Plant Physiol. (1968) 43, 1953–1958

Stomatal Opening in Isolated Epidermal Strips of Vicia faba. II. Responses to KCl Concentration and the Role of Potassium Absorption[1]

R. A. Fischer[2] and Theodore C. Hsiao

Laboratory of Plant-Water Relations, Department of Water Science and Engineering, University of California, Davis, California 95616

Received February 16, 1968.

Abstract. The stimulation by KCl of stomatal opening in isolated epidermal strips of *Vicia faba* was examined. In dark + normal air the opening response was maximal at 100 mM KCl while in light + CO_2-free air it was maximal at about 10 mM KCl. CO_2-free air was more influential than light in reducing the KCl concentration required for maximal opening. K^+ was essential while Cl^- seemed to be of secondary importance in these processes.

The use of $^{86}Rb^+$ as a tracer for K^+ showed that the increase in stomatal aperture under various conditions was well correlated with K^+ uptake. The estimated amount of K^+ taken up by guard cells, along with a counter ion, was sufficient to account for the changes in solute potential associated with opening. It is suggested that the absorption of extracellular solutes, such as K^+, may be the primary mechanism of stomatal opening. Both opening and K^+ absorption are stimulated by light + CO_2-free air.

The increase in stomatal aperture was also well correlated with the decrease in stainable starch in guard cells under all conditions. It is suggested that this is a secondary change, although perhaps closely linked to K^+ absorption.

It has been shown in the preceding paper (5) that stomata in isolated epidermal strips of *Vicia faba* open in response to light and CO_2-free air when the strips are floated on dilute KCl solutions. Further, the response was similar to that of non-isolated stomata on leaf discs floating on water as judged by some important criteria. It was proposed that uptake of KCl may be an integral part of the stomatal opening process. The nature of the response to KCl and the quantity of solute taken up carry important implications for proposed mechanisms of stomatal opening (5) and were examined in this study. Some of the results have been reported in a preliminary communication (4).

Materials and Methods

Growth of the plants (*Vicia faba,* var. Long Pod), handling of epidermal strips, and experimental conditions and techniques have been described (5). Immediately after stripping, however, epidermal strips were always floated in the dark at 22° to 26° firstly for 30 min on 0.1 mM $CaCl_2$, then for a few seconds on deionized distilled water before their transfer to test solutions. This pretreatment was used to leach solutes out of the broken cells, and to permit any mechanical adjustment in aperture which may occur as a result of stripping. Thus aperture immediately after the pretreatment (initial aperture) should represent a stable base level. This level varied from experiment to experiment depending on the tissue and its growth history. As before (5), strips were given 3 hr on the test solution under controlled conditions before measurement of final stomatal apertures. Unless otherwise stated, test solutions were tris/maleate/Ca buffer (5) containing various amounts of KCl.

When estimating K^+ uptake, KCl was labeled with $^{86}Rb^+$ (2.4×10^8 to 7.5×10^8 cpm per mmole of K^+). After the uptake period (3 hr) strips were floated briefly on water, transferred to unlabeled buffered 100 mM KCl for 5 min to remove exchangeable $^{86}Rb^+$, then washed twice more by immersion in buffered 100 mM KCl. Desorption and washing were carried out in the light (about 100 ft-c) and temperature of the laboratory. Washed strips were placed in the center of planchets, dried, and counted. Standard solutions were similarly placed, dried, and counted. In estimating K^+ uptake, it was assumed that $^{86}Rb^+$ and K^+ moved identically as has been shown in other higher plant systems (19, 20).

Starch in guard cells was stained and scored as before (5).

[1] Supported in part by grant B-029-CAL from the Office of Water Resources Research, United States Department of the Interior, and by a grant from the Water Resources Center, University of California.

[2] Present address: Research School of Biological Sciences, Australian National University, Canberra, A.C.T., Australia.

