THE REGULATION OF STOMATAL APERTURE IN TOBACCO LEAF EPIDERMAL STRIPS

I. THE EFFECT OF IONS

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Summary

A simple apparatus has been developed whereby the extent of stomatal opening on isolated epidermal strips can be monitored by measuring the flow rate of bathing solution through the stomata. This apparatus has been used to study the opening and closing response of stomata to various treatments. A supply of ions, particularly K^+, was found necessary to initiate and maintain the opening of stomata in the light. In the presence of K^+ stomata could be opened in the light and closed in the dark. This cycle could be repeated. The pattern of stomatal opening in the light and closing in the dark showed a similar shape with time to that shown for stomata on leaves. Similar results could be obtained in a bathing medium consisting of KH_2PO_4–Na_2HPO_4 buffer. The best pH found for opening was 8·0. No specificity of stomatal opening was found for the anion associated with K^+. The addition of Ca^{2+} or Mg^{2+} to the bathing medium caused reductions in aperture. The extent of stomatal opening in response to the concentration of K^+ in the range 0–10 mM gave a saturation curve with a concentration for half maximal opening of 0·32 mM. Increasing the concentration of K^+ past 10 mM resulted in reductions of aperture.

The response of stomata when only NaCl was present in the bathing medium was different from that when K^+ was present. Opening in the light was slower and in the absence of K^+ stomata would open in the dark. When stomata, opened in the dark in a NaCl solution, were exposed to light there was a reduction in aperture. The addition of small concentrations of K^+ to stomata opened in the dark in the presence of NaCl solution alone resulted in stomatal closure. The closing effect of light on stomata opened in the dark in the presence of NaCl solution alone could be converted to an opening effect after the addition of small concentrations of K^+. When epidermal strips with partially open stomata were quickly transferred to the bathing solution, KCl in the light and NaCl in the dark, further rapid stomatal opening could take place. Experiments with epidermal strips of *Kalenchoe marmorata*, a plant which opens its stomata in the dark and closes them in the light, indicate that the opening in the dark is caused by a diffusional influx of Na^+ in the dark, and closing in the light by a light-stimulated Na^+ efflux. The addition of ATP gave the same result as exposure to light. No sensitivity of *K. marmorata* stomata to K^+ was found.

The results are discussed with reference to the regulation of ion fluxes in plant and animal cells and how these might regulate stomatal aperture.

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It is considered that the opening in the light of stomata on tobacco epidermal strips is connected to a specific, light-stimulated, accumulating mechanism and that the permeability of the guard cell membranes increases as the cells swell. The equilibrium aperture reached is then determined by the balance between the influx and efflux of K^+.

I. Introduction

The concept that osmotic swelling of the guard cells is responsible for stomatal opening is probably best supported by the experiment of Heath (1938) in which he showed that the puncturing of a guard cell of an open stoma caused its shape to return to that typical of a closed guard cell.

Recently interest has been revived in earlier work which considered a possible relationship between the mechanism controlling stomatal opening and closing and the influx and efflux of ions in the stomatal guard cells (Fujino 1967; Slatyer 1967; Fischer 1968; Fischer and Hsiao 1968; Humble and Hsiao 1969; Willmer and Mansfield 1969).

Basic to these studies is the idea that a metabolically controlled influx of ions into the stomatal guard cells reduces the water potential in these cells with respect to the surrounding water which then moves into the guard cell down the water potential gradient and leads to swelling of the guard cells and opening of the stomatal pore. For many years it has been considered that the changes in water potential in the guard cell were associated with the interconversion of sugar and starch.

The interest in the mechanism regulating the stomatal aperture has been stimulated by recent theoretical considerations which indicate that a controlled reduction of the stomatal aperture may reduce transpiration to a greater extent than photosynthesis (Slatyer 1967).

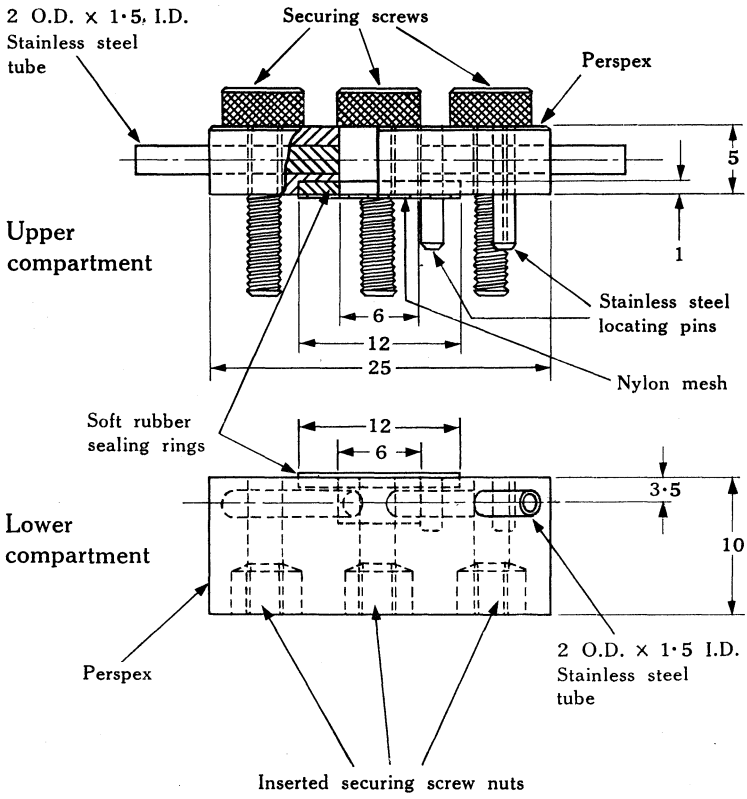
Much work has been carried out on the response of stomata on leaves to environmental changes such as the radiation flux, availability of water, and carbon dioxide concentration. These studies give little insight into the cellular mechanisms involved in the control of the stomatal aperture.

In this work a new method is used to study the response of stomata on bathed epidermal strips. The results using this method are compared to those obtained by other workers using epidermal strip material. The effect of ions, particularly K^+ and Na^+, on stomatal opening in the light and dark are reported.

II. Materials and Methods

To study the *in vitro* reactions of stomata on isolated epidermal strips, a Perspex solution-flow porometer was built (Fig. 1). It had an upper and lower compartment, each with stainless steel inlet and outlet tubes. Flat soft rubber sealing rings were inserted in the lip of both the upper and lower chamber. The opening of the upper chamber was covered with a nylon mesh. Epidermal strips were carefully dissected from the under surface of the third or fourth leaf, numbered down from the unfolded apical leaves of tobacco (*Nicotiana tabacum* L. cv. Virginia Gold).

