Plant Physiol. (1969) 44, 1350-1354

Short Communication

Direct Determination of Potassium Ion Accumulation in Guard Cells in Relation to Stomatal Opening in Light

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Received April 23, 1969.

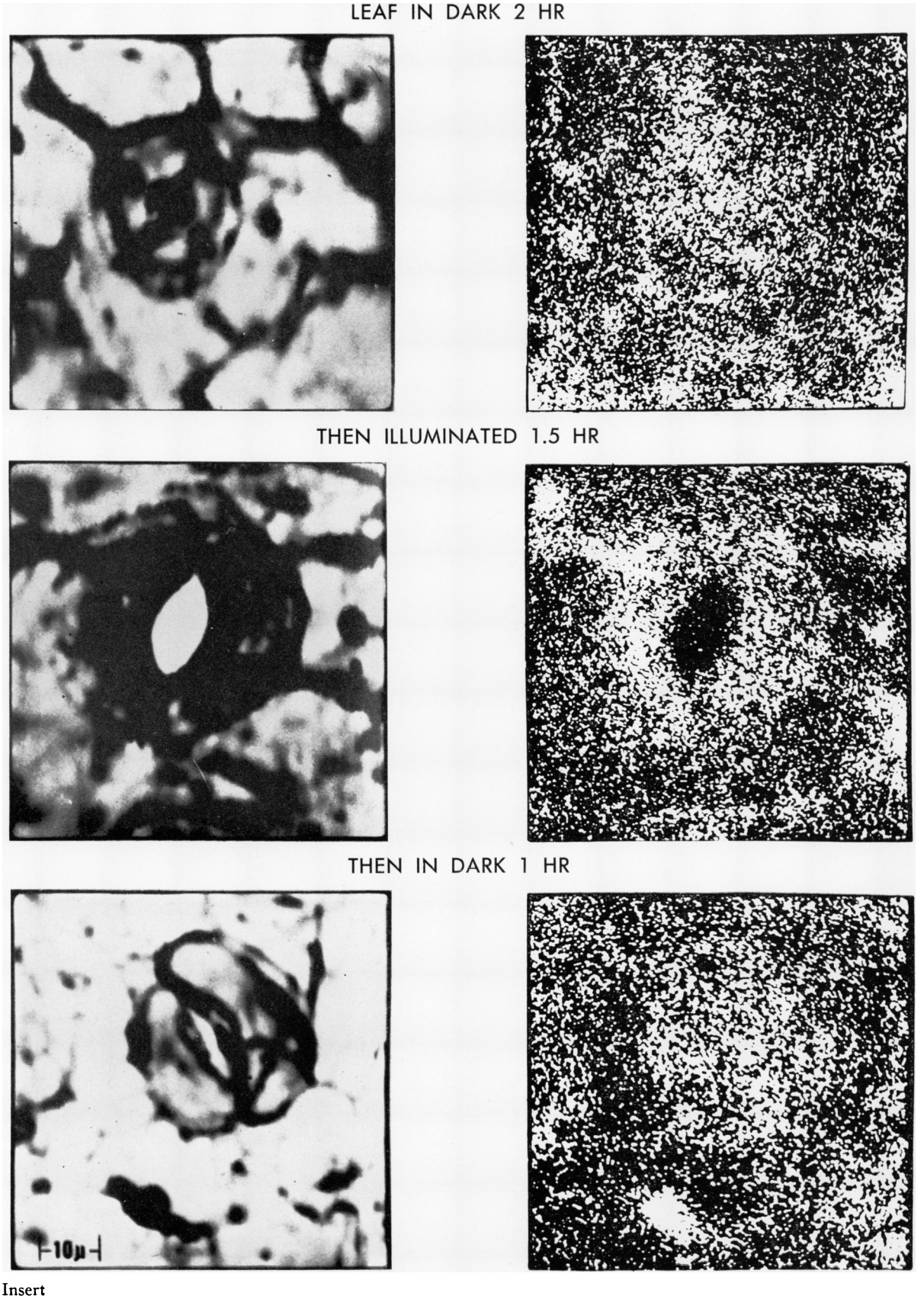
Understanding the mechanism of stomatal opening in leaves is important because stomata are the avenues for CO_2 and H_2O diffusion. Stomata in most species open in the light when the guard cells that encompass the pores take up water and increase in turgor relative to adjacent epidermal cells (1). Thus stomatal opening is likely an osmotic phenomenon that depends upon the accumulation of solute in the guard cells, and during opening this accumulation has been reported to range from 0.1 M to 0.5 M in various experiments (2). For over 100 years botanists have believed that the primary solute accumulating in the light was soluble carbohydrate produced during photosynthesis, because guard cells possess chloroplasts and other epidermal cells do not. However careful quantitative work [reviewed in (3)] failed to provide strong support for this view.

