PREPARATION OF ROLLED EPIDERMIS OF *VICIA FABA* L. SO THAT STOMATA ARE THE ONLY VIABLE CELLS: ANALYSIS OF GUARD CELL POTASSIUM BY FLAME PHOTOMETRY

By W. G. ALLAWAY* and T. C. HSIAO*†

[Manuscript received 13 September 1972]

Abstract

A rolling technique is described with which cells in epidermal strips of *V. faba* were differentially broken so that only guard cells remained alive and functional. In rolled epidermis potassium was retained only in live guard cells, as judged by staining with cobaltinitrite. Potassium in rolled and rinsed epidermal strips, analysed by flame photometry, showed a fivefold increase on the average when stomata opened in leaves under light. Guard cell solute potential, estimated plasmolytically, decreased 12 bars for an increase of 10 μm in stomatal aperture. The corresponding increase in guard cell potassium was sufficient to account for a solute potential change of as much as 11 bars. Thus, the results are in general agreement with previous studies with radioactive label or electron microprobes indicating that potassium is the major osmotic solute involved in stomatal movement of *Vicia*.

I. INTRODUCTION

Accumulation of potassium in guard cells, first observed by Macallum (1905), has been suggested by a number of workers (Imamura 1942; Fujino 1959, 1967; Fischer 1968*a*) to be the mechanism by which turgor pressure of guard cells is increased, causing stomatal opening. Previous workers (Fischer and Hsiao 1968; Humble and Raschke 1971) have found that estimated K^+ uptake can account for a major proportion of the observed osmotic changes during stomatal opening and suggested that the total osmotic changes in guard cells during stomatal movement may be due to the accumulation of K^+ and of a balancing anion—probably organic in nature. Others have confirmed in various species the effectiveness of K^+ in inducing stomatal opening and the accumulation of K^+ in guard cells during opening (e.g. Sawhney and Zelitch 1969; Willmer and Mansfield 1970; Raschke and Fellows 1971).

Studies of stomatal mechanisms would be greatly facilitated by the development of procedures to obtain isolated guard cells, thus making feasible the determination of guard cell potassium by standard chemical procedures and the study of guard cell metabolism by biochemical techniques. Hitherto guard cell K^+ has been determined directly only with the elaborate and expensive electron microprobe

*Department of Environmental Biology, Research School of Biological Sciences, Australian National University, P.O. Box 475, Canberra City, A.C.T. 2601.

†Present address: Department of Water Science and Engineering, University of California, Davis, California 95616, U.S.A.

method (Humble and Raschke 1971). Since with this method guard cells must be analysed one by one and there are pronounced variations in osmotic contents from guard cell to guard cell (Fischer 1968*a*; Humble and Raschke 1971) and from leaf to leaf (Hsiao *et al.* 1972), data representative of the population are hard to obtain. The indirect method of determining K^+ uptake into epidermal strips with radioactive tracers, on the other hand, is complicated by the presence of many viable cells other than guard cells in the strip (Humble and Hsiao 1970). In this paper we describe a technique for preparing "quasi-isolated" guard cells from *Vicia faba* L. epidermal strips (so that guard cells are intact and viable, but no epidermal cells are intact) and the results of K^+ analysis of such strips by flame photometry.

II. Materials and Methods

(*a*) *Tissue*

V. faba cv. Early Long Pod was grown in solution culture in a glasshouse, minimum temperature 20°C, during the summer. Leaflets were collected early in the morning from leaves fourth to sixth from the shoot apex of plants with more than 10 leaves. Leaflet bases were freshly cut under 0·1 mM $CaCl_2$ in the laboratory and kept in this solution during dark storage until treatment. Closed or open stomata were obtained concurrently by placing leaflets, abaxial surface upwards and with base in 0·1 mM $CaCl_2$, either in darkness or in light from a Philips HPLR high-pressure mercury fluorescent lamp, usually 120–140 W m^{-2} and of saturating intensity for stomatal opening (Hsiao *et al.* 1972). Atmosphere above the leaflets was maintained by passing humidified air of normal CO_2 content (pumped from outside the building) through boxes covering the samples (100–300 ml min^{-1}; box volume 7–9 litres). Treatments were at room temperature (22–26°C) and lasted 3 hr. Leaflets were put into treatments sequentially with dark and light treatments alternating. After treatment, abaxial epidermal strips were taken (Hsiao *et al.* 1972) between major veins from the leaflet and floated immediately on 0·1 mM $CaCl_2$. The strips were cut with a U-shaped template and were about 20 mm^2 in area. The area gripped by the fine forceps used to pull off the strip contained numerous crushed guard cells and was trimmed off with a razor blade in most experiments. Strips so prepared were, perhaps surprisingly, rather uniform in area during any one day (e.g. a range of 17·8–23·1 mm^2 for one experiment).