The plants were grown in a glasshouse at temperatures controlled between the range 21–27°C. In a few experiments epidermal strips were obtained from other species of plants also raised in a glasshouse. The epidermal strip was floated, cuticle surface uppermost, in a Petri dish containing the basal solution to be used in an experiment. The strip was then floated over the submerged lower chamber of the solution-flow porometer and the level of the liquid in the Petri dish lowered so that the strip rested on the rubber sealing ring on the surface of the lower



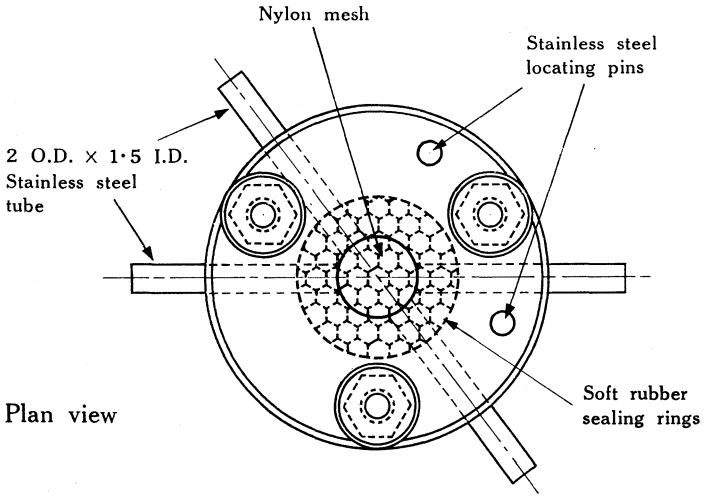


Fig. 1.—Construction details of the solution-flow porometer. All measurements given in millimetres.

chamber. The upper chamber was then bolted to the lower so that the epidermal strip separated the solution contained in the upper and lower chambers. The solution-flow porometer was clamped above the stage of a binocular microscope. The inlet to the lower chamber was connected by plastic tubing to a constant-head bottle. Both upper and lower chambers were flushed with the basal solution to be used in an experiment after which the outlet to the lower chamber and the inlet to the upper chamber were closed so that any flow of solution had to pass through the stomata of the epidermal strip. Almost all experiments were started in the dark to see whether there was a normal light response in stomatal opening and as a check against leakage through the epidermal strip due to small tears or holes that might be made in the strip during preparation.

The opening and closing of the stomata were then monitored by measuring the flow rate through the strip. This was done by collecting the flow in small volumetric flasks and recording the volume collected over a given time interval, usually 10 min. No automatic means of measuring the flow rate, such as by measuring the rate of convective heat loss from a heated thermistor, were found to be stable enough to give accurate results. A relation between flow rate and stomatal aperture was obtained. When a constant flow rate through the stomata was recorded, the epidermal strip was quickly removed from the solution-flow porometer by refloating it on the solution used in the Petri dish and the mean aperture was determined by the method of Fischer and Hsiao (1968). A regression analysis gave a relation between the stomatal aperture, A, and the flow rate, F, of $A = 10 \cdot 7F^{0 \cdot 39}$, $n = 27$ with a correlation coefficient of $0 \cdot 79$.

A continuous check of the distance between two marker stomata during an experiment indicated that there was no stretching or shrinking of the epidermal strip as a whole during the course of an experiment.

The advantages of using the solution-flow porometer method over that of microscopically measuring the stomatal aperture of a sample of stomata on a section of epidermal strip at given time intervals, the method that has been used previously, seem to be:

(1) An almost continuous record can be made of time of the response of stomata to changes in the experimental conditions.

(2) All experimental treatments can be carried out on the same epidermal strip.

(3) The response of a greater number of stomata is sampled, i.e. approximately $2 \cdot 8 \times 10^3$ stomata are exposed in the solution-flow porometer.

The disadvantages are:

(1) It would be difficult to study the effect of a large number of treatments at the same time.

(2) It would be difficult to run simultaneous independent controls.

It seems that the two methods are essentially complementary.

When used, light was supplied by a 50 W, 8 V tungsten projector lamp. Unless otherwise stated the total flux used in the 400–3000 nm waveband was $22 \cdot 8$ mW cm^{-2} and $4 \cdot 4$ mW cm^{-2} in the waveband 400–700 nm, i.e. photosynthetically active radiation. This flux was found to be more than adequate for the light saturation of stomatal opening. Flux measurements were made using a differentially filtered solarimeter.

Experiments were carried out in a constant-temperature room set at 22°C; all experimental solutions were equilibrated at this temperature. The temperature of the solution-flow porometer was maintained within +1°C of this temperature by blasting air across it by means of a high-speed fan. All solutions used were prepared using distilled water that had been equilibrated with air and then freshly deionized to remove carbon dioxide.

As with the results of Seidman and Riggan (1968) it was found that stomata opened more quickly, reached larger apertures, and the ouabain sensitivity was greater in epidermal strips prepared from plants grown from mid-spring to mid-autumn than in those grown in winter. Hence most experimental work was done during the mid-spring to mid-autumn period.

III. Experimental

(a) *Effect of KCl*

Figure 2 indicates that illuminated closed stomata on a tobacco epidermal strip show little tendency to open when the strip is supplied only with distilled deionized water, but when the strip is supplied with 1 mM KCl, stomatal opening is initiated

and maintained. Therefore, the opening of stomata in the light is associated with the presence of the K^+ ion and closing follows darkening. Opening in the light and