Results

Opening in Response to KCl. The response of stomatal opening to a range of KCl concentrations is shown in figure 1, with KCl concentrations on a logarithmic scale. Also shown is the mean response with KCl concentrations on a linear scale (insert, fig 1). Light + CO_2-free air had a marked effect on the response of stomata to KCl concentration; the opening response was saturated at about 10 mM KCl under these conditions whereas in dark + normal air, approximately 100 mM was required. Opening in both light and dark was reduced markedly at 200 mM and 400 mM KCl. Maximal openings in light + CO_2-free air were 2 to 3 microns greater than those in dark + normal air. At zero KCl, there was still a small response (2.1 microns) to light + CO_2-free air.

Stomatal apertures were measured after 3 hr and may not have represented steady state values although 3 hr in the light was sufficient time for maximal aperture in non-isolated stomata (3). In dark + normal air on 100 mM KCl, sampling beyond 3 hr showed that the aperture did not reach maximal value until at least 7 hr after the strip had been placed on the solution. At 3 hr the aperture was 7.6 microns; at 7 hr, it was 9.5 microns, almost as great as that to be expected in light.

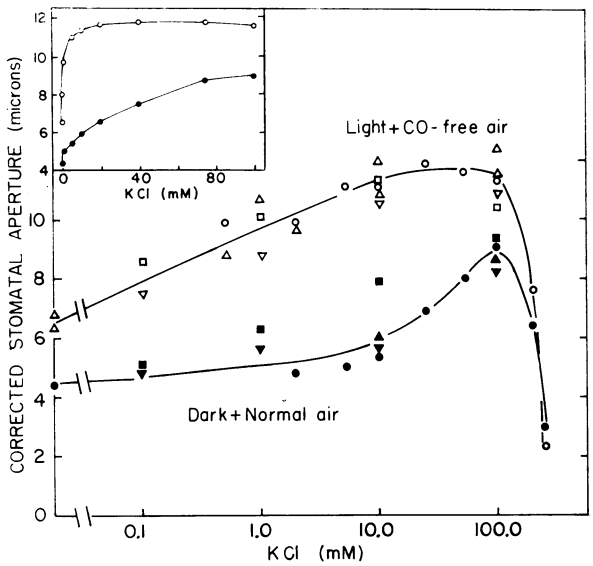

FIG. 1. Stomatal opening in response to KCl concentrations in dark + normal air and light + CO_2-free air. Results are from various experiments, each designated by a different symbol (solid, dark + normal air; open, light + CO_2-free air) and involving 5 to 16 replicates. Results were corrected in each experiment to give identical mean apertures in dark + normal air at zero KCl (*i.e.*, 4.4 microns). Before correction this mean aperture ranged from 2.9 to 5.7 microns for the various experiments; correction involved the subtraction of the difference between this value for each experiment and 4.4, from all the mean apertures at other KCl concentrations in the experiment. Initial stomatal apertures averaged 1.0 microns less than final values in dark + normal air at zero KCl. Insert shows the mean response to KCl on a linear scale, which was plotted from arbitrary points taken off the 2 composite curves drawn in the main figure.

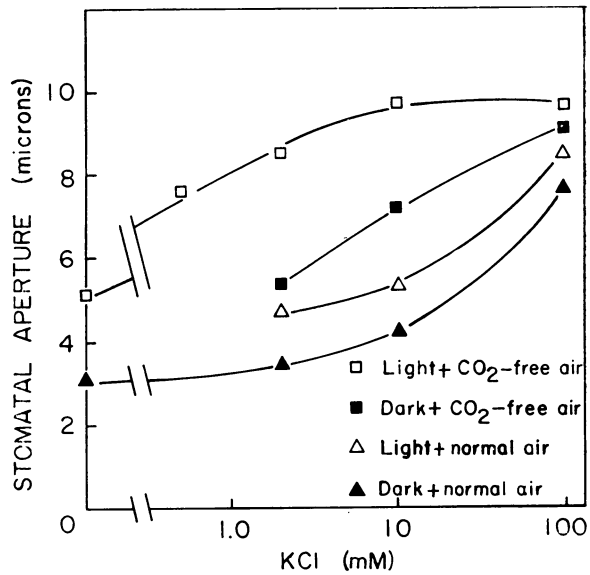

FIG. 2. Response of stomatal aperture to light and CO_2-free air, given separately and together, at various KCl concentrations. 8 replicates.

