ASPECTS OF POTASSIUM ACCUMULATION BY STOMATA OF *VICIA FABA*

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Abstract

Potassium accumulation by guard cells during the light opening of *V. faba* stomata in epidermal strips was determined quantitatively with ^{42}K and ^{86}Rb tracers. The sodium cobaltinitrite stain for potassium was also used. Particular attention was paid to errors arising from the presence of intact epidermal cells.

When epidermal strips obtained from darkened leaves were floated on potassium chloride solution (usually 10 mM) in light plus CO_2-free air, guard cell potassium content and stomatal aperture increased in parallel, both approaching a maximum after about 300 min. Flux measurement suggested that the steady maximum potassium content (and aperture) arises because of a decline in the initially high potassium influx. With opening of stomata in the light, external calcium reduced in a parallel manner both opening and potassium uptake at potassium concentrations ranging from nearly zero to 50 mM. Also, maximum stomatal opening and potassium accumulation in the presence or absence of calcium bore linear relationships to log external potassium concentration. These results are discussed in relation to potassium accumulation in other plant systems.

In experiments with epidermal strips, guard cell potassium content showed a consistent linear relationship to stomatal aperture. For 16 experiments spanning two years and many treatments affecting aperture, the mean slope of this relationship was 2·6 nmoles cm^{-2} μm^{-1}. This is equivalent to a change in potassium concentration of 40 mM μm^{-1}. The increase in guard cell osmotic pressure attributable to potassium plus anion accumulation is estimated to be 1·5 bars μm^{-1}, which is a major portion of the observed increase in osmotic pressure with opening (2·0 bars μm^{-1}).

I. INTRODUCTION

Recently it has been suggested that accumulated potassium and an accompanying anion are the major osmotically active solutes involved in the increase in guard cell osmotic pressure generally observed with opening of stomata in the light (Fujino 1967; Fischer and Hsiao 1968; Sawhney and Zelitch 1969; Humble and Raschke 1971). Histochemical staining for guard cell potassium indicated substantial accumulations with stomatal opening (Imamura 1943; Fujino 1967; Humble and Hsiao 1970). Quantitative estimates with the electron microprobe have shown in both tobacco (Sawhney and Zelitch 1969) and *Vicia faba* (Humble and Raschke 1971) very substantial increases in guard cell potassium concentration with stomatal opening.

Guard cell potassium content has also been estimated by the uptake of the radioactive tracer ^{86}Rb by isolated epidermal strips taken from leaves of *V. faba* (Fischer and Hsiao 1968; Humble and Hsiao 1970). When these epidermal strips are

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floated on dilute potassium chloride solutions, stomatal aperture and guard cell starch respond to light and CO_2-free air as they do in intact leaf pieces (Fischer 1968). Guard cell potassium concentration was estimated to increase about 300 mM with the opening of stomata in light plus CO_2-free air. This was considered sufficient to account for the corresponding increase in guard cell osmotic pressure (Fischer and Hsiao 1968). Also potassium appears to accumulate in the guard cells of stomata opened in the light in leaves of *V. faba* in similar quantities to those observed in isolated epidermal strips (Humble and Hsiao 1970; Fischer 1971).

The use of radioactive tracers and isolated epidermal strips represents the most feasible method of quantifying potassium accumulation by guard cells under various conditions. However, several potential sources of error had become evident in the approach, including tracer uptake by epidermal cells which remain intact in the epidermal strips (Humble and Hsiao 1970), and the unsuitability of ^{86}Rb as a tracer for potassium (Hiatt 1970). Also conclusions about the role of potassium required accurate estimations of changes in guard cell potassium content and hence knowledge of equilibration times for internal specific activity. This paper considers these aspects of the tracer method and presents improved values for the quantities and fluxes of the potassium accumulated during stomatal opening in *V. faba* under a wide range of conditions. Particular attention is given to the effects of time, and of external potassium and calcium concentrations.

II. Methods

These studies were conducted in Canberra, A.C.T. (lat. 35°S.). Plants of *V. faba* (cv. Early Long Pod) were grown separately in 2-litre containers of aerated nutrient solution (half-strength Hoagland's solution, changed weekly). Plants were grown in a glasshouse, or in growth cabinets (21 ± 3°C, 14-hr photoperiod, 3000 f.c.). Glasshouse air temperatures were usually within the range 20–30°C, but extremes as low as 15°C and as high as 35°C were occasionally recorded. On rare occasions plants grown entirely out of doors were used.

Expanded leaves two to six positions below the shoot apex of 4–8-week-old plants were used. Experimental material was collected in the mornings. Leaflets were cut off plants and carried to the laboratory abaxial surface upwards in darkened closed Petrie dishes containing a little water. Rectangular epidermal strips ($\simeq 3$ by 5 mm^2) were taken from the abaxial surface of leaflets $\frac{1}{2}$–2 hr later as described before (Fischer 1968) and immediately floated cuticle upwards on distilled water in the dark. Light or other experimental treatments commenced 15–30 min later.

Experiments usually studied the response of stomatal aperture to light plus CO_2-free air in epidermal strips floated on a range of solutions (but always without buffer, in contrast to Fischer 1968). Unless otherwise stated the chloride salts of potassium and calcium were used; solution pH ranged from 4 to 7 and temperature from 28 to 32°C without effect on responses. For experiments not involving radioactive isotope uptake, illumination was from below at an intensity of 1·8 mW cm^{-2} (0·4–0·7 μm, Sylvania Gro Lux/VHO/WS lamps). In isotope experiments illumination was from above at an intensity of 4–6 mW cm^{-2} on the same basis (mercury vapour lamp). The difference in lamps and light intensities with the two systems of illumination did not affect the magnitude of stomatal opening. CO_2-free air (< 1 p.p.m. CO_2) passed continually over the experimental material. Controls in the dark and ventilated with normal air ($\simeq 300$ p.p.m. CO_2) were usually included.