Fig. 2

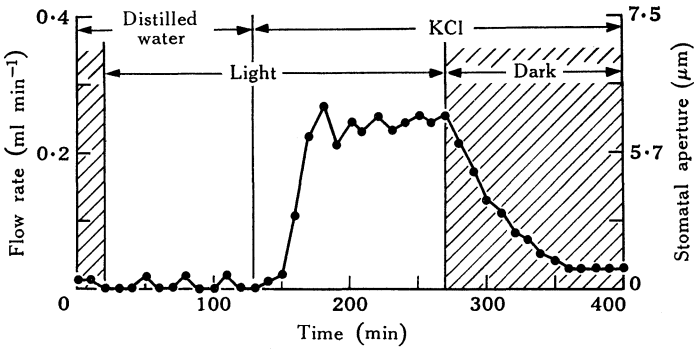


Fig. 3

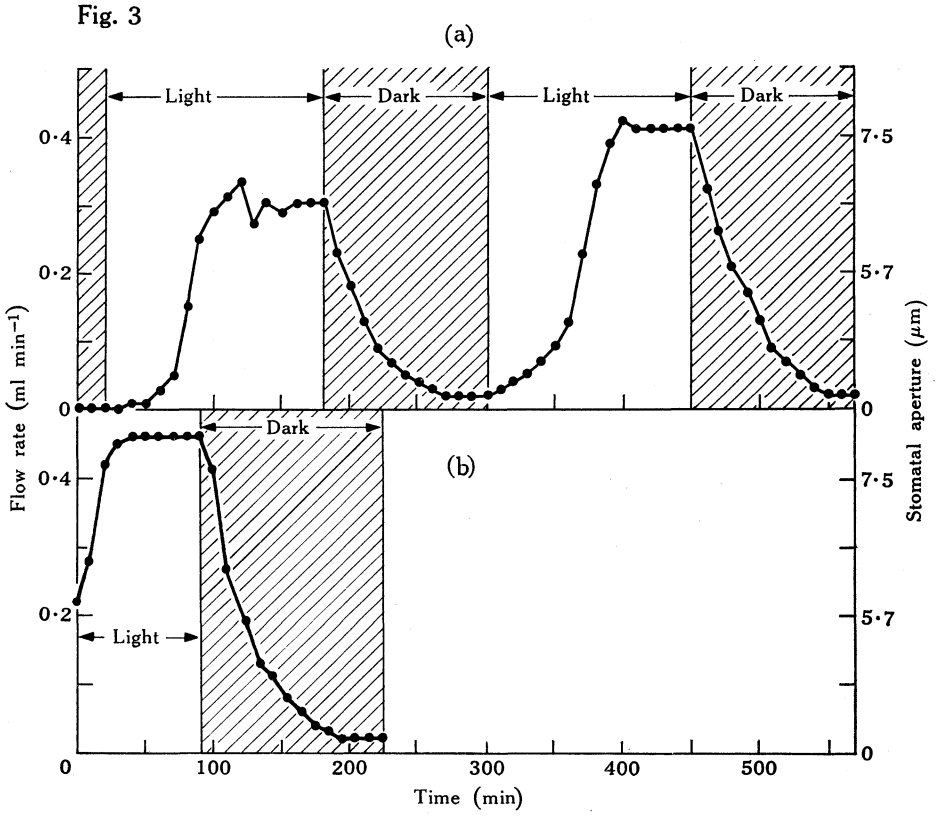


Fig. 2.—Effect of the addition of 1 mM KCl on stomatal opening.

Fig. 3.—Tobacco epidermal strips bathed in 10 mM KCl. Effect of light and dark on stomata initially closed (*a*) and on stomata initially partially open (*b*).

closing in the dark was the first response of stomata observed with a change in environmental conditions.

The result is similar to that of Fujino (1967) who showed that there was no stomatal opening on isolated illuminated epidermal strips of *Commelina communis* unless KCl was supplied, and of Fischer (1968) who found that in epidermal strips of *Vicia faba* only KCl was essential for stomatal opening, the presence of Tris–maleate buffer or 0·5 mM $CaCl_2$ together with KCl making little difference to the stomatal opening obtained in the light. Similar results have been obtained by Fischer and Hsiao (1968) and Humble and Hsiao (1969).

Figure 3(*a*) shows that stomata on tobacco epidermal strip bathed in 10 mM KCl can be opened in the light and closed in the dark and that the stomata will respond to these changes over a period of 9 hr. Similar results have been obtained by Humble and Hsiao (1969) for stomata on *V. faba* epidermal strips.

Use of the solution-flow porometer enables the course of stomatal opening and closing to be followed more closely with time than by methods such as the microscopic determination of stomatal aperture on samples of epidermal strips taken at half-hourly to hourly intervals.

The time course of opening of stomata on tobacco epidermal strip bathed in 10 mM KCl on illumination shows many similarities to stomatal opening on leaves (e.g. Raschke 1966). The pattern of opening shows in general a sigmoidal type curve with time. There is an initial slow "transient phase" of opening followed by a rapid "steady-state" phase, and then a slowing down before an "equilibrium state" of opening is reached. Often stomata show a series of "oscillations" in opening which are "damped" with time before an equilibrium opening is attained [e.g. Figs. 2 and 3(*a*)]. Oscillations with a similar time period have been found in the opening of stomata on *Zea mays* leaves (Raschke 1966). Oscillations in stomatal aperture were also observed after changes in the environmental conditions. It has been found by Kuiper (1961) that the stomatal apertures of a bean leaf show oscillations with a mean period of about 9 min.

Once stomatal opening has started, the half-time for opening lies in the range of 20–40 min. Similar half-times have been found for the opening of stomata on *Z. mays* leaves (Raschke 1966).

The slow initial transient phase shown on the opening of essentially closed stomata was lost when an epidermal strip on which the stomata were already open to a considerable extent was quickly exposed to 10 mM KCl in the light [Fig. 3(*b*)]. This effect will be discussed later in relation to the considered increase in guard cell membrane permeability that could occur with the swelling of the guard cells and stretching of the membranes [see Sections III(*h*) and IV].

(*b*) *Effect of pH and Phosphate Buffer*

It was found that the stomata on tobacco epidermal strip could be opened in the light and closed in the dark in a bathing solution consisting of KH_2PO_4–Na_2HPO_4 buffer. Willmer and Mansfield (1969) showed that the stomata on *V. faba* epidermal strip will open in the light while bathed in 10 mM KH_2PO_4–K_2HPO_4 buffer. The stomata on epidermal strips of *C. communis* will not open in 66·6 mM phosphate buffer unless 66·6 mM KCl is also added (Fujino 1967; Willmer and Mansfield 1969).

The equilibrium stomatal apertures on tobacco epidermal strip in phosphate buffer at pH 6·0, 7·0, and 8·0 were 3·3 (55%), 5·2 (87%), and 6·0 μm, respectively, the percentage opening at the two lower pH's relative to that at pH 8·0 being given in parenthesis. The relationship between pH and stomatal opening is similar to that shown by stomata on *C. communis* epidermal strips bathed in 66·6 mM KH_2PO_4–K_2HPO_4 buffer plus 66·6 mM KCl (Willmer and Mansfield 1969).

In buffers consisting of ions that can be absorbed it seems impossible to determine whether the change in aperture is due to the pH change or to the change in ion concentration, i.e. at the pH's used the concentration of K^+ in the KH_2PO_4–Na_2HPO_4 buffer are 37·1, 11·2, and 1·4 mM at pH 6·0, 7·0, and 8·0 respectively. It will be shown later that increasing the K^+ concentration in the bathing medium past 10 mM causes a reduction in aperture in tobacco epidermal strip stomata. It was found by Fischer (1968) that in epidermal strips of *V. faba* incubated on a solution of 10 mM KCl buffered in the pH range 6–8 by Tris–maleate buffer the stomata showed little change in aperture with respect to the pH of the incubating solution.

(c) *Effect of Divalent Ions*

As with stomata on epidermal strips of *C. communis* (Fujino 1967; Willmer and Mansfield 1969), the presence of Ca^{2+} at a concentration of 1 mM effectively causes complete closing of the stomata of tobacco epidermal strips bathed in 10 mM KCl solution and opened in the light. The equilibrium stomatal apertures were 1·7, 2·7, 3·8, and 6·4 μm for $CaCl_2$ concentrations of 1·0, 0·5, 0·1, and 0 mM respectively, representing percentage inhibitions of 74, 58, 40, and 0 respectively. This differs from the response to Ca^{2+} of stomata on *V. faba* epidermal strips where Ca^{2+} at a concentration of 0·5 mM (Fischer 1968) and 1·0 mM (Willmer and Mansfield 1969) did not reduce or increase the stomatal aperture on epidermal strips floated on KCl solution.

The effect of Ca^{2+} on the uptake of ions by cells is not well understood. Various workers have shown that the presence of Ca^{2+} may either stimulate the uptake of K^+ (Elzam and Hodges 1967), have no effect (Johansen, Edwards, and Loneragan 1968), or decrease its uptake (Elzam and Hodges 1967; Grignon and Salec 1969). The effect of Ca^{2+} on K^+ uptake differs in different species. In maize roots it stimulates while in soybean roots it depresses K^+ uptake (Kahn and Hansen 1957).