Figure 2 shows the response to KCl concentration under all 4 combinations of light and dark, and CO_2-free air and normal air. Light and CO_2-free air, showing independent stimulation of opening, tended to be synergistic in their combined effect at KCl concentrations of 10 mM or lower.

Requirement for K^+. As substitutes for K^+ and for Cl^-, Ca^{2+} and SO_4^{2-} were tested since they are taken up only slowly by other plant systems (2). The results (table I) showed that K^+ could not be replaced by Ca^{2+}. Replacing Cl^- by SO_4^{2-} caused a

Table I. *The Role of K^+ and Cl^- in the Response of Stomata to Light + CO_2-free Air*

Strips floated on solute listed, in tris/maleate buffer.

Condition	Solute	Stomatal aperture microns: Mean	Stomatal aperture microns: Opening response[1]
Light + CO_2-free air	10 mM KCl	12.1	8.0
	5 mM K_2SO_4	9.8	5.7
	5 mM $CaCl_2$	4.8	0.7
Dark + normal air	100 mM KCl	10.3	6.2
	50 mM K_2SO_4	4.7	0.6
	5 mM $CaCl_2$	1.1	—3.0
LSD, 5 %		1.3	1.9

[1] Difference between mean and the initial stomatal aperture (4.1 microns).

30 % reduction of the opening response in light + CO_2-free air; however, in dark + normal air the reduction was 90 %. Although Cl^- appears to have an important role in the dark, overall. K^+ is clearly the more important component of KCl. Replacement by other monovalent ions is now being examined.

Opening and Uptake of K^+ as Indicated by $^{86}Rb^+$. The extent of K^+ uptake was estimated using $^{86}Rb^+$ as a tracer for K^+. It was essential that uptake be confined mainly to guard cells since radioactivity on the whole epidermal strip was determined. Care was taken to insure that only traces of broken mesophyll cells and a few of their chloroplasts remained on the strips. Strips were selected so that intact epidermal cells (5) always averaged less than 5 % of the area of the strip; guard cells constituted about 7 % of this area.

Initially, the exchangeability of $^{86}Rb^+$ which went into the strips was studied. Strips floated on labeled KCl took up $^{86}Rb^+$ and retained the label when floated on unlabeled KCl (fig 3). Thus the retained $^{86}Rb^+$ was not exchangeable and presumably was taken into cells.

Figure 3 also shows that light + CO_2-free air markedly stimulated $^{86}Rb^+$ uptake and that this was associated with a large increase in aperture. The