For measurement of stomatal aperture epidermal strips were mounted in immersion oil on a glass slide as before (Fischer 1968). Unless otherwise mentioned, aperture measurements were made at $\times 1000$ magnification on 10–20 stomata whose guard cells were not in direct contact with intact epidermal cells, but which were otherwise selected at random. Under such conditions aperture should be a direct reflection of guard cell turgor pressure and therefore internal

solute concentration. Intact epidermal cells are readily distinguished at lower magnification by being more refractive, while at high power their protoplasm has an even consistency with only the nucleus, protoplasmic strands, occasionally streaming particles, and leucoplasts faintly visible. Neutral red uptake, staining for potassium, and the cutting open of epidermal cells confirmed the distinction made between intact (living) and broken epidermal cells.

Experiments involving the uptake of radioactively labelled potassium (^{42}K) and of ^{86}Rb were carried out as before (Fischer 1968). However, the time for free-space washout was lengthened to 10 min and took place under exactly the same conditions and concentrations as the loading phase. In addition, at the end of the washout period, each strip was submerged briefly in distilled water in order to wash off any isotopically labelled salt deposits on the cuticular surface of the strip. The submerging procedure was shown to remove a small but significant amount of isotope. Strips were then mounted on a glass slide in oil and stomatal aperture was measured microscopically as above. Strips were scored at this time for intact epidermal cells, this being expressed as the percentage of the strip's area occupied by such cells. The exact area of the strip was also measured. Each strip was then placed in the centre of a planchet which was counted in a gas-flow counter. Passage of the strip through oil on the slide for the duration of the measurements (about 5 min) did not alter significantly its content of radioactive isotopes. Duplicate strips were processed for every treatment observation in each replicate. Potassium contents are expressed per unit surface area of epidermal strip (nmoles cm^{-2}).

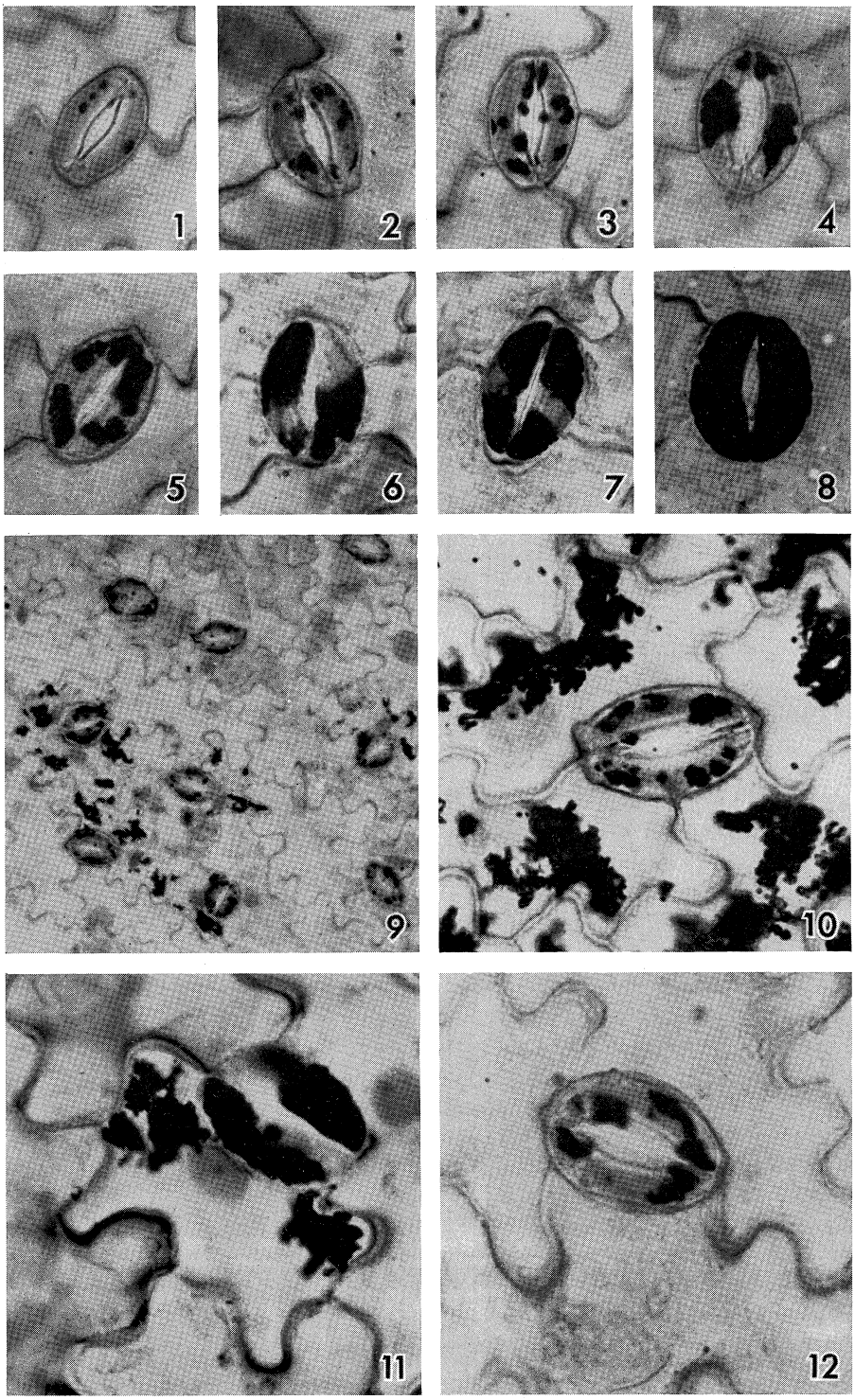
In some experiments, potassium in the guard cell of stomata was stained with sodium cobaltinitrite exactly as described before (Fischer 1971). Strips were then mounted on glass slides in a 1:1 mixture of concentrated ammonium sulphide and 50% glycerine. The amount of black cobaltous sulphide precipitate was scored under 400-fold magnification as the percentage of the guard cell area covered by precipitate. Scoring was aided by the preparation of photographic standards (Figs. 1–8).

III. Results

(*a*) *Interference from Intact Epidermal Cells*

Staining of epidermal strips for potassium shows that intact epidermal cells contain considerable amounts of potassium (Figs. 9–12). This supported earlier suspicions that intact epidermal cells might accumulate labelled potassium from the external solution either by net uptake or exchange. In order to avoid this complication, the results of the uptake experiments already reported (Fischer and Hsiao 1968) always referred to epidermal strips with less than 3% of their total area having intact cells. This has posed a considerable limitation on the rate of progress since the percentage of intact epidermal cells in any strip is very variable, depending in an inconsistent fashion on the position on the leaflet, the way in which the strip is taken, and particularly on the growing conditions of the plants. Epidermal strips containing no intact epidermal cells have only, but not always, been obtained when plants were grown in the glasshouse under summer conditions of high radiation, high air temperatures (up to 35°C), and low relative humidities (down to 40%).