Recent independent evidence from 2 laboratories suggests that the accumulation of potassium ions in guard cells provides the essential solute for stomatal opening. Fujino (4) used cytochemical staining methods and found that open stomata in several species contained more potassium in their guard cells than closed stomata. Opening of stomata in epidermal strips of *Commelina communis* in the light was stimulated by added potassium salts, and qualitative tests showed that the potassium concentration increased in the guard cells. Fischer (5-7) showed that potassium and rubidium salts stimulated opening in epidermal strips of *Vicia faba*. Calculations made from the uptake of $^{86}Rb^+$ by such tissues indicated that if all of the accumulation were in the guard cells there would be sufficient solute (with an accompanying anion) to account for stomatal opening. Thus, although precise quantitative data were lacking, it seemed plausible that stomatal opening was mediated by a potassium ion pump that accumulated these ions in the guard cells (8).

We have carried out a quantitative test of this hypothesis on intact leaf tissue by taking advantage of the electron microprobe technique, which permits an accurate determination of the concentration of an element in a 1 to 2 μ diameter region (9). Essentially, a beam of electrons of desired diameter is focused on the specimen surface. The chemical elements irradiated by the beam emit their characteristic X-ray spectra. The emitted X-rays are then analyzed according to wavelength and intensity, permitting both qualitative and quantitative chemical analysis of micron-sized volumes.

Tobacco leaves (*Nicotiana tabacum*, variety Havana Seed) were excised from greenhouse grown plants and placed in darkness for several hr so that the stomata closed. Leaf disks 3.2 cm in diameter were cut with a sharp punch and floated upside down on water in the air at 30° in 1000 ft-c illumination. Following illumination the disks were left in the dark for various periods. After these treatments, portions of the lower epidermis were quickly stripped off. They were placed on a glass slide and rapidly

FIG. 1. Specimen morphology (left) and potassium X-ray images (right) of closed and open tobacco stomata obtained by scanning with an electron beam. The images of freeze-dried tissue were obtained with an Acton microprobe. To minimize loss of potassium by volatilization the electron beam current was adjusted to 50 nA on a reference mineral, scheelite. The voltage used was 20 KV. All exposures were for 608 sec. The intensity and number of white spots indicate that potassium accumulates precisely in the guard cells of the illuminated open stoma. Fewer white spots surrounding the guard cells of the open stoma as compared to the completely closed stoma suggest a depletion of potassium in the adjacent epidermal cells during opening.



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frozen by immersion in a mixture of dry ice and isopentane for about 30 sec (10). The tissue was then freeze-dried under a high vacuum for about 20 min. Control experiments showed that the mean stomatal widths in the freeze-dried preparations were similar to those in silicone rubber impressions (11) made before freezing.

Approximately 1 mm^2 samples of freeze-dried tissue were then mounted on a carbon block with a little Apiezon N stopcock grease. Samples were then covered by evaporating a thin layer of carbon upon them and the concentrations of potassium, calcium, and phosphorous in guard cells, as well as in the surrounding epidermal cells, were determined simultaneously with an electron microprobe. Further details are given in the legend to Fig. 1.

For qualitative estimates, a 60 μ × 60 μ area containing a pair of guard cells enclosing a stoma was scanned with an electron beam. The characteristic X-radiations displayed on an oscilloscope were photographed. These X-ray images represent the relative distribution of the element in the tissue scanned, since the concentration is directly related to the number and intensity of the white spots. Congruent display pictures of absorbed electrons were also obtained and illustrate the micromorphology of the tissue. The 2 pictures together show where the elements are (Fig. 1).

For the quantitative estimation of an element, the electron beam was focused on a fixed 1 to 2 μ diameter region of tissue and the emitted X-radiations were measured as counts per 10 sec. This brief exposure time was used because exposures longer than 50 sec with a focused electron beam decreased the potassium concentration slightly by volatilization. Counts were corrected for background radiation of the carbon block (10 counts per 10 sec) and were then converted to molar concentrations from a standard curve obtained from impregnated membrane filters prepared as follows: 5 μl of 0.05 M to 0.50 M KCl solution was placed in the center of a 2.5 cm diameter Gelman membrane filter (Type AM-5, mean pore size 0.65 μ). The solution uniformly wet an area about 0.8 cm in diameter within a few seconds. The membrane filter was quickly frozen and freeze-dried and the potassium concentration measured with the electron microprobe. The thickness of the membrane filter was 0.0089 cm, and thus the volume occupied by the 5 μl of KCl solution could be calculated.