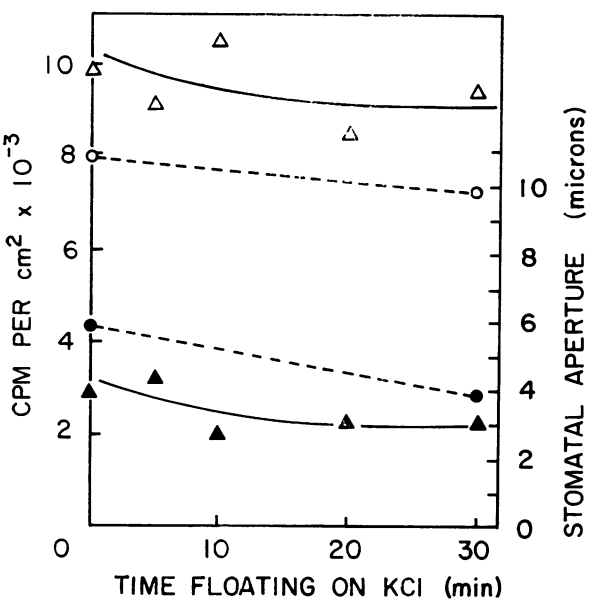

FIG. 3. The $^{86}Rb^+$ content of labeled epidermal strips floating on unlabeled KCl. Strips of known area were exposed for 3 hr to 6.5 mM KCl labeled with $^{86}Rb^+$ in either light + CO_2-free air (open symbols) or dark + normal air (solid symbols), removed, rinsed a few seconds in unlabeled buffered 100 mM KCl, then floated on buffered 100 mM KCl at low light intensity (100 ft-c) for various lengths of time. Radioactivity retained by the strip (triangles) was determined after washing and drying and is given as cpm per cm^2 of strip area. Stomatal aperture (circles) was measured on other strips treated similarly. The initial aperture was 2.7 microns.

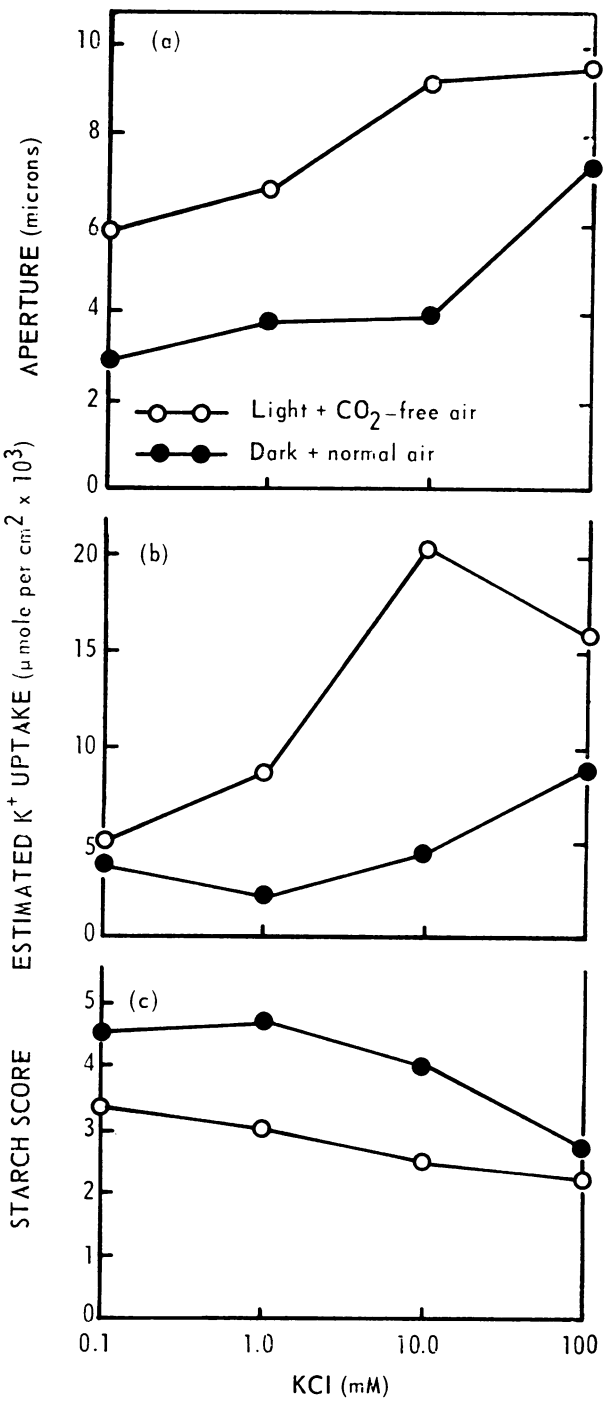

FIG. 4. The response of stomatal aperture (a), K^+ uptake as estimated with $^{86}Rb^+$ (b), and guard cell starch score (c) to light + CO_2-free air and to KCl. Stomatal aperture and guard cell starch were measured in similarly treated material exposed to unlabeled KCl solutions. Intact epidermal cells were estimated to be 2.5 % of the area of the epidermal strip. Initial stomatal aperture was 1.8 microns; initial guard cell starch score was 5.0. Four replicates.