Many methods of breaking intact epidermal cells without damaging the guard cells were tried without consistent success. The general procedure adopted therefore was to take epidermal strips and discard those which from their appearance floating on water could be judged to have moderate (20%) or higher percentages of intact cells. The remaining strips showed low but variable numbers of intact cells. By running duplicate strips for all measurements it was possible to discard the results for epidermal strips with more than 5% intact epidermal cells while usually retaining a complete data matrix. In some experiments, the difference in content of label for duplicate strips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after a period on labelled potassium was consistently related to the chance difference in percentage of intact epidermal cells for the same duplicates. In such cases linear regression analysis, using the former difference as the dependent variable, provided a relationship which could be used to correct the tracer uptake of each strip to the condition for no intact epidermal cells (Table 1).

TABLE 1

UPTAKE OF POTASSIUM BY INTACT EPIDERMAL CELLS OF *V. FABA*

Results are expressed as the differences in duplicate epidermal strips in content of potassium label per unit area of epidermal strip (nmoles cm^{-2}) per percentage of epidermal strip area occupied by intact epidermal cells (linear slope), assuming a linear relationship between these parameters. The ratio was obtained from many replicates of duplicate strips which were floated on labelled 10 mM KCl + 0·1 mM $CaCl_2$ (see text). Results subjected to linear regression analysis

Expt. No.	Sampling times	Conditions	Linear slope (nmoles cm^{-2} per 1% area)	Correlation coefficient
B62	At 390, 630, and 890 min	Light plus CO_2-free air	0·27±0·13	0·507*
		Dark plus normal air	0·33±0·050	0·899**
37	At 400 min	Light plus CO_2-free air	0·58±0·060	0·908**
		Dark plus normal air	0·22±0·058	0·727**

*, ** Significant at 5% and 1% level of probability, respectively.

The accumulation of potassium label by guard cells in the light under the conditions of Table 1 is usually greater than 10 nmoles cm^{-2} (see later). Thus the amounts of potassium label accumulated by intact epidermal cells are sufficiently small relative to guard cell accumulation for the 5% upper limit on intact epidermal cells to appear satisfactory, even considering the fact that uptake of label by intact cells was increased significantly in the light in experiment 37 (Table 1). Results from strips with greater proportions of intact cells should be viewed with caution; epidermal cells in strips in the light with 100% intact epidermal cells could contain up to 60 nmoles cm^{-2} of potassium label or 4–6 times that contained in the guard cells. If potassium tracer content estimates potassium content (see later), these results illustrate the difficulty of detecting changes in potassium content of guard cells by analysing the potassium content of epidermal strips when many epidermal cells remain intact.

It should be pointed out that in some experimental material intact epidermal cells did not participate in potassium uptake to the full extent indicated in Table 1.

Figs. 1–8.—Representative standards for scoring of the degree of potassium staining in guard cells: 1, 5%; 2, 10%; 3, 20%; 4, 35%; 5, 50%; 6, 60%; 7, 80%; and 8, 100%. In Figure 7 the unstained region is that occupied by the guard cell nucleus; generally, however, the intracellular location of the stain is not a good indicator of the original location of the potassium (Macallum 1905).

Figs. 9–12.—Epidermal strips stained for potassium (see text) to show potassium in intact epidermal cells (black precipitate within the cell boundary): 9, low-power view showing a patch of intact epidermal cells; 10, 11, and 12, respectively, are high-power views of stomata surrounded entirely, on one side, and not at all by intact epidermal cells.

(b) *Time Courses of Uptake and Aperture Change*

The major aim of this work has been to understand the response of stomata to light plus CO_2-free air (Fig. 13), this response being equivalent, it is believed, to the most common opening movement of stomata under natural conditions. Stomata at the commencement of such experiments appear to be slightly or moderately open because this represents the resting aperture of most darkened stomata when epidermal cell back pressure has been eliminated. The light response is very obvious in Figure 13, but there is also a small response of aperture to time in the dark. Moderate to complete opening has been reported earlier to occur on 100 mM KCl solutions in the dark (Fischer and Hsiao 1968); its occurrence to a lesser degree at 10 mM KCl is usual, with potassium content showing a parallel increase.

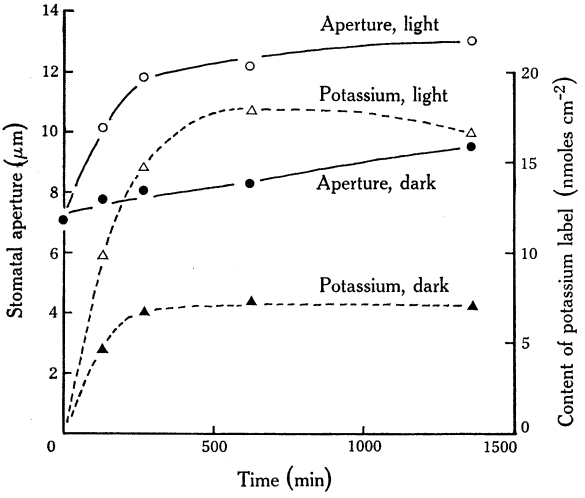

Fig. 13.—Response of stomatal aperture and content of potassium label to time in light plus CO_2-free air (light) and dark plus normal air (dark). Epidermal strips floated on 10 mM $^{42}KCl + 0{\cdot}5$ mM $CaCl_2$. Experiment 36.

Five other experiments conducted on various widely separate occasions provide essentially similar data to that of Figure 13 on the stomatal response to time in light. Although the uptake of labelled potassium and stomatal opening may require 500 min to reach a maximum, generally one-half of the response has occurred in the first 100 min. Aperture and uptake parallel one another closely although in some experiments, after a long period of time, the content of potassium label tended to fall while aperture was steady or increasing very slowly. For this reason experiments were usually confined to the first 500 min in light plus CO_2-free air.