(*b*) *Differential Destruction of Epidermal Cells by Rolling*

Strips were floated for at least 2 min to ensure full turgor and then were placed on a Perspex spatula and rolled gently with a roller made by covering a Perspex rod (4·6 mm diameter) with resilient Tygon tubing 1·5 mm thick. To ensure even pressure, the ends of the roller were supported on Perspex blocks the same thickness as the spatula. Depending on the pressure used and the conditions under which plants were grown, strips could be produced with guard cells and many epidermal cells intact, with guard cells only intact, or with all epidermal cells and many guard cells destroyed. The pressure applied has to be carefully gauged with the fingers to produce strips without intact epidermal cells but with virtually all guard cells intact and active; this required some practice. It was desirable and sometimes necessary to use the first one or two strips from each leaf to test out the rolling pressure, but once this had been done an experienced operator could roll reproducibly as many strips as needed. The pressure required may vary from leaf to leaf and from day to day (see below). After rolling, strips were again floated in 0·1 mM $CaCl_2$.

(*c*) *Potassium Extraction and Analysis*

K^+ from the cells broken by rolling was removed by immersing strips sequentially in four beakers of 0·1 mM $CaCl_2$. The solutions were changed for fresh after about every 10 strips. The rinsing time for each strip was about 7–10 min, and the total time from leaf removal from treatment conditions to the end of rinsing was 20 min or less. After rinsing, the strips were put in 1–2 ml of water in a covered 10-ml beaker. More than 40 strips from at least five leaflets constituted a sample for analysis. Parallel samples without strips were made to check for contamination. Samples were

evaporated to dryness at about 100°C and then extracted for 1 hr in 5% HNO_3 at about 100°C. After drawing off the extract with pipettes, the strips in the beaker were rinsed twice with 5% HNO_3 and the rinsing solutions added to the extract to make a total of about 3 ml. It was necessary to work with such small volumes because of the sensitivity limit of the measuring instrument and the small amount of K^+ present.

K^+ concentration in extracts was measured with a Baird–Atomic type KY3 flame photometer, modified for small samples by the Department of Geophysics and Geochemistry, Australian National University. The usual precautions for microanalysis were taken. Pyrex or similar glassware was soaked overnight in 50% analytical grade HNO_3 and extensively rinsed with glass-distilled water before each use.

(*d*) *Potassium Stain, Stomatal Aperture, and Solute Potential*

To demonstrate the location of K^+, epidermal strips were stained in a modified Macallum's (1905) stain (R. A. Fischer, personal communication) made by stirring 27·5 g sodium cobaltinitrite in 38 ml water, adding 5·0 ml glacial acetic acid, and making up to 55 ml total volume with water. Fresh stain was made up every 2 weeks. Strips were floated cuticle up on the stain for 1 min at room temperature, thoroughly rinsed in four changes of water at 0°C for a total of 5 min, and then mounted in a medium consisting of equal parts of glycerol and ammonium sulphide solution (Analar, 10% H_2S w/v). A black sulphide precipitate is formed where cobalt-containing products of the reaction of the stain with K^+ are present (Macallum 1905).

