gs}. It was clear that P'_{gs} fell very steeply with increasing π_o, i.e., a reduction in the external osmotic pressure produced a much greater reduction in P_{gs}. Thus by adding 79 mosmol kg^{-1} sucrose to the bathing solution, the mean aperture increased from 6.4±0.5(8) μm to 8.7±0.6(8) μm, and P_{gs} fell by 294 mosmol kg^{-1}, from 657 ±40(8) mosmol kg^{-1} to 363±45 mosmol kg^{-1}; the ratio $\frac{\Delta P_{gs}}{\Delta \Psi}$ had an average value of 3.7±0.5 (8). This implies that $\frac{\Delta P_{gs}}{\Delta P_s}$ is greater than 3.7, since in the

subsidiary cell we have $dP_s = \left(\frac{\varepsilon}{\varepsilon+\pi}\right)_s d\Psi$. Thus a pressure reduction in the subsidiary cell is magnified in its effect in the guard cell by a factor of more than 3.7. The decrease in P_{gs} is all the more striking since the volume of the guard cells has increased, and P_{gs} would be expected to increase on this account, by about 76 mosmol kg^{-1} if the dependence of P_{gs} on aperture is similar in strip 7 to that calculated from the other strips. (It was not possible to get a good estimate of the slope of the relation between P_{gs} and A_i in strip 7, as the range of aperture was not large, but the slope was higher rather than lower than that for the other strips.) This would suggest that the effect of declining subsidiary cell turgor at constant guard cell volume should be expressed by the ratio of $\frac{\Delta P_{gs}}{\Delta \Psi} = \frac{294+76}{79} = 4.7$. This implies that $\left(\frac{\Delta P_{gs}}{\Delta P_s}\right)_{V_g \text{constant}}$ is greater than 4.7.

This calculation is an alternative method of demonstrating the so-called "mechanical advantage" of the guard cell over the subsidiary cell, discussed by De Michele and Sharpe (1973), Edwards and Meidner (1975), Cowan (1977), Meidner and Bannister (1979), and renamed the "antagonism ratio" by Cooke et al. (1976).

A figure for the antagonism ratio may also be calculated. It was defined by the equation: $A = b_o + b_g P_g + b_s P_s$, and is equal to the ratio of the coefficients $-b_s/b_g$. We have (from Table 2) $\frac{dA}{dP_g} = b_g = 5.6\,\mu m\ MPa^{-1}$. For $\Delta\Psi_o = \Delta\Psi_g = \Delta\Psi_s = -0.19$ MPa, we have an aperture increase of 2.3 µm; $b_g \Delta P_g = \frac{dA}{d\Psi}\Delta\Psi_g$ has the value -1.1 µm, and therefore $b_s \Delta P_s$ must equal 3.4 µm. This implies that $\left(\frac{dA}{d\Psi}\right)_s = \frac{b_s \Delta P_s}{\Delta \Psi_s} = -17.6\,\mu m\ MPa^{-1}$. Hence $b_s = -17.6 \left(\frac{\varepsilon+\pi}{\varepsilon}\right)_s \mu m\ MPa^{-1}$, and we have the antagonism ratio equal to 3.1 $\left(\frac{\varepsilon+\pi}{\varepsilon}\right)_s$ i.e., greater than 3.1.

Discussion

The purpose of this work was to determine the osmotic changes in guard cells of *Commelina* which are required to change the aperture, for comparison in two later papers with measured potassium changes. The results show that the internal osmotic changes required are 83 mosmol $kg^{-1}\,\mu m^{-1}$ below 10 µm, 129 mosmol $kg^{-1}\,\mu m^{-1}$ in the range 10–15 µm, and 180 mosmol $kg^{-1}\,\mu m^{-1}$ above 15 µm, with standard errors in the order of 5–10% of these values. (In pressure units these figures are about 0.20, 0.31 and 0.44 MPa μm^{-1}, respectively.) These figures represent the increase in internal osmotic pressure required to increase the volume of the guard cell, and open the pore, against the resistance to expansion of the guard cell wall, and therefore apply to "isolated" guard cells, or states in which the subsidiary cell turgor has been released. There is no significant difference between the values measured on intact guard cells at pH 6.7, and those measured on 'isolated' guard cells at pH 3.9 or pH 6.7. Thus the low pH treatment used to "isolate" guard cells does not appear to have changed the properties of the cell walls, at least up to 44 hr of exposure to low pH. In the following paper results on the ion content of "isolated" guard cells suggest that cell wall properties change with prolonged incubation at low pH, but not up to 45 hr.