During uptake experiments, such as shown in Figure 13, it is important to know when the specific activity of guard cell potassium reaches the external specific activity, since in this situation guard cell content of potassium label estimates guard cell potassium content. In an earlier paper (Fischer and Hsiao 1968), it was assumed that this was the case once the content of potassium label had reached a plateau (e.g. after about 500 min). It may occur, however, at an earlier stage, depending on how much potassium is present in the guard cells at time zero and on the magnitude of the potassium fluxes. Using the data of all the above experiments and plotting content of potassium label for all times against increase in aperture from that at time zero gives a linear relationship ($r = 0{\cdot}849$, slope $= 2{\cdot}60$ nmoles cm^{-2} μm^{-1}). Samplings before

100 min do not fall noticeably below this relationship. The relationship extrapolates to 3·4 nmoles cm^{-2} of potassium at zero aperture change; this approximates the initial content of potassium in guard cells, which is seen to be quite low. Staining of epidermal strips at the commencement of experiments also showed very little potassium in the guard cells. Since the guard cell pool of potassium initially is small relative to the rate of net tracer uptake (for example, the latter was about 10 nmoles cm^{-2} in 120 min in Fig. 13), it can be concluded that equality of specific activity was approached rapidly. Since tracer influx and efflux in this initial period were not measured it is not possible to estimate exactly how rapidly this equality was reached. However, within 100 min is considered a safe assumption for the experimental conditions here (i.e. starting with material from darkened leaves).

It is interesting to examine the pattern of potassium fluxes associated with a steady-state aperture in light. Owing to the nature of the experimental system it was not possible to measure anything approaching instantaneous fluxes; in fact a period of 60–90 min plus 10 min for washout was chosen for flux determination. In open stomata, the proportion of vacuole relative to cytoplasm is large and with the time periods chosen there is no doubt that fluxes to and from the vacuole were being measured (Pitman 1969). If the vacuole potassium pool is large relative to the fluxes and its specific activity known, which was the case for stomata which have reached a steady open state, it is possible to distinguish influx, efflux, and net flux of potassium under such conditions. For influx open stomata were transferred from unlabelled KCl to labelled KCl, for efflux the opposite transfer was carried out, and for net flux stomata floated at all times on labelled KCl. The fact that change in tracer content of the guard cells over a 90-min period after transfer was linear with time, supports the argument that vacuolar influx and efflux of potassium were being measured in the respective cases.

For comparative purposes, fluxes were also measured in the case of stomata immediately after transfer to KCl and light+CO_2-free air. Again, fluxes into and from the vacuole would dominate the picture with the long measurement periods adopted. However, in this case the measured net flux of tracer will lie somewhere above net flux and below influx of potassium (Cram 1969). In view of the small internal pool of potassium initially, the relatively large tracer influxes, and the likely small efflux relative to influx, it is probably that the difference initially between the three parameters net tracer flux, net flux of potassium, and influx of potassium is small.

The above measurements of tracer flux were made in two experiments (Table 2). Initial tracer flux (i.e. upon transfer to light+CO_2-free air and labelled KCl) was measured on two groups of material in each case: for one group (A) this was done at time zero, while for the other (C) it was done at 200 (expt. 86) or 300 min (expt. 84), the strips having been kept until this time on distilled water in the dark. Fluxes are presented per unit surface area of guard cell (pmoles cm^{-2} sec^{-1}) where the effective surface area per guard cell was taken to be $1 \cdot 4 \times 10^{-5}$ cm^2 (area of inner wall surface as calculated from microscopic measurements of living cell dimensions for material with a stomatal density of 6500 cm^{-2}).

It is apparent in Table 2 that the steady state in aperture and potassium content after 200–300 min in light plus CO_2-free air is the result of a substantial reduction in the potassium influx component, if it is recalled that net flux of tracer in groups A and C

(Table 2) underestimates potassium influx. The efflux component in open stomata was small in contrast to suggestions of Thomas (1970). The cause of the reduction in influx observed here was not time *per se* since the stomata stored in dark on distilled water retained to a large degree their ability to take up potassium. Thus the cause is no doubt related to time in light plus CO_2-free air floating on KCl, or directly to opening itself. It is interesting to speculate that there is a feedback inhibition of influx by guard cell internal potassium content or by turgor itself (Gutknecht 1968). In experiment 86, measurement of influx over 60 min in the presence of 0·20M sucrose to reduce guard cell turgor, and carried out also after 200 min in light plus CO_2-free air, gave a value

TABLE 2

POTASSIUM FLUXES IN GUARD CELLS OF *V. FABA*

Independent measurements were made in light plus CO_2-free air with epidermal strips floating on 10 mM KCl+0·1 mM $CaCl_2$ (see text). ^{42}K was used in experiment 84 and ^{86}Rb in experiment 86. Fluxes are averages over a period of 90 min (expt. 84) and 60 min (expt. 86) after times specified. There was a 10-min washout period in both experiments

Expt. No.	Conditions	Parameter	Flux (pmoles cm^{-2} sec^{-1})
84	A. Time zero	Net flux (tracer)	+16·5
	B. After 300 min in light+CO_2-free air on 10 mM KCl+ 0·1 mM $CaCl_2$	Influx	+ 2·0
		Efflux	− 3·7
		Net flux	− 1·8
	C. After 300 min in dark on water	Net flux (tracer)	+12·2
86	A. Time zero	Net flux (tracer)	+11·0
	B. After 200 min in light+CO_2-free air on 10 mM KCl+ 0·1 mM $CaCl_2$	Influx	+ 2·7
		Efflux	− 2·6
		Net flux	+ 1·6
	C. After 200 min in dark on water	Net flux (tracer)	+ 8·5

of 3·2 pmoles cm^{-2} sec^{-1} which, however, was not significantly higher than the value of influx in the absence of sucrose (2·7 pmoles cm^{-2} sec^{-1}). Corroborating the evidence for reduced influx of potassium with stomatal opening are measurements of potassium influx into stomata, opened to a steady-state aperture on leaves in light, then removed as epidermal strips free of intact epidermal cells and transferred to labelled potassium solutions; low values similar to those of Table 2 were obtained (4·7 and 2·7 pmoles cm^{-2} sec^{-1}, expts. 45 and 63b respectively). Also the results of Fischer and Hsiao (1968, fig. 3) indicate a low efflux of potassium from open stomata (approx. 5 pmoles cm^{-2} sec^{-1}).