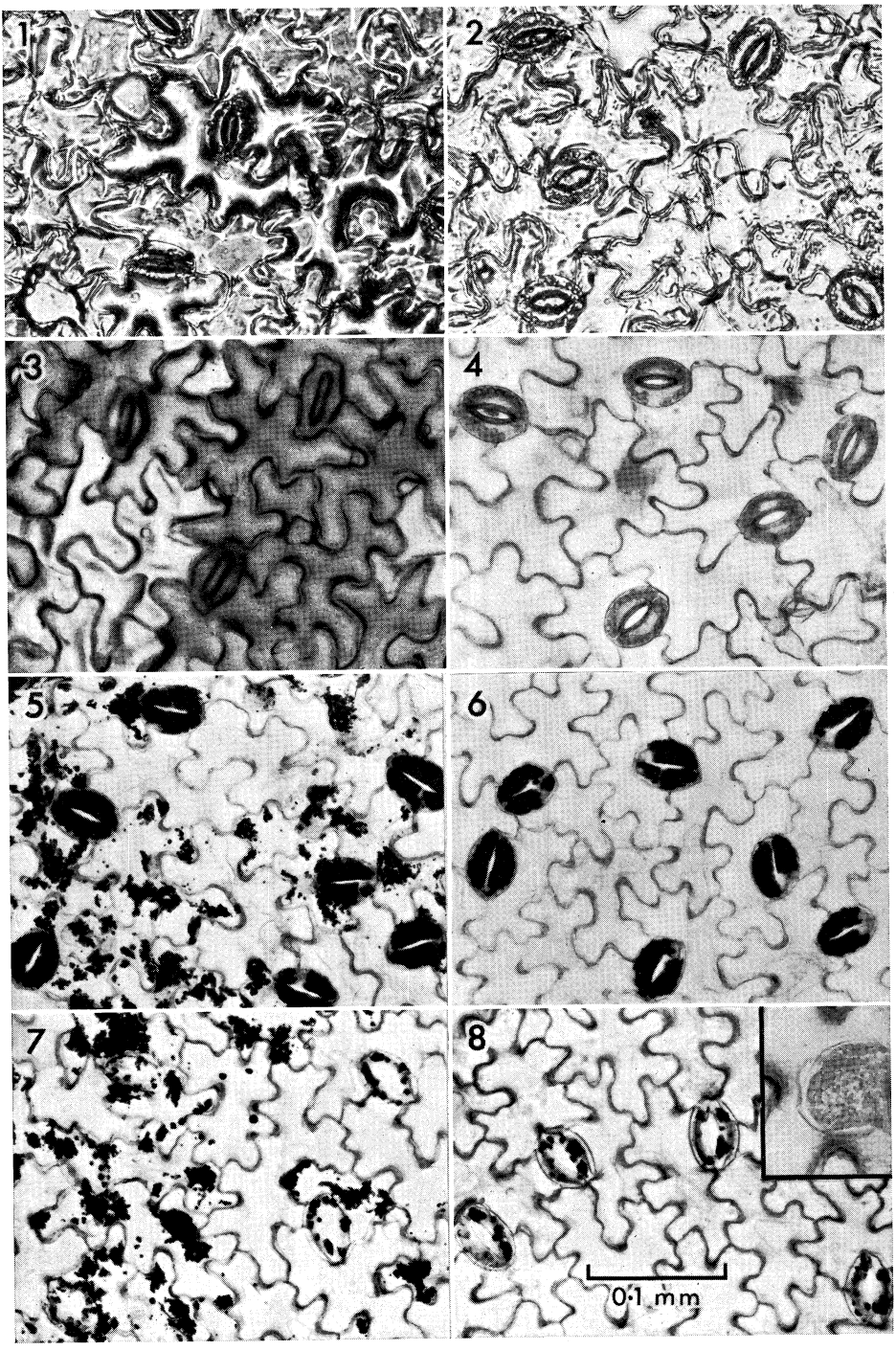
In the potassium-analysis experiments, stomatal aperture, epidermal strip area, and proportion of dead guard cells were determined (Hsiao *et al.* 1972) on one strip of each leaflet, taken at random after rolling and rinsing. In each strip mounted in immersion oil (Fischer 1968*b*), 20 apertures of living stomata were measured. Apertures in whole leaf pieces were also measured microscopically under immersion oil (Hsiao *et al.* 1972).

Solute potential of guard cells was estimated plasmolytically. After rolling, strips were taken from 0·1 mM $CaCl_2$ and floated at 25°C for 20 min on a series of sucrose solutions graded in solute potential in steps of 2 or 4 bars and all 0·1–0·2 mM in $CaCl_2$. Solute potentials of the solutions were calculated from tables (Lang 1967; Wolf and Brown 1969). Each strip was mounted in the same sucrose solution and the number of guard cells plasmolysed out of 20 examined was found. The solute potential for 50% plasmolysis (Crafts *et al.* 1949) was obtained by graphical interpolation. With two people working, the total time from stripping to microscopic examination of each strip varied from about 23 to 35 min within a replicate. In view of this, the order of solutions was reversed for alternate replicates.

III. Results and Discussion

(*a*) *Assessment of the Rolling Technique*

Several criteria (Fischer 1968*b*) were used to judge the vitality of cells. The routine technique for epidermal cells was to close the iris diaphragm of the microscope, when, with the strip mounted in immersion oil, intact epidermal cells presented a bulging or refractive appearance (Fig. 1) in contrast to the flattened and wrinkled look of broken epidermal cells (Figs. 1 and 2). Live cells took up neutral red, a vital stain (Pallas 1966), and appeared pink or light red (shown grey in Fig. 3). Broken cells apparently adsorbed neutral red, probably on their wall, and appeared weakly brownish yellow or reddish brown (low contrast in Figs. 3 and 4). Another criterion is the degree of organization of the cell contents. Dead cells were usually discernibly disorganized even under only × 400 magnification, and showed excessive Brownian motion in the cytoplasm (these criteria were routinely used for guard cells). Also, viable cells generally (but not always) exhibited cytoplasmic streaming. Applying the different criteria to the same cells in various strips yielded mostly the same conclusion as to cell vitality.