It seems that the range of response shown by stomata from different plants to the presence of Ca^{2+} may be similar to the effect of Ca^{2+} on K^+ uptake in different plant species.

Mg^{2+} was also found to reduce stomatal aperture, the addition of 0·5 mM reducing the aperture by 37% (Thomas, unpublished data). This is similar to the findings of Willmer and Mansfield (1969) who found that 1·0 mM reduced the final aperture by 50% in *C. communis* epidermal strips. In the same species Fujino (1967) found that Mg^{2+} caused no reduction in stomatal aperture until the external concentration reached 10 mM.

(d) *Effect of Change in Anion Associated with K^+ on Stomatal Aperture*

Figure 4 shows the effect on stomatal aperture of changing the bathing solution from 10 mM KCl to 5 mM K_2SO_4. As found by Fischer and Hsiao (1968) there is only a small change in aperture on substituting sulphate for chloride. It seems

that the anion associated with K^+ makes little difference to stomatal opening in tobacco epidermal strips as it has already been shown that stomata will open when the only anion present is phosphate. The anions Cl^-, Br^-, and NO_3^- associated with K^+

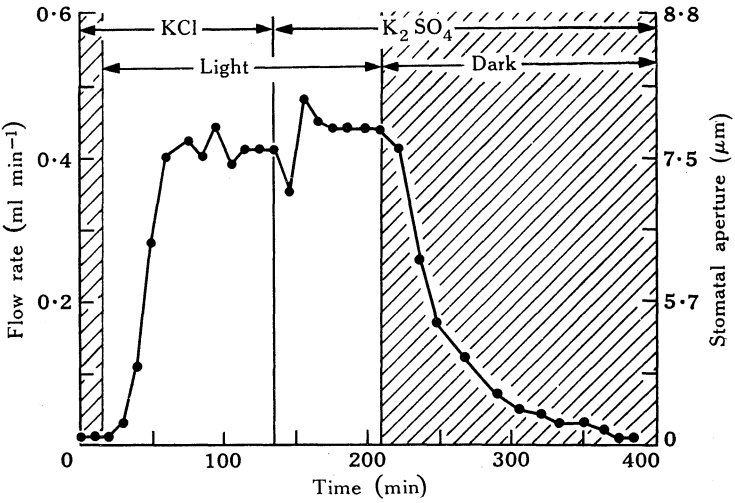


Fig. 4.—Effect of change in anion in the bathing medium from Cl^- (10 mM KCl) to SO_4^{2-} (5 mM K_2SO_4) on stomatal aperture.

did not give differences in stomatal aperture on *V. faba* epidermal strips (Humble and Hsiao 1969). These results indicate that the influx of K^+ is not connected to a specific anion uptake process.

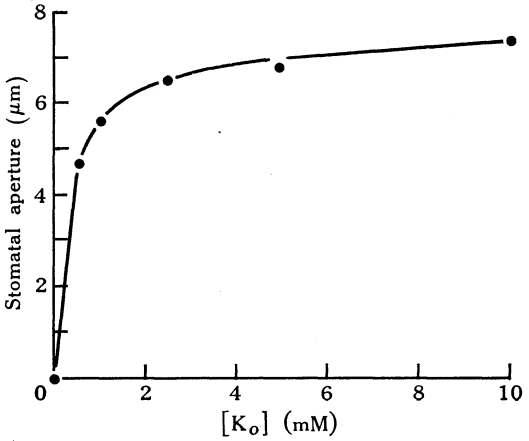


Fig. 5.—Effect of concentration of K^+ in the bathing medium on stomatal aperture.

(e) Opening of Stomata in Response to Varying K^+ Concentration in the Bathing Solution

Figure 5 shows the response of stomatal aperture in the light to increases in K^+ concentration supplied to the epidermal strip in the range 0–10 mM. When this type of saturation curve is obtained in relation to ion uptake by tissues and cells with

increasing concentration it is considered that there may be a specific mechanism, e.g. a pump, associated with the transfer of ions across the cell membrane. The concentration to give half maximal stomatal opening is 0·32 mM. This value lies

Fig. 6

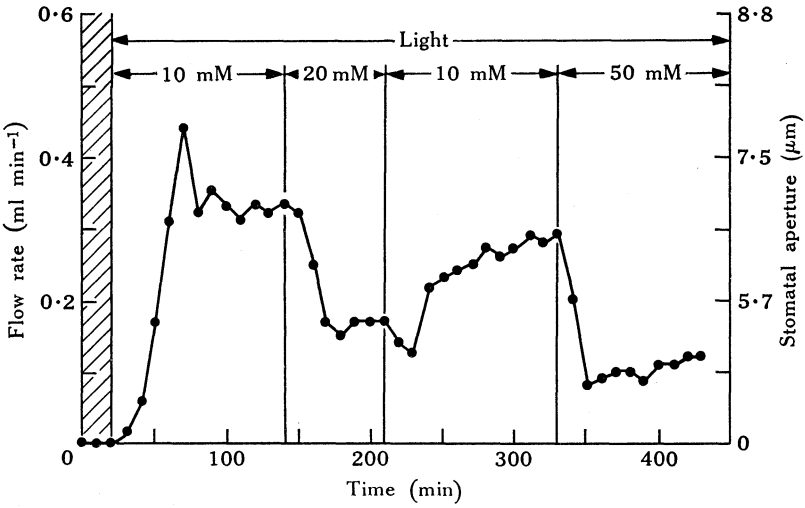
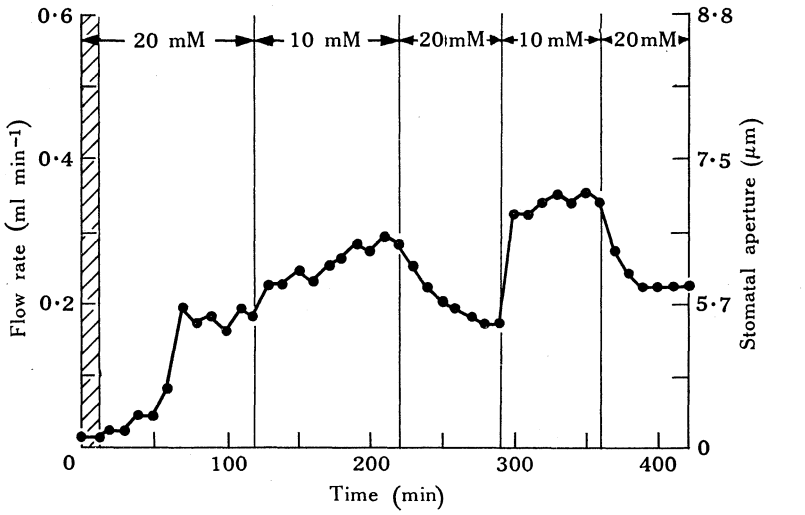


Fig. 7



Figs. 6 and 7.—Effect of varying the concentration of KCl in the bathing medium from 10 to 20 mM (Fig. 6) and from 20 to 10 mM (Fig. 7).

between the value for the high-affinity mechanism of 0·02 mM and the low-affinity mechanism of 17·3 mM for the absorption of K^+ by barley roots as defined by Epstein (1966).