(c) *Effect of Calcium and of Concentration of Potassium*

It was originally reported that calcium has no effect on stomatal opening in isolated epidermal strips of *V. faba* floating on KCl solutions (Fischer 1968).

Subsequently calcium was found to have a small inhibitory effect on this opening (Pallaghy 1970); similar results have been observed in *Commelina communis* (Fujino 1967; Willmer and Mansfield 1969*a*), and tobacco (Thomas 1970).

Figure 14 shows the results of many more recent experiments on the response to calcium in *V. faba*. The presence of calcium reduces the aperture reached after several hours in light plus CO_2-free air by 1–3 μm. This effect, although becoming significantly less at higher KCl concentrations, is still quite obvious at 10 and 50 mM KCl. The increase in stomatal aperture of 1·7 μm for each 10-fold increase in potassium concentration in the presence of calcium was similar to that observed before (Fischer and Hsiao 1968). Stomatal opening in the apparent absence of potassium has been noted before (Fischer and Hsiao 1968; Pallaghy 1970); the calcium effect was also significant under such conditions.

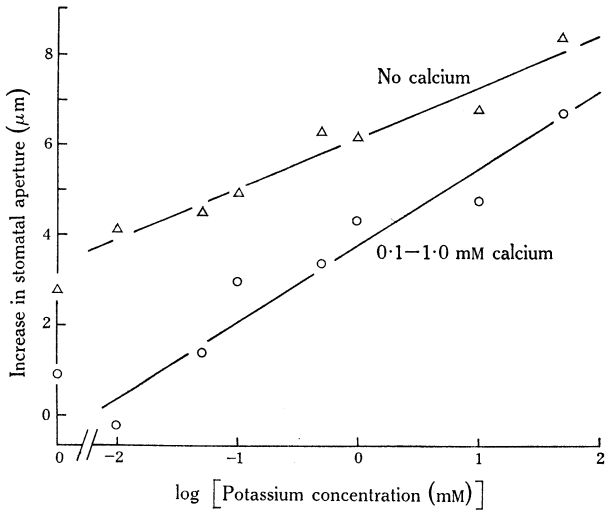

Fig. 14.—Increase in stomatal aperture after 150–240 min in light plus CO_2-free air in the presence and absence of calcium and potassium. Epidermal strips floated on solutions. Results are averaged for experiments over a period of 4 months, each point being the mean of determinations on from 4 to 19 separate leaf samples of material; calcium concentrations from 0·1 to 1·0 mM. Linear regression analysis (excluding zero potassium data): no calcium, $y = 6·2+1·1\,x$ ($r = 0·976$); with calcium, $y = 3·8+1·7\,x$ ($r = 0·967$). Correlation coefficients (r) significant at the 1% level; regression slopes significantly different at the 5% level.

The effect of calcium on potassium uptake was examined in a further experiment. At the concentrations of potassium tested (0·0075 mM, 0·029 mM, and 0·74 mM), 0·1 mM calcium significantly reduced the stomatal aperture reached after 300 min in light plus CO_2-free air by an average of 2·4 μm, paralleled by a significant reduction in the potassium uptake by an average of 5·4 nmoles cm^{-2}. There was no interaction between the effects of calcium and the external potassium concentration. In both the presence and absence of calcium, potassium uptake was linearly related to log potassium concentration (see also Fig. 15). The relationship between potassium content and stomatal aperture, including treatments with and without calcium, was also linear, with a correlation coefficient of 0·987 ($P < 0·01$). In a similar experiment (Fig. 15), potassium content and stomatal aperture were linearly related with equal precision over the range of external potassium concentrations tested. One treatment without calcium was included and the results coincided exactly with this relationship.

The evidence indicated that the effect of calcium on stomatal opening at various potassium concentrations is being mediated by a change in potassium uptake and not

by, for example, effects of calcium on the elasticity of the guard cell wall. However, the small opening of stomata in distilled water (Fig. 4), and the effect of calcium on this opening, suggested that under such conditions there may be a non-potassium uptake component to stomatal opening and this may also be sensitive to calcium. Levitt (1969) has also pointed to this problem. On the other hand, guard cells can accumulate substantial amounts of potassium from very dilute solutions. The experiment shown in Figure 15 included treatments of supposedly zero external potassium (distilled water with and without 0·1 mM $Ca\,Cl_2$). The increase in stomatal aperture on these solutions for the light plus CO_2-free air period were 0·5 and 2·4 μm respectively. Extrapolating

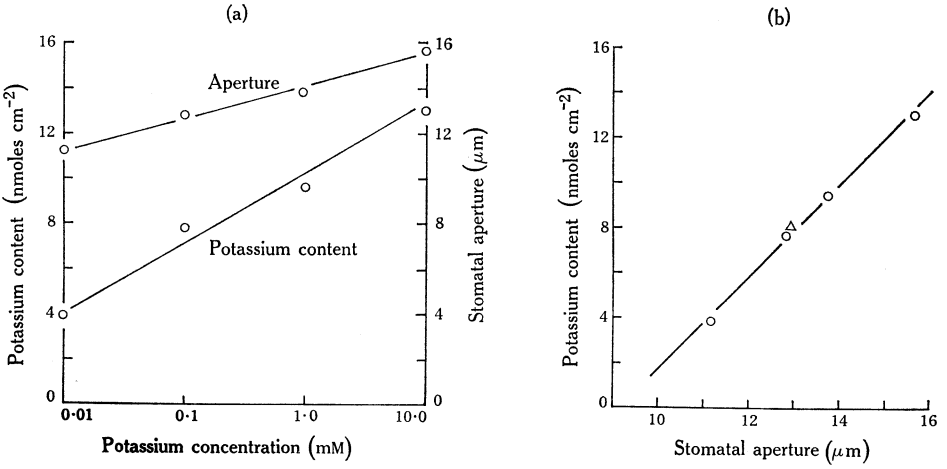

Fig. 15.—(*a*) Effect of external potassium concentration on stomatal aperture and content of potassium after 315 min in light plus CO_2-free air. Experiment 63; labelling with ^{42}K; all solutions with 0·1 mM $CaCl_2$; initial aperture 9·8 μm. (*b*) Relationship of potassium content to stomatal aperture for treatments of (*a*), including in addition data on treatment without 0·1 mM $CaCl_2$ (0·010 mM KCl alone, open triangle). Linear regression analysis; correlation coefficient = 0·996, significant at the 1% level.

the relationships of Figure 15, such increases suggest that the supposedly zero external potassium treatments actually contained approximately 0·003 mM potassium. Such concentrations of extraneous potassium are not considered unlikely although an alternative explanation still remains. This is that there is a non-potassium uptake component to opening at zero external potassium concentration but this component is completely suppressed at 0·010 mM external potassium concentration.