The electron beam penetrates the specimen only a few μ (9). The plant tissue and the membrane filter are assumed to be of similar density, hence the depth of penetration into the 2 would be equal and less than the thickness of the specimen. Any small differences in penetration between the 2 would produce only a slight error in the potassium analyses in comparison with the large differences in potassium concentration in the guard cells of open and closed

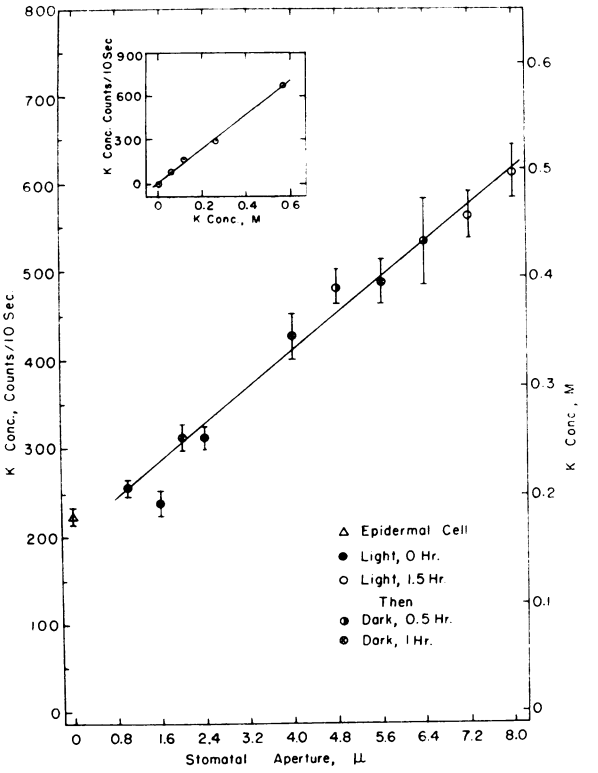

FIG. 2. Relation of potassium concentration in guard cells and stomatal aperture in intact tobacco leaf tissue. Each point shows the mean of 6 to 8 determinations, and the standard deviation of the mean is indicated. The potassium concentration in a typical epidermal cell is also shown. The insert illustrates the relation between counts per 10 sec and potassium concentration of a KCl-impregnated membrane filter that was used to calculate the potassium content of the leaf cells.

stomata. The potassium concentration in the filter membrane was linearly related to the counts per 10 sec between 0.05 M and 0.50 M, and this curve was used to determine the potassium concentration in the guard cells and adjacent epidermal cells (Fig. 2). Calcium and phosphorous were not estimated quantitatively because the concentration of these elements, unlike potassium, did not show any regular change in the guard cells during stomatal opening and closing.

Fig. 1 shows the micromorphology and potassium content of freeze-dried epidermis from a tobacco leaf taken after a period of darkness, then after exposure to light to open the stomata, and finally after a second dark period. Clearly, potassium ions increase in the guard cells as stomata open and decrease as stomata close.

In another experiment, quantitative determinations of potassium concentration were made in guard cells that differed in width because of different exposures of the leaf to light and darkness. These measure-

ments of potassium were made on 6 to 8 different locations in each pair of guard cells and in epidermal cells. The stomatal width and mean potassium concentration in the guard cells were linearly related as shown in Fig. 2. The concentration of potassium in closed stomata (width about 1 μ) was 0.21 M; in fully open stomata (width of 8 μ), 0.50 M; and in adjacent epidermal cells, 0.19 M. Hence an increase in concentration of 0.29 M potassium was observed in the guard cells during stomatal opening. Similar results were obtained in 2 other experiments. On the assumption that there is an accompanying anion, the increase in solute concentration would be 0.58 M, an amount sufficient to account for the largest accumulation of solute reported in the literature (2).

The potassium ions are probably supplied from the adjacent epidermal cells which occupy 90 % of the volume of the leaf epidermis. Therefore no single epidermal cell need decrease greatly in potassium concentration to supply the needs of the guard cells. Although this decrease could not be demonstrated in 10 sec exposures, the potassium image in Fig. 1, obtained with longer exposures, suggests a decrease in potassium in the epidermal cells surrounding the open stoma.

Stomatal opening in the light is temperature-dependent, requires oxygen in the atmosphere, and is inhibited by substances known to inhibit ATP formation (3, 8). Thus a mechanism of active transport of ions can reasonably account for stomatal opening in the light. The data presented here provide strong evidence of accumulation of potassium ions, suggesting that a potassium ion pump functions in guard cells of intact leaves to transport and concentrate this ion. Hence factors that affect ATP synthesis by non-cyclic photophosphorylation in the guard cells (including the oxidation of glycolate) will influence stomatal opening because of their effect on the potassium ion pump (8). Therefore the search for the solute responsible for stomatal opening in the light may be considered to be half-completed, and studies on the nature of the anion (transported to or synthesized within the guard cell) and on the mechanism of action of the ion pump can now begin.

Acknowledgments

We thank Bonnie Gambs for technical assistance and M. D. McConnell, Geology Department, Yale University for his assistance with the electron microprobe.

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