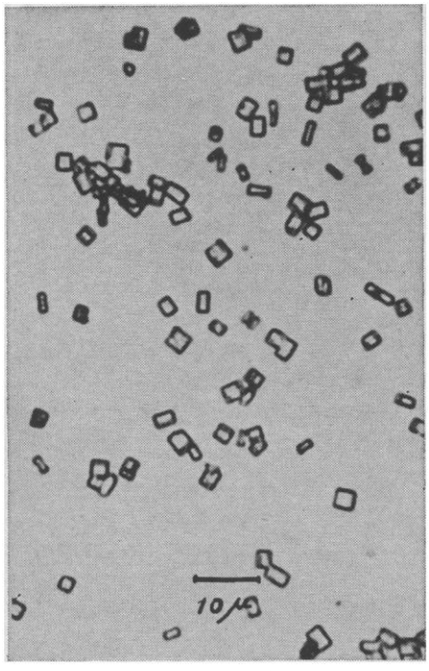

Fig. 1. Crystals from the cocoons of *Malacosoma neustria testacea.*

The crystals originate from the contents of Malpighian tubes and have erroneously been considered to be uric acid by other workers (*6*). The yellow pigment is assumed to be impregnated after the crystallization of calcium oxalate within the lumen of the tube. The oxalate amounts to more than 5 mg per individual. The indications are that this compound is concerned with the regulation and excretion of calcium by this insect.

EIJI OHNISHI
SUSUMU Y. TAKAHASHI
HARUYUKI SONOBE
Biological Institute, Faculty of Science, Nagoya University, Nagoya, Japan
TSUYOSHI HAYASHI
Tokyo Institute of Technology, Tokyo, Japan

References and Notes

1. J. Dewitz, *Zool. Jahrb. Physiol.* **38**, 365 (1921).
2. These analyses were done at the laboratory of Dr. K. Yamazaki, to whom our thanks are due.
3. Product of Katayama Chemical Co.; grade extra pure; lot No. 670113. We thank Drs. Y. Hirata and S. Takata for infrared spectrophotometry.
4. E. S. Dana and C. S. Hurlbut, in *The System of Mineralogy*, C. Palache, H. Berman, C. Frondel, Eds. (Wiley, New York, ed. 7, 1951), vol. 2, pp. 1099–1101.
5. American Society for Testing and Materials, x-ray powder data file, card No. 16-379 (1966).
6. R. G. Busnel and A. Drilhon, *Compt. Rend. Soc. Biol.* **135**, 1009 (1941); R. Gerstaecker, *Sitz. Ber. Ges. Naturforsch. Freunde Berlin*, 138 (1873), cited in V. B. Wigglesworth, *The Principles of Insect Physiology* (Methuen, London, ed. 6, 1965), p. 519.
7. We thank Dr. S. Fukuda for valuable suggestions.

4 March 1968

Stomatal Opening: Role of Potassium Uptake by Guard Cells

Abstract. *Stomata in isolated epidermal strips open in response to light plus air free of carbon dioxide when the strips are floated on potassium chloride solutions of low concentrations. This opening depends on the stimulation of active accumulation of potassium in quantities sufficient to account for the observed changes in solute potential of the guard cells.*

This study of stomatal opening in isolated epidermal strips provides new evidence that opening is due to active accumulation of potassium by guard cells. Essential to the relevance of this finding was the prior demonstration that stomata in such strips are living and respond normally to natural stimuli such as light and changing concentration of CO_2; normal opening had not been demonstrated during extensive earlier work with epidermal strips (*1*, *2*).

Abaxial epidermal strips (0.3 by 0.4 cm) of *Vicia faba* were used, being largely free of entire mesophyll cells although some mesophyll cell fragments and chloroplasts usually adhered to the strips. The proportion of epidermal cells remaining intact, judged by accumulation of neutral red and appearance of the protoplasm, was kept below 20 percent and did not appear to influence stomatal movement. Almost all guard cells were living as judged by accumulation of neutral red and presence of protoplasmic streaming.

Stomata in strips floated on certain solutions responded to light plus CO_2-free air in the same manner as did unisolated stomata (Table 1); response applied to changes in aperture, guard-cell solute potential, and guard-cell starch content (determined by scoring after iodine staining). The treatment with light plus CO_2-free air was used because it is generally thought that the opening by light of stomata depends partly on the reduction in concentration of intercellular CO_2, and thus that the light response of stomata in isolated epidermal strips is not fully expressed unless the CO_2 concentration of the environment is reduced similarly. For convenience, CO_2-free air (<0.0001 percent CO_2 by volume) was used; in fact other experiments (*3*) showed that, although stomata in strips responded independently to light and to CO_2-free air, only with both factors together did stomatal apertures equal those of unisolated stomata under comparable conditions.

These results demonstrate normal function of stomata in isolated epidermal strips and justify further studies with this system. It was found that the only component of the buffer solution (Table 1) that was essential for the opening of isolated stomata in light plus CO_2-free air was KCl; without KCl the opening response is slight (Fig. 1). Replacement of potassium with calcium inhibited opening completely, whereas substitution of sulphate for chloride reduced opening by only 30 percent. The important component thus appears to be potassium.