Staining for potassium overcame the above limitations of the isotope technique for measuring potassium contents and provided the clearest evidence that stomatal opening on distilled water and the associated response to calcium were in fact closely paralleled by changes in potassium content of the guard cells (Table 3). Other evidence had indicated that the potassium staining score was linearly related to guard cell potassium content (Fischer 1971) and a linear regression based on the mean results of Table 3, and including the initial reading, gave a correlation coefficient of 0·999 between stomatal aperture and guard cell stain score. Thus practically all of the variation in

aperture over the range zero to 10 mM external potassium in the presence and absence of calcium can be related to changes in guard cell potassium content.

The responses at zero external potassium of two replicates of experiment 74 (Table 3) with no intact epidermal cells were no different from those of the other two replicates which had moderate percentages of intact epidermal cells. Thus it seems unlikely that the potassium accumulated by guard cells in this condition came from neighbouring intact epidermal cells. The staining technique may not have detected potassium bound to the thick guard cell walls in the initial condition for, although the high concentration of sodium (3·7N) and low pH involved should have caused displacement and hence precipitation of this potassium, the extracellular precipitate would probably have been lost in washing of the strip. Plots based on tracer uptake (e.g. Fig. 15) would also have failed to detect such potassium because of the 10-min washout period involved. It is concluded that the potassium accumulated in the zero external potassium case arose from initially wall-bound potassium or very small concentrations of extraneous potassium in the supposedly zero potassium concentration solutions used, or both.

TABLE 3

STOMATAL OPENING IN *V. FABA* AND STAINING FOR POTASSIUM IN THE PRESENCE AND ABSENCE OF POTASSIUM AND CALCIUM

Experiment 74. "Final measurements" refer to measurements after 230 min in light plus CO_2-free air on the various solutions. Each result is the mean of 20 stomata (aperture) and two epidermal strips (stain score) for each of four replicates

Measurements	KCl concn. (mM)	Stomatal aperture (μm)			Potassium stain score*		
		No calcium	0·1 mM calcium	Mean	No calcium	0·1 mM calcium	Mean
Initial				8·0			5·5
Final	0	12·0	9·8	10·9	27·3	12·7	20·0
Final	0·10	12·2	11·4	11·8	32·7	14·4	23·5
Final	10·0	14·6	12·9	13·8	33·1	33·6	33·3
Standard error of mean		0·6	0·6		2·7	2·7	

* Percentage guard cell area.

Examining the time course of potassium uptake in the absence of calcium and in dilute KCl solutions (Fig. 16) it is seen that in both experiments the maximum uptake of potassium was approached at or before 300 min as was also the case with strips on 10 mM KCl+0·1 mM $CaCl_2$ (see also Fig. 13). Absence of calcium or the reduction of potassium concentration both appear to alter the final equilibrium content of potassium (and aperture) by changing the initial rates of net uptake of potassium rather than changing the times for which these rates proceed. The zero calcium treatment of Figure 16(*b*) is somewhat anomalous in that very rapid initial uptake of label was followed by a net loss of label after 100 min.

(d) General Occurrence of the Relationships of Uptake of Potassium to Aperture

From the preceding sections, it is apparent that the potassium content of stomata is closely related to the stomatal aperture in any given experiment. This applied where aperture varied in response to time of floating in light plus CO_2-free air (Fig. 13) and to external concentration of potassium and of calcium (Fig. 15). Many other experiments involving stomatal responses to other factors have given similar linear relationships between potassium content and stomatal aperture. Table 4 summarizes all experiments where content of potassium label in the strips could be expected to estimate guard cell potassium content, i.e. experiments with samplings after 100 min, or usually longer, on labelled potassium solutions. For no other reason were experiments excluded from Table 4. The correlation coefficients of Table 4 with the sole exception of experiment 38 are significant at the 5% level; many are so at the 1% level of probability or better. Usually a major portion (>80%) of the variation in stomatal aperture is linearly related to changes in potassium content.

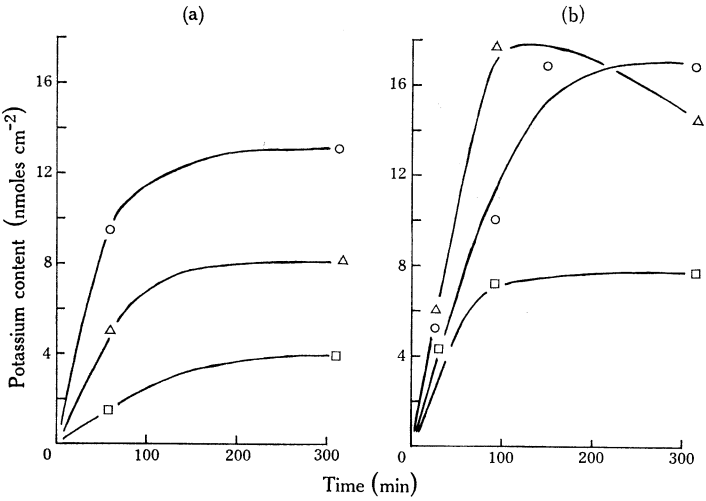

Fig. 16.—Time course of uptake of ^{42}K in light plus CO_2-free air and the effects of low potassium concentration and of zero calcium. (*a*) Experiment 63: ○ 10·0 mM potassium plus 0·1 mM calcium. △ 0·01 mM potassium. □ 0·01 mM potassium plus 0·1 mM calcium. (*b*) Experiment 84: ○ 10·0 mM potassium plus 0·1 mM calcium. △ 0·04 mM potassium. □ 0·04 mM potassium plus 0·1 mM calcium.