Similar results in relation to K^+ concentration and stomatal aperture have been obtained by Fischer and Hsiao (1968) and Humble and Hsiao (1969) using *V. faba* epidermal strips. Using $^{86}Rb^+$ as a tracer for K^+, Fischer (1968) showed that the increase in aperture of stomata on *V. faba* epidermal strips could be correlated to the amount of K^+ taken up by the guard cells.

Considering the above results and those of Section III(*a*) it seems that the opening in the light of stomata on epidermal strips of *V. faba* and tobacco is associated with a light-stimulated K^+ uptake process.

Figure 6 shows the result of a change in concentration from 10 to 20 mM and shows that this decreases the aperture, while reverting to a concentration of 10 mM increases the aperture. Raising the concentration to 50 mM causes an even more marked decrease in aperture. Figure 7 shows that stomata on tobacco epidermal strip will open in the light in a 20 mM KCl bathing solution, but that decreasing the concentration to 10 mM results in an increase in aperture and returning to a concentration of 20 mM again decreases the aperture.

Similar results were obtained when 50 mM KCl was added to and subsequently removed from stomata on tobacco epidermal strip opened in the light while being bathed in a solution of 17·6 mM phosphate buffer, pH 8·0. With the buffer alone the equilibrium stomatal aperture was 8·0 μm. When 50 mM KCl was added and then removed the aperture decreased to 5·6 μm (70% of initial) and then increased to 7·2 μm (90% of initial), respectively.

The stomatal aperture on *V. faba* epidermal strips showed little change when the K^+ concentration in the bathing medium was 10–100 mM, reduction in aperture only occurring when the concentration was increased past 100 mM (Fischer 1968), though Pallaghy (personal communication) has found that increasing concentrations of K^+ decrease the stomatal aperture in *V. faba* epidermal strip material.

It has been found by Etherton (1967) that increases in the external concentration of K^+ result in an increased efflux of K^+ from oat and pea roots. In *Chlorella pyrenoidosa* (Barber 1968), *Chara australis* (Hope and Walker 1960), and tobacco guard cells (Pallaghy 1968) it has been found that increasing the external K^+ concentration results in a depolarization of cell membranes. Depolarization produced by increases in the external K^+ concentration causes an increased permeability of squid axon membranes that leads to an increased efflux of K^+ (Sjodin and Mullins 1967).

In tobacco the closing response of stomata to increasing external K^+ concentrations above 10 mM around the guard cells may be important in decreasing stomatal aperture with a decreasing water supply to the plant. With such a restriction the internal concentration of K^+ could increase and result in a reduction of stomatal aperture.

(*f*) *Effect of Na^+ on Stomatal Opening*

Figure 8 compares the reaction in the light and dark of stomata on tobacco epidermal strips taken from the same leaf and bathed in either 10 mM KCl or 10 mM NaCl over the same period of time. The response shows marked differences. Opening when the epidermal strip is bathed in 10 mM NaCl is much slower and the aperture

reached over the same time period is much less. The opening in the light in 10 mM NaCl proceeds in small steps and remains constant over relatively long periods and does not show the rapid rise, once stomatal opening has started, which is a feature of the opening when the bathing medium is supplied with K^+. On darkening, the

Fig. 8

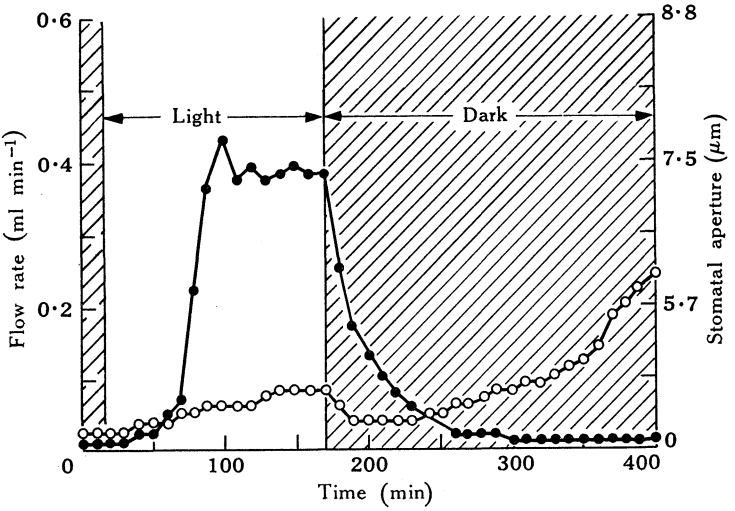


Fig. 9

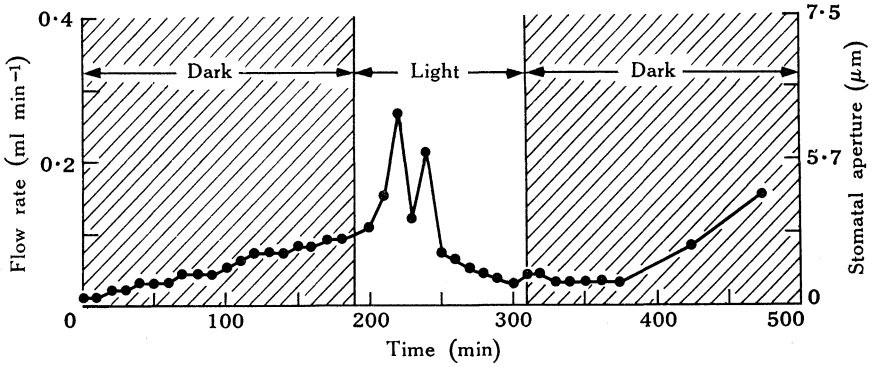


Fig. 8.—Effect of a 10 mM KCl (●) or 10 mM NaCl (○) bathing medium in light and dark on stomatal opening.

Fig. 9.—Effect of exposure to light on stomata opened in the dark and bathed in 10 mM NaCl.

stomata bathed in NaCl show a small initial decrease in aperture which remains constant for some time and then is followed by a slow increase in aperture, whereas in the KCl solution once the aperture is reduced in the dark there is no subsequent increase in aperture. This indicates that in a bathing medium supplied with K^+, at least at concentrations not greater than 10 mM, there is a specific opening pattern

in the light and closing pattern in the dark. This difference in response of stomatal opening when supplied with K^+ or Na^+ may indicate that Na^+ is entering the guard cell by a slow diffusion process, whereas K^+ is entering by at least a facilitated diffusion mechanism if not an active one.

In a study of the effect of monovalent cations on stomatal opening in epidermal strips of *V. faba* in the light, Humble and Hsiao (1969) found that only K^+ and Rb^+ stimulated near maximal opening at concentrations around 10 mM. Increasing concentrations of Li^+, Cs^+, and Na^+ resulted in small steady increases in aperture, but even at external concentrations of 100 mM the increase was less than that attained in 10 mM K^+ or Rb^+.