relationship between stomatal opening and K^+ uptake was further delineated by estimating K^+ uptake at various KCl concentrations in light + CO_2-free air and dark + normal air (fig 4a and b). Again light + CO_2-free air stimulated both K^+ uptake and opening. Furthermore, wider apertures were associated with greater uptake of K^+ at the various levels of KCl. When KCl concentration was sufficiently high (10 mM), neither aperture nor estimated K^+ uptake was affected by a further 10-fold increase in KCl. Data from all experiments, summarized in figure 5, show that the increase in stomatal aperture correlated closely with the amount of K^+ taken up. The data from 4 separate experiments and from light + CO_2-free air and dark + normal air treatments fit approximately the same curve.

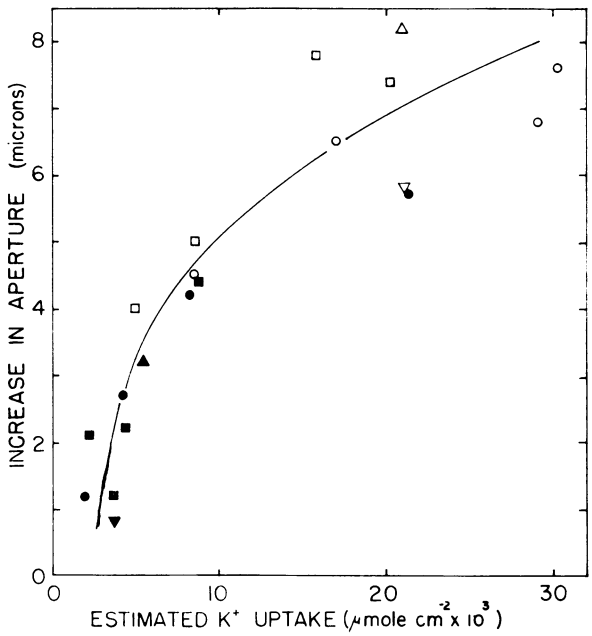

FIG. 5. The relationship of increase in stomatal aperture to estimated K^+ uptake over a 3 hr period. Data from various experiments similar to and including that of figure 4 (squares), each experiment being represented by a different symbol (open symbols, light + CO_2-free air; closed symbols, dark + normal air). Increases in stomatal aperture were obtained by subtracting the initial value from final values in each case.

Guard Cell Starch in Relation to Stomatal Aperture. Increases in stomatal aperture in response to KCl and light + CO_2-free air were closely related to decreases in starch score for guard cells as well as to increases in K^+ uptake (fig 4). Figure 6, which summarizes the results of similar experiments, reveals an apparently linear inverse relationship between changes in starch score and in aperture. Data from 2 experiments (circles and squares) fit one relationship, while data of the third experiment (triangles) are described better by another line.

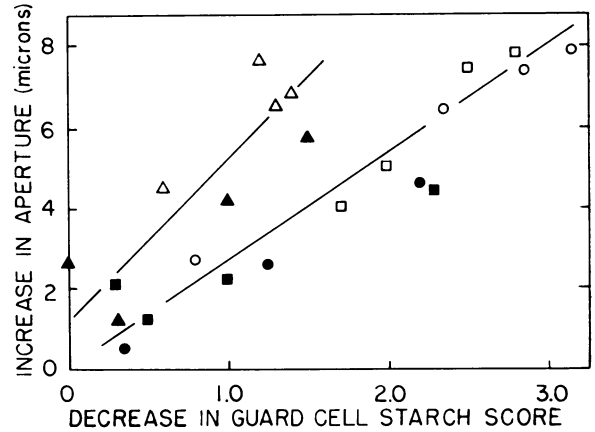

FIG. 6. The relationship of increase in stomatal aperture to decrease in guard cell starch score over a 3 hr period. Data from various experiments similar to and including that of figure 4 (squares), each experiment being represented by a different symbol (open symbols, light + CO_2-free air; closed symbols, dark + normal air). Decreases in starch score were obtained by subtracting final values from the initial value in each case.