Figs. 1–4.—Two criteria used in judging vitality of cells in epidermal strips of *V. faba*. In Figure 1, before rolling the strip and with iris diaphragm stopped down, intact epidermal cells appear bulging and refractive in contrast to the flat appearance of broken ones. In Figure 2, a strip is shown after

Epidermal strips freshly taken from *V. faba* leaves usually had many epidermal cells intact (Figs. 1 and 3). These cells contained considerable quantities of K^+, both from light- (Fig. 5) and dark-treated (Fig. 7) leaves. In some material, most epidermal cells can be destroyed by bending back the epidermis sharply when stripping (Fischer 1968*b*); but this seldom gives complete breakage. The technique of rolling was therefore devised, and has permitted the use of strips from material with apparently tough epidermal cells.

After correct rolling, strips showed no intact epidermal cells (Figs. 2 and 4). Rolled strips were rinsed and stained for K^+ (Figs. 6 and 8). No stain was present in epidermal cells of these strips, although guard cells from light-treated leaves (Fig. 6) still showed dense staining and from dark-treated leaves, weak staining (Fig. 8). The guard cells had survived rolling, presumably because of their thickened walls, and had evidently retained much of their original K^+ (compare Figs. 5 with 6, and 7 with 8). Often a few mesophyll cells remained attached to a strip. After the strip had been rolled and rinsed, they always appeared disorganized and with all their chloroplasts broken. Such cells did not stain for K^+ (Fig. 8, inset). Staining experiments were repeated at various times, and always gave similar results. It was concluded that in correctly rolled and rinsed strips, most or all of the K^+ was contained in the guard cells, and that guard cell K^+ changes rather little as a result of the preparation procedure. Guard cells in rolled strips are functional in that they take up $^{86}Rb^+$ and open in response to light and show action spectra similar to those for stomata in leaf pieces (Hsiao *et al.* 1972). A preparation of rolled strips consists, therefore, of viable essentially isolated guard cells, and as such should be useful for metabolic studies.

Occasionally, guard cells which did not show any K^+ stain were observed. It was supposed that these were the guard cells with disorganized cytoplasm and showing Brownian movement of small organelles, seen before staining. Counts, made on the same 15 strips before and after staining, showed that unstained guard cells were about 120% of the "dead" ones detected before staining. There was either an under-

---

rolling, with the iris diaphragm stopped down; no intact epidermal cells are visible. In Figure 3, neutral red taken up by viable epidermal and guard cells in an unrolled strip appears as greyer areas (high contrast print from Kodachrome transparency). In Figure 4, a rolled strip stained with neutral red shows stain confined to guard cells. All × 250 approximately.

Figs. 5–8.—Cobaltinitrite stain for potassium in epidermal strips of *V. faba*, showing the effect of rolling and rinsing the strips on potassium content of the cells. Treatments are as follows: Figures 5 and 6—light-treated leaflet; Figures 7 and 8—dark-treated leaflet. Figures 5 and 7 show unrolled, unrinsed strips, and Figures 6 and 8 show strips stained after they had been rolled and rinsed. Potassium (shown as black staining deposit) in guard cells was only slightly affected by rolling and rinsing, but rolling and rinsing removed all stainable potassium from the epidermal cells. This result applied to strips from both light and dark treatments. Heavy potassium stain is evident in guard cells from the light treatment, and only small amounts of stain are present in guard cells from the dark treatment. Inset (Fig. 8) shows a crushed mesophyll cell on a rolled, rinsed strip; there is no staining deposit in the mesophyll cell. (All photographs are of strips from the same experiment; strips for Figures 5 and 6 were from the same leaflet; strips for Figures 7 and 8 were from the same leaflet; treatments in light and darkness lasted 3 hr. Stomata on the leaflet from the light treatment were open before staining, and from the dark treatment stomata were closed; the staining procedure causes all stomata to appear closed. All ×250 approximately, except inset to Figure 8, which is approximately ×650.)

estimation of the number of "dead" guard cells, or damage during transfer from the slide to the staining solution. In any case, a count of "dead" guard cells approximated the number which did not contain K^+.