The high specificity of stomatal opening for K^+ in *V. faba* epidermal strips was considered by Humble and Hsiao (1969) to indicate that the guard cells must have an ion uptake mechanism with the highest specificity for K^+ shown in higher plants.

Nevertheless Willmer and Mansfield (1969) have shown that larger apertures in epidermal strips of *C. communis* can be obtained when these are supplied only with 66·6 mM NaCl compared with 66·6 mM KCl. Using the same species Fujino (1967) reports that NaCl did not support stomatal opening.

Differences in the response of stomata to K^+ or Na^+ may depend on the species [Section III(*k*)].

(*g*) *Effect of Light after Na^+-supported Opening in the Dark*

The stomatal opening that occurs in the dark when epidermal strips are bathed in 10 mM NaCl can be reduced on exposure to light. Two distinct effects on exposure to light were found. One effect (Fig. 9) showed a rapid increase in aperture after illumination, then a rapid decrease in aperture followed by another increase and decrease in aperture. These fluctuations then die down followed by a steady decrease in aperture which is maintained while the epidermal strip is illuminated. In other cases illumination resulted in a steady decline in aperture [cf. Fig. 11(*b*)]. Similar results were obtained either by exposure to light or by the addition of ATP to a NaCl solution in which the stomata had opened. ATP reduced the stomatal aperture and prevented any further increase in the light or dark (Thomas, unpublished data).

Such results suggest that there might be an energy-requiring Na^+ efflux mechanism present in tobacco guard cells similar to that found in many other plant cells, or that when the guard cell membranes are supplied with a readily available energy substrate such as ATP, changes are brought about in the guard cell membranes which enable them to discriminate between Na^+ and K^+. This discrimination could either lower the influx of Na^+, increase its efflux, or do both and result in a decreased aperture. Where exposure to light results in an initial transitory stimulation of stomatal opening (Fig. 9) it seems that a transitory stimulation of Na^+ influx could occur.

(*h*) *Effect on Stomatal Opening of Bathing Epidermal Strips having Partially Open Stomata with NaCl*

When an epidermal strip with partially open stomata was exposed to a 10 mM NaCl in the dark, a further rapid increase in aperture resulted. Exposure to the light after this rapid increase only caused a transitory small reduction in aperture

[Fig. 10(*a*)]. In other cases [Fig. 10(*b*)] exposure of epidermal strip with partially open stomata led to an initial reduction in aperture, though prolonged exposure to NaCl again resulted in stomatal opening. Exposure to light when the stomatal opening was quite large after exposure to NaCl again only resulted in a transitory small reduction in aperture. Even when large stomatal openings have occurred when strips are bathed with NaCl the addition of ATP to the solution causes a marked reduction in aperture and no further stomatal opening occurs while ATP remains in the bathing solution (Thomas, unpublished data).

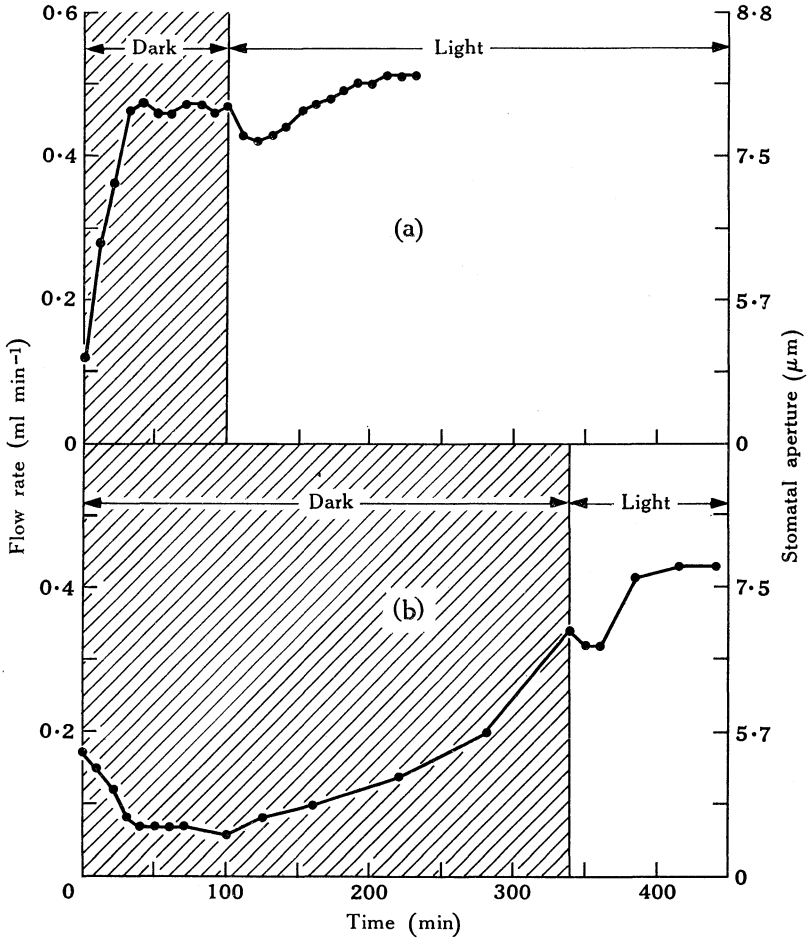


Fig. 10.—Showing effect of exposing partially open stomata to 10 mM NaCl and resulting in further opening (*a*) or closing (*b*).

These results have been interpreted to mean that the permeability of the guard cell membranes increases with the swelling of the guard cells and that at a certain critical stage of stomatal opening the permeability of the membranes is increased to an extent where discrimination with respect to the entering ion is lost and ions can flood into the guard cell.

(*i*) *Effect of Adding K^+ to Na^+-stimulated Stomatal Opening*

The effect of the addition of K^+ even at a concentration of 0·1 mM to the stomatal opening which results when epidermal strips are bathed in NaCl in the dark is to reduce the aperture [Fig. 11(*a*)].

The addition of K^+ also resulted in no further increase in aperture, even after 4 hr. This time period in the presence of Na^+ alone could be expected to give an increase in aperture.