Discussion

Absorption of K^+. Various aspects of the data point toward uptake of KCl into the guard cells in the isolated epidermal strips. The results obtained with $^{86}Rb^+$, however, constitute the strongest evidence. This ion was absorbed by living cells since it was non-exchangeable. A number of points make it unlikely that epidermal cells were involved to any extent in the uptake. The number of intact epidermal cells was small (they constituted <5 %, and about 2.5 % in fig 4, of the strip area) and in various instances, high contents of $^{86}Rb^+$ were measured in strips which contained no observable intact epidermal cells. Further it seems unlikely that light would stimulate ion uptake by epidermal cells of *Vicia* since they have no chloroplasts.

Changes in K^+ concentration in the guard cells can be estimated from data on K^+ uptake if one assumes that the uptake measured is net influx of K^+. This assumption appears reasonable in light of the lack of substantial tracer efflux (fig 3). The number of stomata averaged 6200 per cm^2 of strip and the volume of a fully open guard cell was 5×10^{-9} cm^3 (estimated from the surface view to be that of a cylinder approx 12 microns in diameter and 44 microns long for stomata 11.3 microns wide). Thus an indicated K^+ uptake of 20×10^{-3} μmole per cm^2 (a conservative value for fully open stomata, see fig 5) represented an increase of about 300 mM in K^+ concentration of the guard cells. Since the solute potential of guard cells in the initial condition (aperture 2 to 4 microns) was about -7 bars (5), it is not possible that their initial K^+ concentration was greater than 150 mM and likely somewhat less.

Thus the K^+ concentration in the guard cell appeared to be more than tripled.

Rates of absorption of 20×10^{-3} μmole per cm^2 of strip per 3 hr are equivalent, for the above dimensions, to approximately 300 μmoles per gram of guard cells per 3 hr or 9 $\mu\mu$moles per sec per cm^2 of guard cell surface. These rates appear to be high relative to light-stimulated absorption in other plant systems (1, 16, 18). Rains (18) reported values approaching 15 μmoles per gram fresh weight per 3 hr for sliced corn leaf tissue in the light in 0.1 mM KCl. Considering the much higher concentration (10 mM) used for stomatal uptake and the likelihood of some non-absorbing tissue in the leaf slices, the difference in rates is not unexpected.

K^+ Absorption and Stomatal Opening. An increase in stomatal aperture of about 7 microns (from initial aperture to final aperture of 11 microns) corresponds to an increase in guard cell K^+ concentration of 300 mM, as calculated above. Assuming that there is corresponding uptake or formation of an anion, this would represent a decrease in solute potential in the order of 12 bars (assuming the solute is KCl, less if anion is not univalent). In a separate but similar experiment (5), an increase in stomatal aperture of 7.2 microns in epidermal strips was associated with a fall in guard cell solute potential of 9.9 bars. Therefore sufficient K^+ was absorbed to account for the changes in solute potential.

Univalent cations have been observed by Iljin (13) and Imamura (14) to cause stomatal opening but neither author measured quantitatively the K^+ taken up. Hence they suggested that K^+ stimulated starch hydrolysis (13) or swelling of cytoplasmic colloids (14). Levitt (15) has suggested that K^+ uptake leads to stomatal opening because it involves exchange for H^+, thereby increasing guard cell pH and stimulating starch hydrolysis. In contrast, we would attribute to K^+ a direct role in stomatal opening, that of the major solute causing the lowered solute potential.

