THE REGULATION OF STOMATAL APERTURE IN TOBACCO LEAF EPIDERMAL STRIPS

II.* THE EFFECT OF OUABAIN

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Summary

The K^+-dependent, light-stimulated opening of stomata on tobacco and *Vicia faba* epidermal strips was found to be rapidly reduced by low concentrations of ouabain. On removing ouabain stomatal aperture rapidly increased. This suggests that the influx of K^+ into the guard cells is associated with a membrane-bound transport ATPase. Experiments with 10^{-5}M *p*-chloromercuribenzoate (PCMB) and ouabain indicate that the considered transport ATPase is not markedly affected by PCMB. The stomatal opening obtained in the presence of Na^+ alone is also decreased on the addition of ouabain, though ouabain does not prevent the longer-term stomatal opening which occurs in the dark in the presence of Na^+ alone. In the light, recovery of stomatal opening on the removal of ouabain from the bathing medium only occurred in the presence of K^+. It is considered that an ATPase-linked K^+ transport system could give the rapid rate of influx that would be necessary to bring about stomatal opening in the times observed. The presence of an ATPase transport system would give an evolutionary link between the stomatal control mechanism and that associated with the function of other excitable cells such as nerve and muscle.

I. INTRODUCTION

Results presented in Part I of this series (Thomas 1970) and the works of others (Fujino 1967; Fischer 1968; Humble and Hsiao 1969) suggested that stomatal opening on epidermal strips may be associated with the uptake of ions, particularly K^+, into the guard cells. As stomatal opening in the light showed saturation when the K^+ concentration was increased from 0 to 10 mM in the bathing solution (Fischer and Hsiao 1968; Thomas 1970) it was considered that there might be a specific "carrier" mechanism associated with the uptake of K^+ into the guard cells.

It has been estimated by Fischer and Hsiao (1968) that K^+ is accumulated against a concentration gradient in *Vicia faba* guard cells and that the extent of stomatal opening could be correlated to the amount of K^+ taken up by the guard cells.

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It has been considered by Fujino (1967) that ATPase systems may be involved in the transport of K^+ into *Commelina communis* guard cells.

The presence of an ATPase-linked transport mechanism for K^+ in guard cells might explain the rapid influx that would be needed to bring about the opening of stomata in the times observed, i.e. around 60–120 min from the start of illumination.

An ATPase-linked transport mechanism would also associate the guard cell opening mechanism with the cation influx and efflux mechanisms found in other excitable cells such as nerve and muscle.

Some cardiac glycosides, e.g. ouabain (G-strophanthin), have been used extensively to show the presence of ATPase-dependent, membrane-bound K^+ and Na^+ active transport systems. In ATPase transport systems it is considered that the energy released on the hydrolysis of ATP is geared by the enzyme to bring about an act of active transport. The work of Glynn (1957) showed that the most probable site at which cardiac glycosides inhibit K^+ and Na^+ transport is at the actual site of linkage with transport mechanism because they do not affect the general energy metabolism of tissues or the supply of energy to the transport system.

The sensitivity of ATPase-mediated transport systems to cardiac glycosides varies greatly. In a study of ATPases connected with cation transport in 21 animal tissues, e.g. nerve, muscle, secretory tissue, erythrocytes, leukocytes, and ascite cells, it was found that the enzymes had wide quantitative differences in pH optima, in affinities for Na^+ and K^+ and in the activating effect of Na^+ or K^+ alone, in the requirement for other cations (e.g. Mg^{2+}), and in the concentration of ouabain required to inhibit their activity (Bonting, Caravaggio, and Hawkins 1962).

The effect of ouabain on cation transport in plant tissues has not been studied as extensively nor is its effect as well understood as it is in animal tissues. It was found by MacRobbie (1962) that ouabain at a concentration of 5×10^{-5}M reduced the K^+ influx into the freshwater characean *Nitella translucens* by 37·8–67·5%. In *Allium* epidermis the transport of K^+ and Na^+ in the same direction is inhibited by low concentrations of ouabain (Brown, Jackson, and Dupoy 1964). In a study of the ion fluxes associated with *Hydrodictyon africanum*, Raven (1967) found that a concentration of 5×10^{-4}M ouabain reduced K^+ influx in the light by 64% and increased the K^+ efflux by 28% and decreased Na^+ efflux by 73%. In the dark the K^+ efflux decreased by 45%. In both *N. translucens* and *H. africanum* the active influx of Cl^- is not inhibited by ouabain.