The reduction in the Na^+-supported stomatal opening on the addition of K^+ might take place as a result of an exchange diffusion process in which internal Na^+ is exchanged for external K^+. If this is so it seems that the exchange could not be

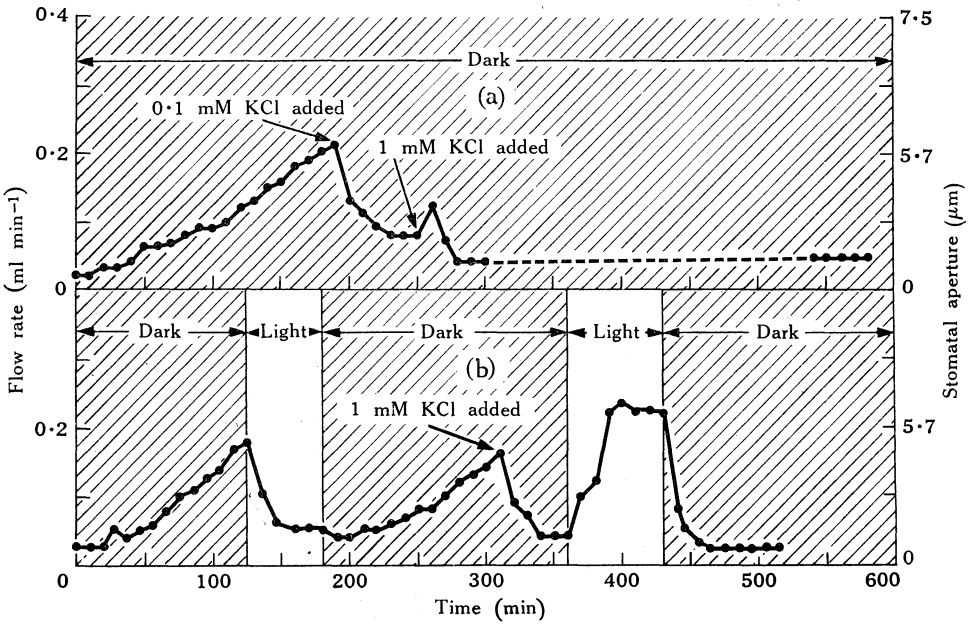


Fig. 11.—Stomata opened in the dark while bathed in 10 mM NaCl solution. (*a*) Effect of adding KCl. (*b*) Effect of light and dark on opening before and after addition of 1 mM KCl.

a one for one exchange of Na^+ for K^+ because if it were so no change in aperture should be expected. It seems more likely that K^+ plays some role in maintenance of membrane integrity and selectivity and that in the presence of K^+ either the influx of Na^+ is substantially reduced or the efflux of Na^+ is increased. The presence of K^+ could effect the membrane permeability to Na^+ by altering either or both the electric mobility of Na^+ in the membrane and the Na^+ partition coefficient. The membrane potential may also be reduced in the presence of K^+ (Barber 1968).

(*j*) *Effect of Light on the Na^+-stimulated Opening in the Dark of Stomata in the Absence and Presence of K^+*

Figure 11(*b*) shows that the closing effect, which occurs when stomata opened in the dark in the presence of Na^+ are exposed to light, can be reversed to give stomatal opening after 1 mM K^+ has been added to the bathing solution. It shows that K^+

is required for the normal light-opening and dark-closing process to occur and that K^+ can bring this about in the presence of an excess of Na^+.

(*k*) *Sodium in Relation to Stomatal Opening in* Kalenchoe marmorata *Epidermal Strip*

It was thought that the opening of stomata in the dark and closing in the light shown by stomata on tobacco epidermal strip when only Na^+ was present in the bathing medium might be a mechanism whereby certain plants, e.g. plants showing a crassulacean acid metabolism, open their stomata during the night and close them during the day. This might occur if the light-stimulated, K^+-specific mechanism was absent in the guard cells of these plants.

Figure 12 shows the response of stomata on epidermal strip of *K. marmorata*, a plant known to open its stomata in the dark and close them in the light (Nishida 1963).

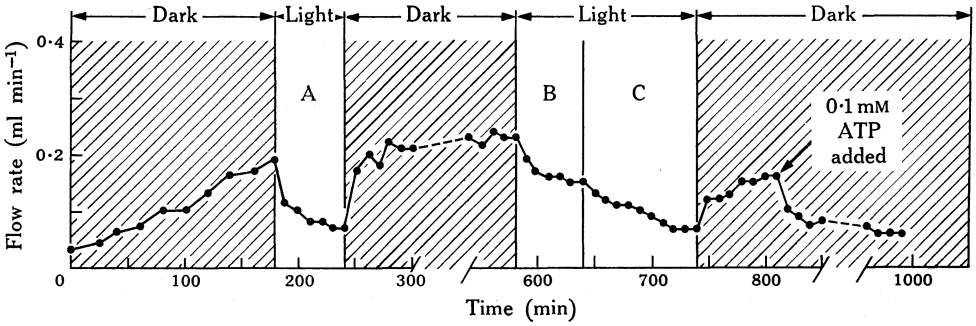


Fig. 12.—Effect of light, dark, and ATP on the opening of *Kalanchoe marmorata* stomata bathed in 10 mM NaCl. Light fluxes at *A* and *B* were 22·6 mW cm^{-2} and at *C* 33·4 mW cm^{-2}.

It shows that in 10 mM NaCl solution the stomata open in the dark and close in the light and that increasing the incident flux increases the stomatal closure. The addition of 0·1 mM ATP is also effective in causing and maintaining closure. Similar results were obtained when ATP was added to tobacco stomata opened in an NaCl solution (Thomas, unpublished data). In three experiments the stomata on *K. marmorata* epidermal strips showed no response in light or dark in a 10 mM KCl bathing solution. Though more experiments are needed to ascertain with certainty that a Na^+-geared mechanism is responsible for the dark-stimulated opening of *K. marmorata* stomata and those of other species which show dark-stimulated opening and light-stimulated closing of stomata, it is interesting to consider that stomata which show this characteristic may do so as a result of a diffusional influx of Na^+ into the guard cells in the dark. This may occur as a result of the increase in negative charge in the guard cells associated with organic acid synthesis. In the light the increased supply of energy substrate available through the action of photosynthesis, e.g. ATP, stimulates a Na^+ efflux mechanism which leads to stomatal closure.

IV. Discussion

From the results of the experiments reported and those of other workers, e.g. Fischer (1968), it is considered that stomatal opening in tobacco epidermal strips is connected to a specific, selective, light-dependent K^+ influx mechanism into the guard cells. K^+ is also probably required by the guard cell membranes to enable them to maintain their integrity and normal differential permeability, e.g. the addition

of small concentrations of K^+ to stomata that have opened in the dark in the presence of NaCl alone results in closure [Section III(*i*)].

It seems that the selectivity of the influx mechanism is a property of the cell membrane because Mitchell (1959, 1961), Keynes (1961), and Glynn (1959) show that compounds that are apparently actively transported into cells are free to move in the cytoplasm and exert their full osmotic effect. Nevertheless there is a contemporary school of thought (e.g. Ling 1962; Ling and Cope 1969), which considers that there are no transport mechanisms located in the membrane, and that distribution ratios of ions and molecules which are higher or lower than unity that exist between the cell and the extracellular fluid are entirely the result of differential binding of compounds by some cellular constituents. Changes in cell volume would then be determined by configurational changes brought about as a result of the binding with these compounds. This would be similar to the hypothesis put forward by Scarth (1926) in which he considered that stomatal opening was connected to the imbibitional swelling of amphoteric colloids.