The results show that the osmotic changes required to open the pore against the resistance of the guard cell walls to expansion, in the absence of subsidiary cell turgor, are equal in the intact strips and in "isolated" strips. However, in intact strips, to open the pore it is also necessary to overcome the resistance of the subsidiary cells to the expansion of the guard cells, the tissue pressure P_{gs}. The results suggest that P_{gs} increases with aperture by about 33 mosmol $kg^{-1}\,\mu m^{-1}$; this is a less certain figure, and there was some indication that the slope increased with aperture to about 50 mosmol $kg^{-1}\,\mu m^{-1}$ above 12 µm. Taken together, the figures suggest that in intact guard cells, with expansion against the subsidiary cell turgor, it is necessary to increase the internal solute by 116 mosmol $kg^{-1}\,\mu m^{-1}$ below 10 µm, by 170 mosmol $kg^{-1}\,\mu m^{-1}$ in the range 10–15 µm, and by 230 mosmol $kg^{-1}\,\mu m^{-1}$ above 15 µm, i.e., by about 0.28, 0.41 and 0.56 MPa μm^{-1}, respectively. These then are the figures to be used to assess the contribution of measured potassium changes to the total solute changes in "isolated" and in intact guard cells, a comparison that will be made in the two following papers.

The present results may also be compared with a number of previous estimates. The estimates of $\frac{d\Psi}{dA}$ obtained here are rather higher than the value of 0.2 MPa μm^{-1} obtained by Fischer (1973) for *Vicia faba*, by the same method of observing aperture changes as sucrose is added, but using long exposures (20 min) to the various solutions. Humble and Raschke (1971) estimated $\frac{d\Psi}{dA}$ in *Vicia faba* as 0.16 MPa μm^{-1}, but again using long exposure to the plasmolysing solutions. These may therefore be under-

estimates. The hazards of solute leakage during long exposure to plasmolysing solutions are shown by Willmer and Beattie (1978). The exposures to plasmolysing solutions were even longer in the work of Glinka (1971), who also observed the opening of stomata in *Vicia faba* as the subsidiary cell turgor was released. Bearce and Kohl (1970) avoided this problem by estimating osmotic pressure by a freezing method; in *Chrysanthemum* they found no correlation between guard cell osmotic pressure and aperture, which may raise questions about the method, but in *Pelargonium* their results show a significant correlation with a slope of $0.32\,\mathrm{MPa}\,\mu\mathrm{m}^{-1}$.

Thus the figures for *Commelina*, *Vicia*, and *Pelargonium* are similar, but differ strikingly from the other species on which good information is available, *Tradescantia virginiana*. Meidner and Bannister (1979) made very careful, rapid estimates of plasmolytic points of guard cells and subsidiary cells in this species, as a function of aperture. They found very low values for the osmotic pressure and for the increase of osmotic pressure with aperture; their value of b_g (or $d\pi/dA$ in the absence of subsidiary cell turgor) is only $0.044\,\mathrm{MPa}\,\mu\mathrm{m}^{-1}$, and the guard cell osmotic pressures were only about 1 MPa even at wide apertures. The difference between species seems to be a genuine one, since Meidner and Bannister (1979) say they often found osmotic pressures of 4 MPa in *Commelina* and 3 MPa in *Vicia* similar to the approximate value of π given in this paper. It would appear that the guard cells of *Tradescantia* open much more readily, with much lower solute contents, than those of *Commelina*.

The influence of subsidiary cell turgor on aperture is very striking, a point made previously by other authors, on theoretical and observational grounds (Glinka, 1971; De Michele & Sharpe, 1973; Edwards & Meidner, 1975; Cooke et al., 1976; Edwards, Meidner & Sheriff, 1976). The antagonism ratio given by the present results, with a value greater than 3.1, is higher than the value of 1.5–1.6 found by Meidner and Bannister (1979) for *Tradescantia*, or the values calculated by Cooke et al. (1976) analyzing previous observations. The recent value for *Tradescantia* is likely to reflect a genuine difference between the species, but the value for *Vicia*, based on Glinka's (1971) results, is rendered uncertain by the likelihood of solute leakage during long exposures to solutions (4 hr). In fact the ratio $\frac{\Delta P_{gs}}{\Delta P_s}$ quoted in this paper is an alternative way of expressing the relative importance of guard cell pressure and subsidiary cell pressure determining the state of the pore. The antagonism ratio is the ratio $-\left(\frac{\partial A}{\partial P_s}\right)_{P_g} \Big/ \left(\frac{\partial A}{\partial P_g}\right)_{P_s}$. But the present ratio $\frac{\Delta P_{gs}}{\Delta P_s}$, for an equal change in the water potential of both cells, may be a more relevant quantity to consider; it has here a value greater than 3.7. ΔP_{gs} is the change in turgor pressure in the guard cell which would be equivalent, in its effect on aperture, to the change of ΔP_s in the turgor of the subsidiary cell. Alternatively the derived ratio $\left(\frac{\Delta P_{gs}}{\Delta P_s}\right)_{V_g \text{ constant}}$, which has a value greater than 4.7, is an even more direct illustration of the mechanical advantage of the subsidiary cell, representing the factor by which a change in subsidiary cell turgor is magnified in its effect on the water relations of the guard cell.