Ouabain does not inhibit the active transport of K^+ in *Chaetomorpha darwinii* (Dodd, Pitman, and West 1966).

The membrane ATPases of plant cells and microorganisms differ from those of animal cells. In no case has an enzyme been demonstrated which is dependent on both K^+ and Na^+ (Rothstein 1968).

II. Material and Methods

The same material and methods were used as those described by Thomas (1970). The sources of other chemicals used in this paper were as follows: ouabain (G-strophanthin)—K.J.K. Laboratories Inc., Plainview, N.Y., and Hollywood, California; *p*-chloromercuribenzoate—Sigma Chemical Company.

III. Experimental

(*a*) *Effect of Ouabain on Stomata Opened in a KCl Bathing Medium*

Figure 1 shows the effect of increasing the concentration of ouabain in the bathing solution (10 mM KCl) on the stomatal opening obtained in the light. It shows that the concentration at which ouabain starts to reduce stomatal aperture seems to be less than that which reduces the light-stimulated K^+ transport into *N. translucens* (MacRobbie 1962) and *H. africanum* (Raven 1967). However, the maximum reduction in aperture is obtained at concentrations which reduce K^+ influx in *H. africanum* and *N. translucens*. At concentrations greater than 10^{-5}M there seems to be a 20% component of the stomatal aperture which is insensitive to further increases in the concentration of ouabain. The transport of K^+ into red blood cells also shows a cardiac glycoside-insensitive component (Glynn 1957). This could mean that there is more than one component in the K^+-dependent mechanism

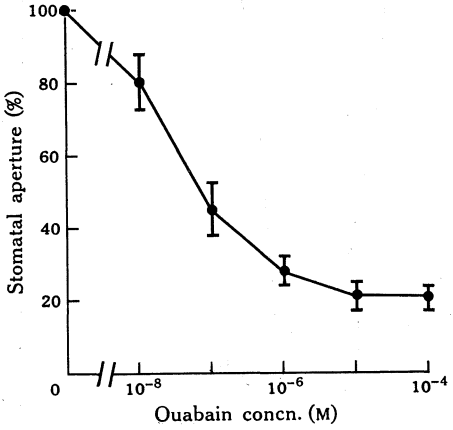

Fig. 1.—Effect of ouabain concentration on stomatal aperture.

of stomatal opening. This component may be another pathway for K^+ transport which is insensitive to ouabain and does not depend on an envisaged ATPase-linked transport system, or alternatively a component of stomatal opening which does not depend on K^+ uptake.

Figure 2 (curve *A*) shows the effect of adding 5×10^{-5}M ouabain to open stomata on a tobacco epidermal strip. The stomata were opened in the light while bathed in 10 mM KCl. Figure 2 (curve *B*) shows a similar experiment using an epidermal strip taken from *V. faba* leaves. Open stomata on both tobacco and *V. faba* epidermal strips show rapid reductions in aperture on the addition of ouabain. This reduction in aperture can be reversed to a considerable extent by flushing the epidermal strip with and returning it to an ouabain-free 10 mM KCl solution. The reversal is also rapid and suggests that ouabain may only block a K^+ uptake mechanism and does not cause a lasting impairment in the functioning of the guard cells. This is consistent with the way in which ouabain is considered to inhibit cation uptake, i.e. by blocking the site of uptake.

These results suggest that the amount of K^+ in the guard cell and hence the extent of stomatal opening may be maintained by a continued light-stimulated influx of K^+ and that an equilibrium opening is determined by a balance between influx and efflux. When the influx is blocked, e.g. by ouabain, the efflux reduces the guard cell content of K^+ and results in a reduction of aperture.