For investigation of the importance of the concentration of potassium (Fig. 1), the opening under light plus CO_2-free air was saturated by approximately 10 m*M* KCl; opening occurred in the dark with normal air also, but required 100 m*M* KCl for saturation. Stomatal opening was markedly reduced with KCl at 200 or 400 m*M*.

The essentiality of potassium and the form of the response to changes in its concentration suggested that stimulated uptake of potassium was implicated in stomatal opening. This uptake was

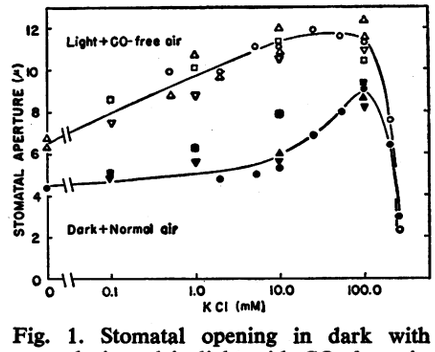

Fig. 1. Stomatal opening in dark with normal air and in light with CO_2-free air, and the response to concentration of KCl. Light intensity, 2150 mphot; temperature, 31° to 34°C; stomata measured after 180 minutes in light or dark. All solutions contained 2.5 m*M* tris-maleate buffer and 0.5 to 0.7 m*M* Ca^{2+}. Results of various experiments, each designated by a different symbol and involving five to 16 replications; all results were corrected to give identical mean apertures in dark plus normal air and zero KCl (that is, 4.4 μ). Before correction, mean aperture under this condition ranged from 2.9 to 5.7 μ for the various experiments; correction involved the addition of the difference between 4.4 μ and this value for each experiment to the mean stomatal aperture at each other concentration of KCl in the experiment. Initial stomatal apertures averaged 1.0 μ less than the final values in the dark with normal air and zero KCl.

measured by use of rubidium-86 as a tracer (*4*). Epidermal strips entirely free of intact mesophyll cells, and containing less than 5 percent of intact epidermal cells, were used in order to ensure that uptake was solely into guard cells.

The uptake of potassium into an unexchangeable form, estimated by use of rubidium-86 and tested over the range of external concentrations of KCl from 0.1 to 100 m*M*, increased with increasing concentration of potassium and was stimulated by light plus CO_2-free air, especially at KCl concentrations of 1 to 10 m*M*. For example, in one experiment for which the KCl concentration was 6.5 m*M*, strips in light plus CO_2-free air took up an average of 20.5×10^{-3} μmole of potassium per square centimeter of strip; for corresponding strips in the dark plus normal air the mean uptake was 5.4×10^{-3} μmole/cm^2 (standard errors, 1.5 and 0.5×10^{-3} μmole/cm^2, respectively). Summary of all experiments made it apparent that the uptake of potassium closely paralleled the concomitant increase in stomatal aperture (Fig. 2). Figure 2 shows also that the data for dark and for light, each of the two conditions involving various concentrations of KCl, were similarly related; this finding suggests that the same basic mechanism of opening operated under both conditions.

The assumption, based on the small fraction of intact epidermal cells, that all the potassium uptake was in guard cells is further supported by the facts that epidermal cells, containing no chloroplasts, are unlikely to be involved in the light-stimulated uptake of potassium, and that strips completely lacking intact epidermal cells showed comparable uptake of potassium. Thus, knowing uptake per square centimeter of strip, stomatal density (6200/cm^2), and guard-cell volume, one could calculate the corresponding increase in the concentration of potassium in the guard cell. An increase in stomatal aperture of 7 μ (from, for example, 4 to 11 μ) was associated with an uptake of at least 20×10^{-3} μmole/cm^2 (Fig. 2). When one considers that guard-cell volume was approximately 5×10^{-9} cm^3 when the mean stomatal aperture was 11 μ, this uptake amounted to an increase in guard-cell potassium of about 0.3*M*. If one assumes an equivalent uptake or formation of anion, this increase represents a decrease in solute potential of about 12 bars (*5*), a figure agreeing well with that determined by the plasmolytic method for similar changes in aperture (Table 1).

Table 1. Stomatal openings and guard-cell solute potentials in isolated epidermal strips and leaf disks of *V. faba* in the dark plus normal air (dark) and in light plus CO_2-free air (light). Light intensity, 2150 mphot; temperature, 31° to 32°C. Stomatal apertures were measured microscopically after 150 to 240 minutes in light or dark; initially they were slightly open (about 2 μ); solute potential was determined by the plasmolytic method, epidermal strips being floated for 25 to 45 minutes on graded sucrose solutions. Six replications.

Openings (μ) in		Potentials (bars) in	
Dark	Light	Dark	Light
	Leaf disk on water		
1.6	10.0	−8.2	−16.2
	*Strip on buffer**		
3.8	11.0	−6.3	−16.2
	Difference for significance†		
2.0	2.0	1.9	**6.1**

* Mixture of 2.7 m*M* tris-maleate buffer (*p*H 6.0), 2 m*M* $CaCl_2$, and 25 m*M* KCl. † Five-percent level of probability.