Growth environment greatly affected the toughness of the cells (guard cells and others) in the epidermis. Leaves grown in low light in the glasshouse required only slight rolling pressure to break the epidermal cells. In fact, it was difficult sometimes to roll lightly enough to avoid damaging the guard cells. Leaves grown outdoors in the spring in Canberra, on the other hand, had to be rolled harder to break all the epidermal cells. The toughest epidermis was from leaves grown at low temperature (19°C maximum) under natural light; several rollings were necessary for complete breakage.

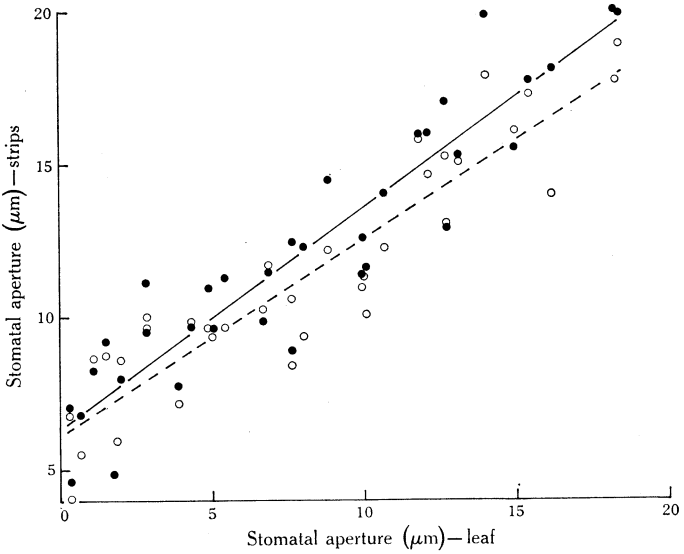


Fig. 9.—Relation between stomatal aperture in rolled epidermal strips, with or without rinsing, and stomatal aperture in the leaf. Leaflets were treated in normal air with darkness, high light, or low light to obtain a range of stomatal apertures. After measuring aperture on the leaf piece, epidermal strips were taken and rolled; aperture was measured without rinsing (solid circles and line) or after rinsing (open circles and dotted line) the strip. The lines are regression lines. Each point represents a single leaf piece. (N.B. vertical scale starts at 4 μm, not zero.)

Breakage of epidermal cells by rolling, releasing the pressure exerted by them on the guard cells, would be expected to cause stomata to open wider (Heath 1938). To relate stomatal aperture measurements in rolled strips to the whole-leaf situation, aperture was measured directly on an area of a piece of leaf pretreated in light or dark and on two strips from an adjacent area of the same leaf piece. One strip was measured after rolling and very brief refloating, another after rolling, refloating, and rinsing. The results are shown in Figure 9. Stomata originally closed in leaves opened substantially in strips after floating and rolling. However, the effect of stripping and rolling on aperture was inversely related to the original aperture in

the leaf, with the effect being almost negligible for wide open (e.g. 18 μm) stomata.) Therefore, assessing stomatal response in leaves by measuring apertures after rolling would lead to an underestimate of the response. Rinsing the strips, taking as long as 20 min in this experiment between stripping and measurement, reduced the aperture generally by perhaps 15%. Presumably some K^+ was lost from the guard cells during this closure, although this was not reliably detectable by staining.

(*b*) *Potassium Content of Guard Cells*

K^+ in extracts of rolled strips ranged between 0·5 and 5·8 p.p.m. Generally the maximum sensitivity of the flame photometer had to be used for analysis. Amounts of K^+ per unit area of epidermis and the corresponding stomatal apertures are shown in Figure 10. The samples averaged about 3% "dead" guard cells.

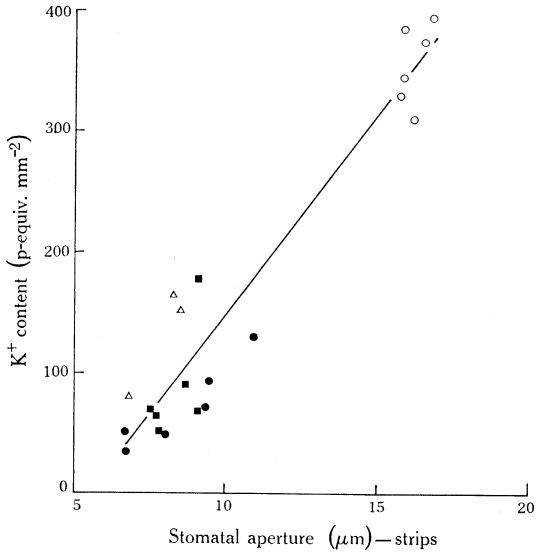


Fig. 10.—Potassium content per unit of strip area as related to stomatal aperture in rolled, rinsed, epidermal strips. Data represent single potassium analyses in three experiments and the regression line is shown. In two experiments data were obtained for initial (■), light (○), and dark (●) treatments. In the third experiment, data were obtained only for a very low light treatment (△). N.B.: stomatal aperture scale on the graphs starts at 5 μm, not zero.