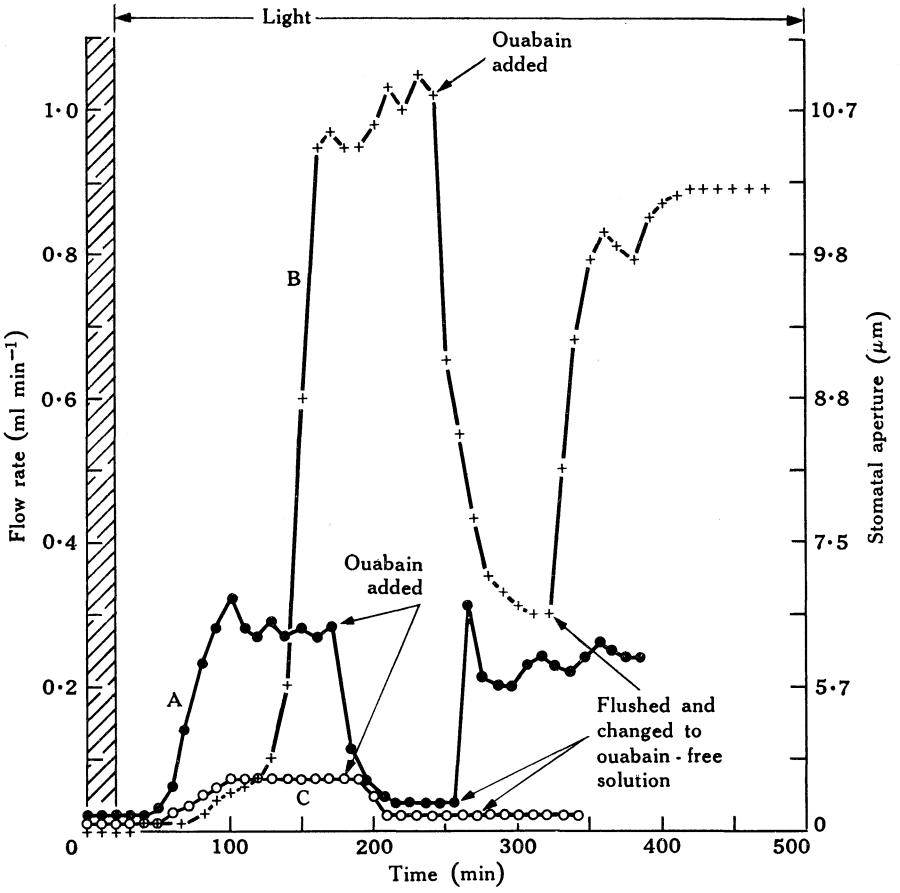

Fig. 2.—Effect of ouabain (5×10^{-5}M) on stomata opened in the light. *A*, tobacco bathed in 10 mM KCl; *B*, *V. faba* bathed in 10 mM KCl; *C*, tobacco bathed in 10 mM NaCl. Stomatal aperture values applicable only to tobacco.

(*b*) *Effect of* p-*Chloromercuribenzoate*

From his histological studies on the guard cells of *C. communis*, *Allium*, and *Tradescantia*, Fujino (1967) concluded that the opening of stomata in the light was connected with an inactivation by light of intracellular ATPases. This inactivation was considered to reduce the breakdown of ATP and result in more ATP becoming available to the mechanism which brought about the uptake of K^+ into the guard cells and thus lead to stomatal opening. In the dark the activity of the intracellular

ATPases is increased and Fujino considered that this was connected with the excretion of K^+ from the guard cells which led to closure.

To substantiate his histological findings Fujino used a known but not specific inhibitor of ATPases—*p*-chloromercuribenzoate (PCMB). When PCMB was supplied at a concentration of 10^{-5}M to *C. communis* epidermal strips it caused marked stimulation in stomatal opening both in the light and dark.

Figure 3(*a*) shows the effect of PCMB on the stomata of tobacco epidermal strip bathed in 10 mM KCl in both light and dark. In the dark the presence of 10^{-5}M PCMB on prolonged incubation (2·5 hr) causes some stomatal opening which does not occur in the presence of 10 mM KCl alone (Thomas 1970). On exposure to light there is only a small increase in aperture and prolonged exposure results in a decrease in aperture.