If an osmotic mechanism is considered as the basis for the swelling of guard cells which leads to stomatal opening, both the movement of solute and solvent must be considered, together with possible mutual interaction of solute and solvent molecules and the interaction of both solute and solvent with and within the cell membrane and the effects of these interactions on the permeability of the membrane. It has been shown by Kedem and Katchalsky (1958, 1961) that the rate and direction of movement of a solvent if it occurs simultaneously with that of the solute can result in a change of permeability of the cell membranes because if solute and solvent interact within the membrane, solute and solvent molecules will exert a frictional drag on one another, resulting in a mutual effect on the movement of solute on solvent and solvent on solute. It is well known that water uptake can be influenced to different degrees by different types and concentrations of ions, at concentrations too low to significantly affect the osmotic driving force (Slatyer 1967).

The effects that ion concentration and type of ion could have on the uptake of water may help to explain the effects of divalent ions [Section III(*c*)], the effect of increasing K^+ concentration greater than 10 mM [Section III(*e*)], and the effect of small concentrations of K^+ on Na^+-supported opening [Section III(*i*)] on reductions in stomatal aperture.

Ca^{2+} may reduce stomatal aperture by either decreasing the influx of K^+ or water or both or increasing their efflux from the guard cells. Ca^{2+} could decrease the influx by blocking or partially blocking the path of entry of K^+ or water or both through the membrane into the cell, or may have direct effects on membrane structure which either decreases the influx or increases the efflux. In cells with elastic walls Ca^{2+} may cross-link various wall components and increase the rigidity of the cell. In a study of the effect of Ca^{2+} on stomatal aperture on epidermal strips of *V. faba*, Pallaghy (1970) found that at concentrations of 10 mM KCl or NaCl the addition of Ca^{2+} reduced the light-stimulated opening in NaCl, but at higher concentrations (50 mM) stomatal opening was increased in NaCl with respect to the openings obtained in the KCl solutions. From his results he considered that Ca^{2+} alters the specific binding of ions to a site responsible for light-stimulated ion uptake into the guard cells.

On the basis of a model in which the extent of stomatal opening depends at any time on the amount of K^+ present in the guard cell, the effect that increasing the con-

centration of KCl past 10 mM has in reducing the aperture [Section III(*e*)] would mean that the content of K^+ in the guard cell is reduced. This could occur through (1) a decrease in influx, the efflux remaining unaltered; (2) an increase in efflux, influx remaining unaltered; or (3) changes in both efflux and influx so that the net flux is decreased. It has been found by Etherton (1967) that increases in the external concentration of K^+ result in an increased efflux of K^+ from oat and pea roots. Etherton considered that the efflux of K^+ brought about by increasing the external concentration was a result of stimulation of an active efflux process, for the electrochemical activity of K^+ in the cells was lower than that in the surrounding solution. Poole (1969) has considered that the external concentration-dependent efflux of K^+ across red beet cell membranes is the result of a stimulated exchange diffusion process where K^+ is exchanged for H^+ via a common transport mechanism. It does not seem that a cation-exchange process could account for the swelling or shrinking of guard cells because a net loss or gain of ions is necessary to cause a change in the water potential.

Increasing concentrations of K^+ may also have direct effects on the characteristics of the guard cell membranes which influence the uptake of water into the cell. From the effect that low concentrations of K^+ have in closing stomata opened while bathed in 10 mM NaCl [Sections III(*i*) and III(*j*)], there is an indication that K^+ is not only connected with the normal light-opening response of stomata but may also play a role in regulating the permeability of the guard cell membranes as it does in *Chara* (Hope and Walker 1960).

In tobacco epidermal strips the stomatal opening which occurs in the light and dark in the presence of only 10 mM NaCl is slower and shows a different pattern from that when K^+ is present in the bathing medium. In the presence of K^+ no opening in the dark occurs. This would suggest that Na^+ is entering the guard cells by a different path from that of K^+ and that it is only with K^+ that there is a specific light-stimulated influx. The opening that occurs in the presence of NaCl alone may be due to a slow diffusion of Na^+ into the guard cells, the membranes being not entirely impermeable to Na^+ in the absence of K^+. The stomatal opening that occurs in the dark in the presence of Na^+ alone can be reversed by light provided the stomatal opening has not become too great, i.e. when the guard cells are probably flooded with Na^+ [Section III(*h*)]. This would indicate that there is a light-stimulated Na^+ efflux mechanism present in the guard cells. The opening that occurs in the light in the presence of NaCl alone may be due to insufficient fuelling of the Na^+ efflux mechanism by light under the experimental conditions because the addition of ATP to the bathing solution causes stomatal closure in the presence of Na^+ alone even when large openings have occurred (Thomas, unpublished data). Nevertheless, in the absence of K^+ the K^+ influx mechanism may show a low affinity for Na^+.

Considering the experiments covered in this work it seems likely that the initial opening in the light of stomata on tobacco epidermal strip material is connected to a specific light-stimulated K^+ influx mechanism (cf. Fujino 1967; Fischer 1968; Fischer and Hsiao 1969; Humble and Hsiao 1969). There is evidence from experiments with *V. faba* that the extent of stomatal opening can be quantitatively related to the amount of K^+ taken up by the guard cells (Fischer 1968). The response of stomatal aperture to increasing K^+ concentration in the range of 0–10 mM [Section III(*e*) and Fischer (1968)] suggests that the K^+ influx mechanism is at least a process of facilitated diffusion and that the influx may be brought about by a

specific "carrier mechanism". Once stomatal opening has reached a certain level, e.g. when partially open stomata are exposed to 10 mM KCl [Section III(*a*)] or 10 mM NaCl [Section III(*h*)] the permeability of the guard cell membranes may increase with the swelling of the cells. An increase in permeability with swelling might be expected on physical grounds as a result of the associated stretching of the membranes or due to the increased concentration of ions in the cytoplasm. With an increase in permeability ions may move rapidly into the guard cells. At this stage diffusion may become important in controlling the influx and efflux of ions. The driving force for the influx of cations under these conditions might be either the generation anionic charges in the guard cell (Pallaghy 1970) or the counterbalancing of fixed intracellular negative charges. Also at this stage selectivity for K^+ may be lost, e.g. Na^+ ions can cause further rapid opening of partially open stomata [Section III(*h*)]. In other excitable tissues, e.g. nerve, there is an increased influx of Na^+ during excitation and a stimulated Na^+ efflux on return to the resting state. It was considered by Bernstein (1902) that during excitation, numerous "pores" opened up in the nerve membranes that allowed indiscriminate penetration of small ions such as Na^+ and Cl^-. Once the intracellular negative charges are balanced the increased permeability of the membrane would facilitate the efflux of ions and the guard cell content of K^+, and hence stomatal opening at any time would be determined by the balance between the influx and the efflux.

If diffusion has a role in stomatal opening in NaCl solutions [Sections III(*f*) and III(*h*)] or in the reduction of opening in more concentrated KCl [Section III(*e*)] and in governing the steady-state level of opening, the electrochemical potential gradient for K^+ and Na^+ must be considered under the prevailing conditions to determine if the flux is passive. Even if there is a net influx or efflux of one of these ion species, it will be necessary to determine whether or not anions accompany the cations.

The possibility that there is a specific light-activated carrier mechanism and an energy supply associated with the mechanism has been investigated. The effect of the bicarbonate ion on the stomatal opening mechanism has also been studied. This work will be reported later.

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