Epidermal K^+ contents shown are corrected values based on 100% intact guard cells. The correctness of the straight line fitted to the points in Figure 10 is somewhat doubtful; in *Nicotiana* guard cells, relative K^+ content determined with an electron microprobe showed a straight line relationship with aperture (Sawhney and Zelitch 1969) although it might perhaps be expected that a greater change in solute concentration would be required per unit opening when stomata are nearly wide open (Humble and Hsiao 1970), because of a likely increase in wall stiffness (by analogy, for example, with a pneumatic tyre). The mean number of guard cells in the strips was 130 mm^{-2}. Hence mean K^+ contents per guard cell, calculated from the data in Figure 10, were 0·55 p-equiv. for closed stomata to 2·72 p-equiv. for open stomata.

(*c*) *Guard Cell Solute Potential as Related to Potassium*

Guard cell solute potentials at 50% plasmolysis and related stomatal apertures are shown in Figure 11. From the regression line, a difference in aperture of 10 μm was calculated to be associated with a difference in guard cell solute potential (for

volume at incipient plasmolysis) of 12·4 bars. The change in solute potential per unit of change in aperture is lower than that measured by Humble and Raschke (1971) but higher than that reported by Fischer and Hsiao (1968) for *V. faba*. A floating time on osmotic solutions of 20 min was used to measure guard cell solute potential. This duration was chosen to make the total time from stripping to examination similar to that used for rinsed strips. Shorter or longer floating time tended to result in slightly fewer or more guard cells being plasmolysed, respectively. This could be due to a slow loss of solutes from guard cells with time, as suggested by the slight reduction in aperture during rinsing (Fig. 9).

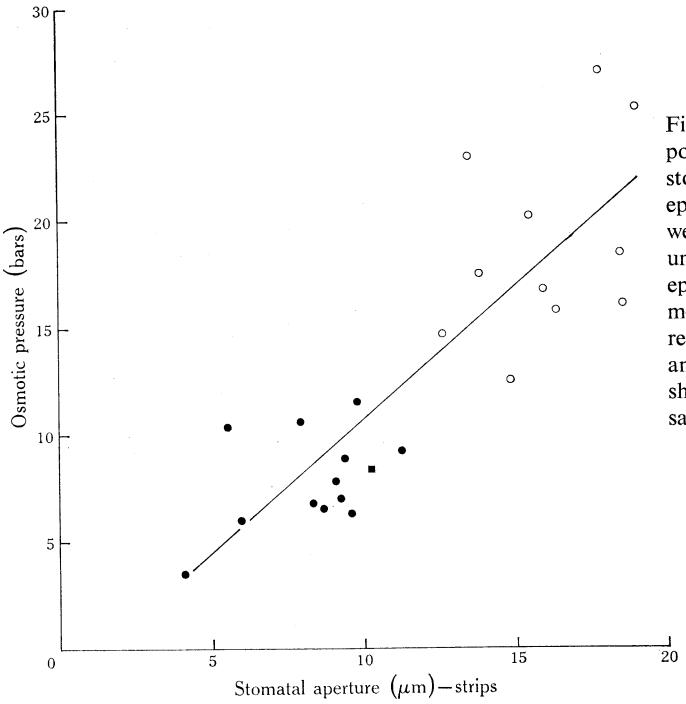


Fig. 11.—Guard cell solute potential as related to stomatal aperture in rolled epidermal strips. Leaflets were treated as described under Figure 9 before taking epidermal strips for measurements. Data represent single leaf pieces and the regression line is shown. Symbols are the same as in Figure 10.