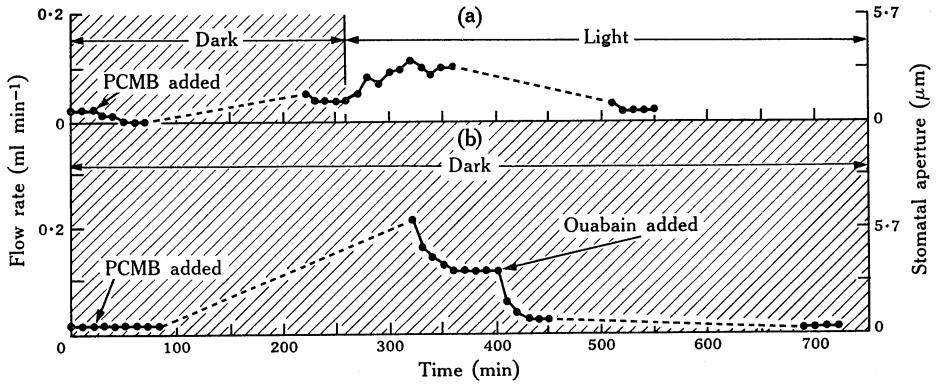

Fig. 3.—Effect of 10^{-5}M PCMB on stomata opened in the dark bathed in 10 mM KCl. (*a*) Effect of exposure to light; (*b*) effect of addition of 5×10^{-5}M ouabain.

Figure 3(*b*) shows that a longer period of incubation in the dark (4 hr) in the presence of 10^{-5}M PCMB can give a larger stomatal opening. However, the addition of 5×10^{-5}M ouabain reduces the stomatal aperture and this reduction is maintained over a further 4-hr period. This suggests that 10^{-5}M PCMB is not markedly inhibiting the suspected ouabain-sensitive, membrane-bound transport ATPase.

The results indicate that 10^{-5}M PCMB does not have the same stimulating effect on the opening of tobacco stomata as it does on those of *C. communis* (Fujino 1967). As PCMB combines with sulphydryl groups its addition to a whole cell system could affect more than specific enzymes. For example, it could combine with the protein components of the cell membranes, alter their structural configuration, and hence change the permeability characteristics of the membrane.

The inactivation of ATPases and the stimulation of stomatal opening by the addition of 10^{-5}M PCMB found by Fujino (1967) may have connections with the findings of Skou and Hilberg (1965). They have found in enzymes prepared from ox brain that low concentrations of PCMB in the presence of ATP inhibit the activity of Mg^{2+}-activated ATPases more than that of $(Na^+ + K^+)$-activated ATPases so that

the activity ratio of the $(Na^+ + K^+)$-activated ATPase to that of the Mg^{2+}-stimulated ATPase is increased. If the $(Na^+ + K^+)$-activated ATPase catalyses cellular cation influx and efflux, as numerous studies have demonstrated (Skou 1965), and the Mg^{2+}-stimulated ATPase is concerned with the hydrolysis of ATP to bring about other cellular functions, it seems that the greater inhibition of the Mg^{2+}-activated ATPase could result in more ATP becoming available to the $(Na^+ + K^+)$-activated ATPase and cause a stimulation of cellular cation fluxes. Membrane-bound ATPases may also be specialized enzymes concerned only with transport so that the way in which they work is possibly very unlike that of intracellular enzymes in that no covalent bond need be formed or split during the transfer of certain substrates (Mitchell 1961).

(c) *Effect of Ouabain on NaCl-induced Stomatal Opening*

When stomatal guard cells are bathed in a medium containing 10 mM NaCl alone, there is a slow stomatal opening in both light and especially in the dark (Thomas 1970). It was considered that this opening could be due to the K^+ influx mechanism showing a low affinity for Na^+ or that Na^+ enters by diffusion to balance intracellular negative charges (Thomas 1970).