After this work was completed, a paper was published by Fujino (10) quoting his earlier papers in Japanese (6, 7, 8, 9) in which histochemical staining for K^+ in guard cells showed that stomatal opening in epidermal strips of *Commelina* and *Allium* was associated with K^+ uptake; opening and uptake of K^+ were both stimulated by light. He concluded that opening was caused by the active movement of K^+. Our estimates of K^+ uptake constitute quantitative evidence in support of his conclusion, particularly demonstrating that probably sufficient K^+ was absorbed to cause the observed changes in solute potential. The fact that Fujino's results were based on the visual scoring of staining in the guard cell lends credence to our conclusion that the radioactivity was accumulated mainly in the guard cells.

Stomatal Responses to Light and to CO_2-Free Air. Variation in stomatal aperture, as effected by light and CO_2-free air, given separately and together (fig 2), may reflect no more than different rates of K^+ absorption under these conditions. This is supported by the fact that in figure 5 data from dark + normal air and light + CO_2-free air fit approximately the same relationship.

The effects of light and air composition on aperture may be examined more quantitatively by plotting the reciprocal of response in stomatal aperture (1/A) against the reciprocal of the external concentration of KCl [1/(KCl)]. Such plots using data of figures 1 (insert) and 2 were linear and showed that the extrapolated maximal response in aperture ($1/A_{max}$) was not changed by light and air composition but that KCl concentration required for one-half of the maximal response was altered. Although plausible mechanisms of light stimulated ion absorption have been proposed (1, 16, 18, 21) the apparent stimulation by CO_2-free air in light and dark presents an intriguing problem.

Stomatal Opening and Starch Changes. K^+ uptake and the decrease in starch content of guard cells were, with 1 exception, well correlated with each other and with changes in aperture in this study, thus providing no evidence to distinguish which is the more significant for stomatal opening. The exception was the small opening response in light + CO_2-free air in strips on buffer without KCl (fig 1 and 2) and on deionized distilled water (5). There was presumably no K^+ uptake whereas the starch score did show a small decrease. On the other hand, there is much evidence in the literature (11) that changes in stomatal aperture occur independently of changes in guard cell starch. Recent reviews (17, 21) have not assigned starch changes a primary role in stomatal opening. Since calculated KCl uptake was sufficient to account for the changes in solute potential, we suggest that K^+ movement may be primary in stomatal opening.

It is possible, however, that starch hydrolysis is an integral part of the K^+ uptake mechanism. Perhaps starch hydrolysis provides energy or is related to the uptake of K^+ *via* the production of organic acid anions. Uptake of cation in excess of anion is known to lead to equivalent increases in organic acids (12).

Stomatal Closing. This discussion implies that stomatal closing involves a net efflux of K^+ from guard cells. In this work with *Vicia*, dark + normal air caused only a gradual closing of open stomata in both epidermal strips and leaf discs (5), making it difficult to examine dark closing in relation to K^+ efflux. In one experiment, strips on labeled KCl (10 mM) were transferred to dark + normal air after 6 hr in light + CO_2-free air at which time they had absorbed 21×10^{-3} μmole of K^+ per cm^2 (estimated with $^{86}Rb^+$), having a mean aperture of 7.5 microns. After a further 9 hr in the dark on labeled KCl, these strips contained only 13×10^{-3} μmole of K^+ per cm^2 (estimated) with a mean aperture of 6.6 microns, while strips remaining in light + CO_2-free air on labeled KCl during the whole

experiment contained 22×10^{-3} μmole per cm^2 (estimated) with a mean aperture of 8.0 microns. These preliminary data suggest that closing was associated with net K^+ efflux.