The drop in guard cell solute potential in light caused by the increase in K^+ was calculated from guard cell volume and K^+ content. Considerable difficulty was experienced in determining the volume of guard cells. Attempts made to fix guard cells with a view of making volume estimates from serial sections failed. Acrolein (10%) and glutaraldehyde (3%) both fixed the cytoplasm immediately; but stomatal aperture decreased in 20 min after fixation. Fixation in ethanol (Lloyd 1908) caused open stomata to close partially and closed stomata to open somewhat. Also, stomatal outline appeared to change from an elliptical to a more squarish form. We have therefore confined our techniques to the usual and crude one of measuring length and estimating the mean width of the guard cells and assuming the cell to be a cylinder. Since guard cells are not cylindrical (Haberlandt 1904), our volume estimates can only be regarded as rough approximations. The pertinent cell volume was that at incipient plasmolysis. Hence, estimates were made on cells mounted in sucrose solutions just sufficiently concentrated to plasmolyse them. From measurements made on 72 guard cells, a

value of $4 \cdot 93 \pm 0 \cdot 09$ pl per cell was calculated (mean and standard error of the mean). Previous estimates for guard cell volume in *Vicia* were 5 pl for open stomata (Fischer and Hsiao 1968), and apparently more precisely $2 \cdot 4$ and $1 \cdot 3$ pl for open and closed stomata, respectively (Humble and Raschke 1971). The substantial difference between the latter values and the present results may be partially attributable to different growing conditions. Our data were obtained with plants grown in nutrient solutions, which had distinctly larger leaves and (based on preliminary measurements) larger guard cells, than plants grown in the same glasshouse in a planting mixture. Plants used by Humble and Raschke were grown in a planting mixture.

Guard cell frequency was determined for each sample of strips analysed for K^+, and the average value was used, together with the mean estimated cell volume, to calculate the contribution of K^+ to solute potential. From the fitted line in Figure 10 an increase in stomatal aperture of 10 μm, for example, would be accompanied by a change in K^+ of 325 p-equiv. mm^{-2}; which is equivalent to about $2 \cdot 5$ p-equiv. per guard cell; this, with the guard cell volume of 5 pl, gives a K^+ concentration change of about $0 \cdot 5$ equiv.l^{-1} in the guard cell. If this K^+ is assumed to have an effect equivalent to one-half of the solute potential of KCl of the same concentration (Lang 1967; Wolf and Brown 1969), it alone could account for a change in solute potential of $11 \cdot 2$ bars. As mentioned, in separate experiments the change in guard cell solute potential corresponding to a 10 μm change in aperture was determined to be $12 \cdot 4$ bars (Fig. 11). This suggests that balancing anions are likely to be mainly di- or polyvalent, in general agreement with the suggestion of Humble and Raschke (1971). Alternatively, K^+ could be bound or complexed substantially in the cell: however, very few K^+ complexes of any stability are known (Johnson 1960; Martell 1960). Some evidence for anion accumulation is presented elsewhere (Allaway 1973).

In spite of the very different methods employed, the present results are in general agreement with previous studies (Fischer and Hsiao 1968; Humble and Raschke 1971) on the amount of K^+ transported and its osmotic effect during stomatal movement in *V. faba*. Stomata of K^+-deficient *V. faba* did not open in response to light (Hsiao and Humble, unpublished data). Stomata in epidermal strips of *Commelina communis* open in response to light with CO_2-free air when floated on moderately concentrated Na^+ solution (Willmer and Mansfield 1969, 1970). These authors felt, however, that since Na^+ is low in most plants, it is unlikely to be of great importance in stomatal movement *in situ*. In view of indications that Na^+, Ca^{2+}, and Mg^{2+} are not transported to any large extent by guard cells (Humble and Hsiao 1970; Humble and Raschke 1971) and that Na^+, NH_4^+, and Mg^{2+} are either inactive or much less effective than K^+ and Rb^+ in inducing stomatal opening (Humble and Hsiao 1969), it seems now to be well established for *V. faba* that K^+ is the principal osmotic cation transported in stomatal movement.

IV. Acknowledgments

We are grateful to Mrs. E. S. Walker for skilled and enthusiastic assistance in these experiments and to Dr. A. E. Ashford for making the photomicrographs. We thank Dr. I. McDougall for the use of the flame photometer, and Mrs. R. Maier for help with its operation. We also thank Mr. C. Chandler for growing the plants. Professor R. O. Slatyer lent generous support and encouragement. T. C. Hsiao gratefully acknowledges the receipt of a Visiting Fellowship from the Australian

National University and a Senior Postdoctoral Fellowship from the United States National Science Foundation.

V. References

ALLAWAY, W. G. (1973).—Accumulation of malate in guard cells of *Vicia faba* L. during stomatal opening. *Planta* **110**, 63–70.

CRAFTS, A. S., CURRIER, H. B., and STOCKING, C. R. (1949).—"Water in the Physiology of Plants." (Ronald Press: New York.)