The stomatal opening that occurs in the dark when epidermal strips are bathed in a NaCl medium is reduced by exposure to light and the addition of ATP causes marked reductions of NaCl-supported stomatal opening in both light and dark (Thomas 1970 and unpublished data). These results suggest that there is a metabolically dependent Na^+ efflux mechanism present in tobacco guard cells.

As ouabain seems to inhibit the K^+ uptake mechanism, if Na^+ enters by the same mechanism the addition of ouabain to stomata that have opened in the presence of Na^+ alone should cause a reduction in aperture. If ouabain inhibits the efflux mechanism its addition should cause an increase in aperture. If Na^+ enters by a process of diffusion, ouabain should have no effect.

Figure 2 (curve *C*) shows the effect of 5×10^{-5}M ouabain on the stomatal opening that can be obtained in the light when epidermal strips are bathed in 10 mM NaCl. Ouabain causes a closure of the stomata under these conditions though the rate of closure is slower than that obtained when ouabain is added to stomata opened in a 10 mM KCl bathing medium (Fig. 2, curve *A*). This suggests that there may be an ATPase-dependent Na^+ influx system in guard cells, or that the K^+ transport system can bring about some influx of Na^+. Unlike the inhibition caused by ouabain in 10 mM KCl solution which is reversible on flushing and returning to an ouabain-free solution (Fig. 2, curves *A* and *B*), the closure caused by the addition of ouabain to stomata opened in a 10 mM NaCl bathing solution cannot be reversed.

Figure 4 shows the effect of 5×10^{-5}M ouabain when added to open stomata bathed in 10 mM KCl, 1 mM KCl plus 9 mM NaCl, and 10 mM NaCl solutions, and the changes found when the system was flushed with and returned to ouabain-free solutions. While K^+ is present in the solution there is recovery in stomatal aperture when the system is returned to ouabain-free solution.

The inability shown by stomata to reopen after the addition of ouabain to a NaCl bathing solution and return to an ouabain-free NaCl solution might be expected

if some ouabain still remained attached to the uptake mechanism and Na^+ had a low affinity for the mechanism.

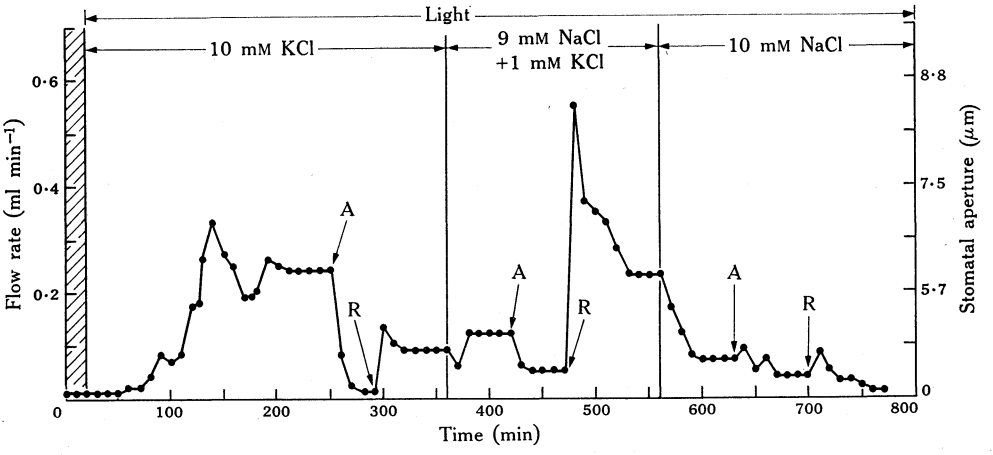

Fig. 4.—Effect of ouabain (5×10^{-5}M) and its removal on stomatal opening in 10 mM KCl, 9 mM NaCl+1 mM KCl, and 10 mM NaCl. Ouabain added at *A*, removed at *R*.