Excluding for reasons discussed later those four experiments where the aperture at zero potassium content estimated by extrapolation exceeded the initial aperture of the experiment and excluding experiment 38, the slopes of the regression lines in the remaining 16 experiments range from 2·0 to 3·5 nmoles cm^{-2} μm^{-1} with a mean value of 2·6 nmoles cm^{-2} μm^{-1} (standard error of mean is 0·10 nmoles cm^{-2} μm^{-1}). In 14 of these experiments ^{42}K (mean slope 2·6 nmoles cm^{-2} μm^{-1}) and in two ^{86}Rb (mean slope 2·7 nmoles cm^{-2} μm^{-1}) were used. Mean stomatal density for experiments ranged from 5500 to 7700 cm^{-2} (overall mean 6300 cm^{-2}) but this variation did not

appear to affect the slope parameter, probably because density appears to be inversely related to guard cell size.

In the 16 experiments considered above the aperture obtained by extrapolation at zero potassium content was less than the initial aperture recorded for the experiment, the difference in aperture ranging from 0·4 to 2·8 μm with a mean value of 1·4 μm. This very likely estimates that portion of the initial aperture which can be ascribed to the osmotic effects of the small amount of potassium initially present in the guard cells (3·4 nmoles cm^{-2} averaged over the 16 experiments).

TABLE 4

RELATIONSHIP OF POTASSIUM CONTENT OF EPIDERMAL STRIPS TO STOMATAL APERTURE IN *V. FABA*

Strips floated on isotopically labelled potassium solutions. Table includes all relevant experiments over 2 years; FCCP = carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone; DCMU = 3-(3,4 dichlorophenyl)-1,1-dimethylurea

Expt. No.	External factors used to vary aperture	Regression analysis*			Stomatal aperture (μm)	
		n	*r*	*b*	Initial	At zero potassium concn.†
B53‡	Light, potassium concn.	8	0·925	4·7	2·9	4·6
B56‡	Light, potassium concn.	8	0·911	2·3	1·8	2·5
B62‡	Light, time	10	0·978	3·1	1·6	1·2
36	Light, time	6	0·977	2·5	7·1	5·6
36	Light, transfer	4	0·981	2·6	7·1	6·9
37	Light, CO_2, 180 min	4	0·988	2·8	5·1	4·5
37	Light, CO_2, 400 min	4	0·995	3·5	5·1	4·2
38	Light, CO_2	4	0·856	1·3	5·0	3·1
43	FCCP, 350 min	12	0·838	2·5	5·6	2·8
43	FCCP, 900 min	6	0·977	2·5	7·7	5·5
44	DCMU, ouabain	9	0·979	2·1	5·0	3·6
45	Leaf source, time	7	0·932	2·8	10·2	8·3
45	Strip, time	5	0·950	2·2	6·1	4·2
46	Light, CO_2	4	0·982	2·7	8·2	5·7
63	Potassium concn., calcium	6	0·963	2·0	9·8	8·9
64, 65	DCMU, light, 105 min	12	0·815	2·4	7·4	5·8
64	DCMU, light, 270 min	12	0·923	2·8	6·7	5·5
71	FCCP, light	12	0·867	2·5	6·2	4·3
81‡	Potassium concn., calcium	6	0·987	2·3	6·6	5·9
84	Potassium concn., time	11	0·933	4·3	5·2	6·3
86‡	Time	6	0·991	5·6	6·4	8·1

* *n* = number of points from which the linear regression was determined; *r* = correlation coefficient from linear regression analysis; *b* = slope of the linear regression in nmoles cm^{-2} μm^{-1}.

† Obtained by extrapolating the linear regression back to a content of zero potassium in the guard cell.

‡ ^{86}Rb used; ^{42}K was used in the remaining experiments.

The four experiments excluded above on the grounds that the aperture at zero potassium content in the guard cells exceeded the initial aperture, all showed high correlations, but with one exception the slopes of the linear regressions were greater than 4·0 nmoles cm^{-2} μm^{-1}. Both phenomena could be the consequence of changes

in the physiology of the stomata (e.g. existence of a non-potassium uptake component in opening plus reduced sensitivity of aperture to potassium uptake). However, it is more likely that both resulted from systematic errors in the labelling of the potassium solutions used. In any case it was considered reasonable to exclude these results as being atypical until further information is obtained.

IV. Discussion

Results presented here have confirmed quantitatively the close relationship between stomatal opening and accumulation of potassium by guard cells that has been observed earlier by various workers. The range of opening factors under the influence of which such a relationship is found has been extended considerably. The very close linearity of the relationship was unexpected although the results of Sawhney and Zelitch (1969) for tobacco stomata also suggest a close linear relationship. In the case of *V. faba*, results (Fischer, unpublished data) suggest that the relationship between aperture and guard cell potassium will be modified somewhat for stomata *in situ* in a leaf by the effects of variable epidermal cell back pressure. Nevertheless potassium uptake by guard cells remains the major component for stomatal opening in light in leaves of *V. faba* (Fischer 1971).

For *V. faba* stomata in epidermal strips and without interference from epidermal cell back pressure, the linear relationship enabled a precise estimate of change in guard cell potassium concentration per micrometre increase in aperture (the regression slope). The method should be somewhat more accurate than earlier calculations (Fischer and Hsiao 1968) although the uncertainty as to guard cell volume still remains. Taking 5×10^{-9} cm^3 as the best estimate of volume per guard cell in *V. faba* (from microscopic measurement of dimensions of the living cell), the mean slope of 2·6 nmoles of potassium per square centimetre of epidermis per micrometre increase in aperture is equivalent to an increase of 41 mM in concentration of potassium in the guard cell (assuming mean guard cell density of 2×6300 per square centimetre of epidermis).