FISCHER, R. A. (1968*a*).—Stomatal opening: role of potassium uptake by guard cells. *Science, N.Y.* **160,** 784–5.

FISCHER, R. A. (1968*b*).—Stomatal opening in isolated epidermal strips of *Vicia faba*. I. Response to light and CO_2-free air. *Pl. Physiol., Lancaster* **43,** 1947–52.

FISCHER, R. A., and HSIAO, T. C. (1968).—Stomatal opening in isolated epidermal strips of *Vicia faba*. II. Response to KCl concentration and the role of potassium absorption. *Pl. Physiol., Lancaster* **43,** 1953–8.

FUJINO, M. (1959).—Stomatal movement and active migration of potassium. [In Japanese.] *Kagaku* **29,** 660–1.

FUJINO, M. (1967).—Role of adenosinetriphosphate and adenosinetriphosphatase in stomatal movement. *Sci. Bull. Fac. Educ. Nagasaki Univ.* **18,** 1–47.

HABERLANDT, G. (1904).—"Physiologische Pflanzenanatomie." (Engelmann: Leipzig.)

HEATH, O. V. S. (1938).—An experimental investigation of the mechanism of stomatal movement with some preliminary observations on the response of the guard cells to shock. *New Phytol.* **37,** 385–95.

HSIAO, T. C., ALLAWAY, W. G., and EVANS, L. T. (1972).—Action spectra for guard cell Rb^+ uptake and stomatal opening in *Vicia faba*. *Pl. Physiol., Lancaster* (In press.)

HUMBLE, G. D., and HSIAO, T. C. (1969).—Specific requirement of potassium for light-activated opening of stomata in epidermal strips. *Pl. Physiol., Lancaster* **44,** 230–4.

HUMBLE, G. D., and HSIAO, T. C. (1970).—Light-dependent influx and efflux of potassium of guard cells during stomatal opening and closing. *Pl. Physiol., Lancaster* **46,** 483–7.

HUMBLE, G. D., and RASCHKE, K. (1971).—Stomatal opening quantitatively related to potassium transport. Evidence from electron probe analysis. *Pl. Physiol., Lancaster* **48,** 447–53.

IMAMURA, S. (1942).—Untersuchungen über den Mechanismus der Turgorschwankung der Spaltöffnungsschliesszellen. *Jap. J. Bot.* **12,** 251–346.

JOHNSON, M. J. (1960).—Enzymic equilibria and thermodynamics. In "The Enzymes". [Eds. P. D. Boyer, H. Lardy, and K. Myrbaeck.] Vol. III. p. 416. (Academic Press: New York.)

LANG, A. R. G. (1967).—Osmotic coefficients and water potentials of sodium chloride solutions from 0 to 40°C. *Aust. J. Chem.* **20,** 2017–23.

LLOYD, F. E. (1908).—The physiology of stomata. *Publs Carnegie Instn* **82,** 1–142.

MACALLUM, A. B. (1905).—On the distribution of potassium in animal and vegetable cells. *J. Physiol.* **32,** 95–118.

MARTELL, A. E. (1960).—The relationship of chemical structure to metal binding action. In "Metal Binding in Medicine". [Eds. M. J. Seven, and L. A. Johnson.] pp. 1–18. (J. B. Lippincott Co.: Philadelphia.)

PALLAS, J. E. (1966).—Mechanism of guard cell action. *Q. Rev. Biol.* **41,** 365–83.

RASCHKE, K., and FELLOWS, M. P. (1971).—Stomatal movement in *Zea mays*: shuttle of potassium and chloride between guard cells and subsidiary cells. *Planta* **101,** 296–316.

SAWHNEY, B. L., and ZELITCH, I. (1969).—Direct determination of potassium ion accumulation in guard cells in relation to stomatal opening in light. *Pl. Physiol., Lancaster* **44,** 1350–4.

WILLMER, C. M., and MANSFIELD, T. A. (1969).—Active cation transport and stomatal opening: a possible physiological role of sodium ions. *Z. PflPhysiol.* **61,** 398–400.

WILLMER, C. M., and MANSFIELD, T. A. (1970).—Further observations of cation-stimulated stomatal opening in isolated epidermis. *New Phytol.* **69,** 639–45.

WOLF, A. V., and BROWN, M. G. (1969).—Concentrative properties of aqueous solutions: conversion tables. In "Handbook of Chemistry and Physics". 50th Ed. pp. D171–D210. (Chemical Rubber Co.: Cleveland.)