The above experiment also provided a check on earlier conclusions. Since tracer content of the strip in light + CO_2-free air was essentially constant over the last 9 hr, internal and external specific activities were presumably equal. Hence total K^+ content of guard cells can be estimated. Assuming univalent anions for charge balance, such a K^+ content would lead to a solute potential of about −13 bars, a reasonable value for an aperture of 8 microns in light of earlier results (5). Again, it is unnecessary to invoke other solutes to explain the solute potential reached.

Acknowledgments

We thank Dr. D. W. Rains for his advice and gift of ^{86}Rb, Dr. H. J. Ketellapper for suggestions, Dr. R. M. Hagan for encouragement, and Miss Jean Koskela for her very capable technical assistance.

Literature Cited

1. Barber, J. 1968. Light induced uptake of potassium and chloride by *Chlorella pyrenoidosa*. Nature 217: 876–78.
2. Briggs, G. E., A. B. Hope, and R. N. Robertson. 1961. Electrolytes and Plant Cells. Blackwell Scientific Publications, Oxford.
3. Fischer, R. A. 1967. Stomatal physiology with particular reference to the after-effect of water stress and to behaviour in epidermal strips. Ph.D. dissertation. University of California. Davis.
4. Fischer, R. A. 1968. Stomatal opening: role of potassium uptake by guard cells. Science 168: 784–85.
5. Fischer, R. A. 1968. Stomatal opening in isolated epidermal strips of *Vicia faba*. I. Response to light and to CO_2-free air. Plant Physiol. 43: 1947–52.
6. Fujino, M. 1959. Correlation among the stomatal movement, pH, salts content, starch content and osmotic value in *Commelina* (in Japanese). Kagaku 29: 147–48.
7. Fujino, M. 1959. Correlation among the stomatal movement, potassium content and pH in *Allium* (in Japanese). Kagaku 29: 424–25.
8. Fujino, M. 1959. Stomatal movement and active migration of potassium (in Japanese). Kagaku 29: 660–61.
9. Fujino, M. 1960. Stomatal movement and enzyme activity in guard cells (in Japanese). Kagaku 30: 89–90.
10. Fujino, M. 1967. Adenosinetriphosphate and adenosinetriphosphatase in stomatal movement. Sci. Bull. Fac. Educ. Nagasaki Univ. 18: 1–47.
11. Heath, O. V. S. 1959. The water relations of stomatal cells and the mechanisms of stomatal movement. In: Plant Physiology, Vol. II. F. C. Steward, ed. Academic Press, New York. p 193–250.
12. Hiatt, A. J. 1967. Relationship of cell sap pH to organic acid change during ion uptake. Plant Physiol. 42: 294–98.
13. Iljin, W. S. 1922. Physiologischer Pflanzenschutz gegen schädliche Wirkung von Salzen. Biochem. Z. 132: 526–42.
14. Imamura, S. 1943. Untersuchungen über den Mechanismus der Turgorschwankung der Spaltöffnungsschliesszellen. Japan. J. Botany 12: 251–347.
15. Levitt, J. 1967. The mechanism of stomatal action. Planta 74: 101–18.
16. MacRobbie, E. A. C. 1966. Metabolic effects on ion fluxes in *Nitella translucens*. I. Active influxes. Australian J. Biol. Sci. 19: 363–70.
17. Meidner, H. and T. A. Mansfield. 1965. Stomatal responses to illumination. Biol. Rev. 40: 483–509.
18. Rains, D. W. 1967. Light-enhanced potassium adsorption by corn leaf tissue. Science 156: 1382–83.
19. Rains, D. W. 1968. Kinetics and energetics of light-enhanced potassium absorption by corn leaf tissue. Plant Physiol. 43: 394–400.
20. Rains, D. W. and E. Epstein. 1967. Preferential absorption of potassium by leaf tissue of the mangrove *Avicennia marina*: an aspect of halophytic competence in coping with salt. Australian J. Biol. Sci. 20: 847–57.
21. Zelitch, I. 1965. Environmental and biochemical control of stomatal movement in leaves. Biol. Rev. 40: 463–82.