The osmotic equivalent of the above potassium accumulation, if accompanied by a univalent anion such as chloride, would be approximately 1·9 bars μm^{-1} at 30°C. If, on the other hand, the counter ion is an internally generated organic acid anion and therefore probably divalent at cell pH (e.g. malate), the osmotic equivalent would be 1·4 bars μm^{-1}. It is assumed that this anion would arise indirectly from the breakdown of guard cell starch, known to parallel stomatal opening under the experimental conditions (Fischer 1968). Results of double-labelling experiments with ^{36}Cl and ^{42}K (Fischer and Pallaghy, unpublished data) suggest that chloride uptake by guard cells is usually about one-quarter of their potassium uptake, with an internal organic acid anion implicated as the other counter ion. Thus the osmotic equivalent of the measured potassium accumulation would be approximately 1·5 bars μm^{-1}. This agrees reasonably well with various direct measurements of the increase in guard cell osmotic pressure with opening (Fischer 1972); the mean value was 2·0 bars μm^{-1} for *V. faba* stomata in epidermal strips and the relationship of aperture to osmotic pressure appeared to be also linear. Thus potassium plus an anion would seem to be the major osmotically active solutes in guard cells of open stomata.

Recent quantitative measurements of potassium in *V. faba* guard cells with the electron-probe microanalyser have led Humble and Raschke (1971) to a similar con-

clusion regarding the major osmotic role of potassium plus an organic acid anion. They estimated approximately 20 and 424×10^{-14} g-equivalents of potassium per guard cell pair of closed and open stomata respectively; results presented here, expressed on the same basis, were about 50 and 300×10^{-14} g-equivalents, respectively. Humble and Raschke (1971), however, measured smaller volumes per guard cell and a substantial change in volume with opening (closed $1 \cdot 3 \times 10^{-9}$ cm^3; open $2 \cdot 4 \times 10^{-9}$ cm^3). As a consequence their calculated potassium concentration in open stomata was considerably greater than reported here (about 900 mM *v.* 300 mM). However, in both studies calculated changes in concentration agreed fairly closely with measured changes in guard cell osmotic pressure; differences in cell volumes and concentrations may therefore reflect differences in the experimental material.

It is interesting to compare briefly the potassium accumulation system of *V. faba* guard cells with other ion-accumulation systems in plants. The guard cells have been shown here to increase their concentration of potassium in the presence of 10 mM KCl from 50 mM or less to as high as 300 mM in a period of 3 hr of illumination. This rate of osmotic work or ion accumulation appears to exceed that observed in other plant systems (roots, storage tissue, leaves, or whole plants). Although concentrations of potassium in leaf cells may reach 200 mM (Allen 1969; Pierce and Higinbotham 1970) and that of *Atriplex* epidermal bladder cells can exceed 300 mM (Osmond *et al.* 1969), it seems that rates of ion accumulation (net fluxes) in both leaf mesophyll cells and in bladder cells are relatively low. The high rates of net flux of potassium into guard cells (10–15 pmoles cm^{-2} sec^{-1} in light from 10 mM external solution) appear at present only to be exceeded under comparable conditions in certain marine algae (Gutknecht 1968). However, salt-transporting glands of the mangrove, *Aegialitis* sp., are known to excrete very concentrated solutions (450 mM) from a leaf with a xylem sap concentration of about 100 mM (Atkinson *et al.* 1967); extraordinarily high ion fluxes have been calculated for these glands.

Insufficient information was collected on the variation of initial rate of ion uptake with change in external ion concentration in order to permit examination of the stomatal system for absorption isotherms. However, it is apparent that stomata have a mechanism which can operate very effectively at low external potassium concentrations. If stomatal opening (and potassium accumulation) at 10 mM external potassium concentration is considered maximal, then half maximal opening (and potassium accumulation) is reached at less than 0·01 mM potassium under calcium-free conditions. This should assist guard cells in competing for whatever potassium is present in the leaf apoplast.

The linear relationship between steady-state potassium content of guard cells (after several hours illumination) and the log of external potassium concentration (Figs. 14 and 15) is in reasonable agreement with data of excised root systems (Jackson and Steif 1965; Pitman 1969). However, the lengthening of the uptake period which occurs in excised root systems as the external concentration of potassium is lowered (Pitman 1969) is not very evident in the stomatal system (Fig. 16). That steady-state potassium content in illuminated stomata resulted from reduced influx into the vacuole rather than stimulated efflux is in agreement with most studies of excised roots systems (Pitman, Courtice, and Lee 1968; Cram and Laties 1971). Reduced influx combined with low efflux would seem an efficient way of maintaining internal ion concentration

(Cram and Laties 1971). Again the closest parallel to stomata may be the osmotic pressure regulatory system of *Valonia ventricosa* described by Gutknecht (1968). There is a further parallel between the stomatal system and unicellular algae; since guard cells appear to have no symplastic connections with neighbouring cells (Meidner and Mansfield 1968) they can be also considered as isolated cells. This distinction could be important when making comparisons with excised tissues since the severing of the symplast involved in excision could lead to artificial effects (Pitman *et al.* 1971).

A major unique feature of potassium accumulation by *V. faba* guard cells is the consistent inhibitory effect of calcium over a wide range of potassium concentrations (0·005 mM to 50 mM). Most other higher plant systems show stimulatory effects of calcium on potassium uptake and especially retention, although there are some exceptions reported for excised roots (Hiatt 1970), stems (Rains and Floyd 1970), and leaves (Osmond 1968). Figure 14 does indicate that as external potassium concentration increases, the inhibitory effect of calcium is reduced, suggesting that potassium and calcium are competing in some way. The exceptionally thick wall of guard cells may enable calcium to exclude potassium from uptake sites at the plasmalemma.

Although this paper has concentrated upon stomatal physiology in *V. faba* there seems little doubt that potassium plays a similar role in at least some other species; there is now direct evidence for substantial movement of potassium into guard cells in the following: *Zebrina pendula* (Imamura 1943), *Commelina communis* and *Allium cepa* (Fujino 1967), tobacco (Sawhney and Zelitch 1969), and maize (Pallaghy, personal communication). Indirect evidence also supports the involvement of potassium in alfalfa (Cooper, Blaser, and Brown 1967) and sugar beet (Graham and Ulrich 1972). Some results indicate that sodium may be similarly involved in stomatal opening in *C. communis* (Willmer and Mansfield 1969*b*), *V. faba* (Pallaghy 1970), and *Kalenchoe marmorata* (Thomas 1970). Thus a new understanding of stomatal physiology, involving inorganic solute accumulation by guard cells, appears to be now well established.

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