One final point may be made. In their analysis of guard cell deformations, using finite element shell analysis, Cooke et al. (1976) reach the conclusion that, on opening, the subsidiary cell should bulge *towards* the interior of the guard cell, rather than the guard cell's bulging into the subsidiary cell. But this is contrary to direct observation. It is quite clear from measurements on prints of photographs taken at different levels of focus, that the guard cell compresses the subsidiary cell as it opens; from a nearly straight wall between the two in closed stomata, the guard cell bulges markedly into the inner lateral subsidiary in open stomata. The regression line quoted in this paper, for the relation between aperture and the ratio of the areas of the susidiary cell at the median level and at the level of surface overlap with the guard cell, shows the extent to which this bulging occurs, but the observation is not a new one. It would seem necessary to modify the theoretical analysis to allow relative movement of the two cells of this kind.

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References

Bearce, B.C., Kohl, H.C. 1970. Measuring osmotic pressure of sap within live cells by means of a visual melting point apparatus. *Plant Physiol.* **46**:515

Cooke, J.R., De Baerdemaeker, J.G., Rand, R.H., Mang, H.A. 1976. A finite element shell analysis of guard cell deformations. *Trans. ASAE* **19**:1107

Cowan, I.R. 1977. Stomatal behaviour and environment. *Adv. Bot. Res.* **4**:117

De Michele, D.W., Sharpe, P.J.H. 1973. An analysis of the mechanics of guard cell motion. *J. Theor. Biol.* **41**:77

Edwards, M., Meidner, H. 1975. Direct measurements of turgor pressure potentials of guard cells. I. *J. Exp. Bot.* **26**:319.

Edwards, M., Meidner, H., Sheriff, D.W. 1976. Direct measurement of turgor pressure potentials of guard cells. II. The mechanical advantage of subsidiary cells, the Spannungsphase, and the optimum leaf water deficit. *J. Exp. Bot.* **27**:163

Fischer, R.A. 1973. The relationship of stomatal aperture and guard cell turgor pressure in *Vicia faba*. *J. Exp. Bot.* **24**:387

Glinka, Z. 1971. The effect of epidermal cell water potential on stomatal response to illumination of leaf discs of *Vicia faba*. *Physiol. Plant.* **24**:476

Humble, G.D., Raschke, K. 1971. Stomatal opening quantitatively related to potassium transport. *Plant Physiol.* **48**:442

MacRobbie, E.A.C. 1977. Functions of ion transport in plant cells and tissues. *Int. Rev. Biochem. Plant Biochem. II* **13**:211.

MacRobbie, E.A.C., Lettau, J. 1980. Ion content and aperture in "isolated" guard cells of *Commelina communis* L. *J. Membrane Biol.* **53**:199

Meidner, H., Bannister, P. 1979. Pressure and solute potentials in stomatal cells of *Tradescantia virginiana*. *J. Exp. Bot.* **30**:255

Meidner, H., Willmer, C.M. 1975. Mechanics and metabolism of guard cells. *Curr. Adv. Plant Sci* **7**:1

Penny, M.G., Bowling, D.J.F. 1974. A study of potassium gradients in the epidermis of intact leaves of *Commelina communis* in relation to stomatal opening. *Planta* **119**:17

Raschke, K. 1975. Stomatal action. *Annu. Rev. Plant Physiol.* **26**:309

Raschke, K. 1977. The stomatal turgor mechanism and its responses to CO_2 and abscisic acid: Observations and a hypothesis. *In:* Regulation of Cell Membrane Activities in Plants. E. Marré and O. Ciferri, editors, p. 173. Elsevier/North Holland, Amsterdam

Raschke, K. 1979. Movements of stomata. *In:* Encyclopedia of Plant Physiology. N.S. W. Haupt and M.E. Feinleib, editors. Vol. 7, p. 383. Springer Verlag, Berlin

Squire, G.R., Mansfield, T.M. 1972. A simple method of isolating stomata on detached epidermis by low pH treatment: Observations of the importance of the subsidiary cells. *New Phytol.* **71**:1033

Willmer, C.M., Beattie, L.N. 1978. Cellular osmotic phenomena during stomatal movements of *Commelina communis*. I. Limitations of the incipient plasmolysis technique for determining osmotic pressures. *Protoplasma* **95**:321

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