These results strongly suggest that the basic mechanism of stomatal opening, in response to light plus CO_2-free air, is stimulation of the uptake of potassium; thus is provided the first clear evidence supporting the hypothesis of solute accumulation for stomatal opening (*6*, *7*). Moreover, stimulation by light of the uptake of potassium by other green plant cells has been reported (*8*). After using chemical staining, workers (*9*, *10*) have reported relatively high concentrations of potassium in guard cells; Fujino (*10*) showed correlation between its concentration and stomatal aperture, and concluded that stomatal opening involved the active accumulation of potassium; his conclusion is confirmed by my estimates of the quantities of potassium taken up.

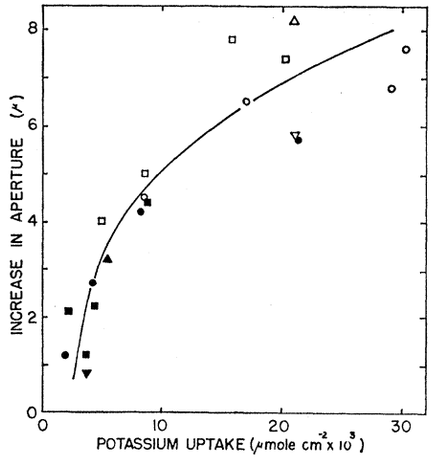

Fig. 2. The relation, during a 3-hour period, of increase in stomatal aperture to uptake of potassium; estimated from uptake of rubidium-86. Results from various experiments are designated by different symbols; open symbols indicate data from light plus CO_2-free air; solid symbols, dark plus normal air. Conditions as for Fig. 1; change in stomatal aperture obtained by subtraction of the initial aperture from the final.

I imply that the conversion of starch to sugar, which is considered to be part of the classic mechanism of stomatal opening (*2*, *6*, *11*), is of secondary importance, since the uptake of potassium accounted for the observed decrease in guard-cell solute potential. In fact the amount of stainable guard-cell starch showed good inverse correlation with stomatal aperture in these experiments (*3*). Nevertheless I consider that starch changes are secondary, although perhaps closely linked, to uptake of potassium. Such linkage may reflect formation of organic acid anions as the products of starch hydrolysis. Epidermal strips of *V. faba* should be very useful for further elucidation of the mechanism of stomatal opening.

R. A. FISCHER*
Laboratory of Plant-Water Relations, Department of Water Science and Engineering, University of California, Davis

References and Notes

1. I. Mouravieff, *Le Botaniste* **40**, 195 (1956); **41**, 271 (1957); P. J. C. Kuiper, *Plant Physiol.* **39**, 952 (1964); J. E. Pallas, Jr., *Quart. Rev. Biol.* **41**, 365 (1966).
2. O. V. S. Heath, in *Plant Physiology*, F. C. Steward, Ed. (Academic Press, New York, 1959), vol. 2, pp. 193–250.
3. R. A. Fischer and T. C. Hsiao, in preparation. The work includes the effects on stomata of metabolic inhibitors, *p*H, and other factors.
4. D. W. Rains and E. Epstein, *Australian J. Biol. Sci.* **20**, 847 (1967).
5. If one assumes that the solute involved is KCl, the molal lowering of the freezing point for KCl at 0.5 molal is 3.3°C [*Handbook of Chemistry and Physics* (Chemical Rubber Publ. Co., Cleveland, Ohio, ed. 37)]. It is assumed also that the change in solute potential equals $-(12.0 \times T)$ bars, where T°C is the lowering of the freezing point. The change in solute potential, represented by an increase in KCl concentration of 0.3*M*, is therefore approximately $-(12.0 \times 0.3 \times 3.3)$ bars, or −12 bars.
6. H. J. Ketellapper, *Ann. Rev. Plant Physiol.* **14**, 249 (1963).
7. I. Zelitch, *Biol. Rev.* **40**, 463 (1965).
8. E. A. C. MacRobbie, *Australian J. Biol. Sci.* **19**, 363 (1966); D. W. Rains, *Science* **156**, 1383 (1967).
9. S. Imamura, *Japan. J. Botany* **12**, 251 (1943).
10. M. Fujino. *Kagaku* **29**, 147, 424, 660 (1959); **30**, 89 (1960); *Sci. Bull. Fac. Educ. Nagasaki Univ.* **18**, 1 (1967).
11. J. Levitt, *Planta* **74**, 101 (1967).
12. Aided by the Office of Water Resources Research, U.S. Department of the Interior (grant B-029-CAL), and the Water Resources Center, University of California (grant to T. C. Hsiao and R. C. Huffaker). I thank especially T. C. Hsiao and D. W. Rains for advice.

* Present address: Research School of Biological Sciences, Australian National University, Canberra, A.C.T.

29 March 1968