Figure 5 shows the effect of adding ouabain to the stomatal opening that occurs in the dark in the presence of 10 mM NaCl alone. As in the case of the opening in the light, the addition of ouabain reduces the aperture. Exposure to light causes

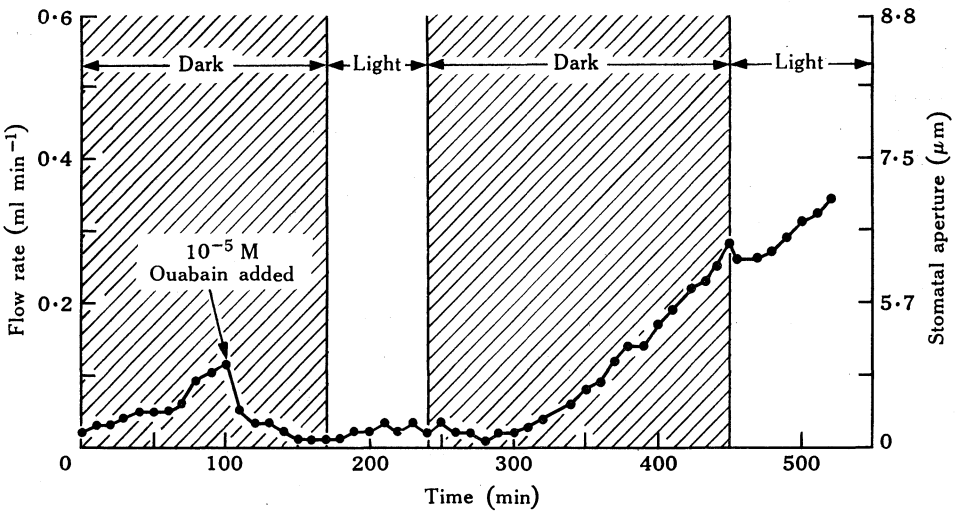

Fig. 5.—Effect of ouabain on stomata opened in 10 mM NaCl in the dark and subsequent exposure to periods of light.

no further decrease in aperture, as it does when the bathing medium consists of 10 mM NaCl in the absence of ouabain (Thomas 1970) and suggests that the light-stimulated efflux process is no longer stimulated. On return to the dark there is further increase

in aperture. This is unlike the effect of metabolic inhibitors, such as carbonyl cyanide-*m*-chlorophenylhydrazone, which reduces the stomatal opening that occurs in 10 mM NaCl alone and also eliminates any further opening (Thomas, unpublished data).

This result may be best explained by considering the findings of Cram (1968). His work on the effect of ouabain on Na^+ fluxes in carrot root tissue showed that the addition of ouabain initially stimulated the efflux of Na^+ and then reduced it over a period to 30% of its initial value.

If ouabain initiated the same sequence in guard cells, the initial decrease in aperture could be due to the stimulation of the efflux mechanism and the following increase in aperture due to subsequent inhibition of the efflux mechanism.

IV. Discussion

Stomata on epidermal strips show a need of ions, particularly K^+, to initiate and maintain the light-opening process. This, together with the low concentration at which ouabain is effective in initiating stomatal closure, indicates that ouabain is acting on stomata in its considered role as a specific inhibitor of cation transport, in this case into the stomatal guard cells. As stomatal opening in the light shows saturation with increasing K^+ concentration (Fischer and Hsiao 1968; Thomas 1970) there is an indication that the accumulation of K^+ into guard cells is brought about by at least a process of facilitated diffusion, if not an active mechanism and that a "carrier" mechanism was involved. The action of ouabain would suggest that the mechanism of transport is an ATPase-linked system in which the free energy of ATP hydrolysis is utilized to transport cations into the cell.

It has been considered that the rate of ion uptake found in other plant tissues and algae would be too slow to account for the rate at which stomata open. An ATPase transport system may give the appropriate rate of transport necessary to account for stomatal opening. For example, Raven (1967) gives the rate of uptake of K^+ into *H. africanum* in the light as 0·97–1·4 pmole cm^{-2} sec^{-1} while the estimates of Humble and Hsiao (1969) give the rate of K^+ uptake into *V. faba* guard cells as 9 pmole cm^{-2} sec^{-1}.

The involvement of an ATPase transport mechanism in guard cell ion uptake gives interesting evolutionary considerations as it would link the guard cell mechanism with that of other excitable cells such as those of nerve and muscle. In these cells ATPase-linked, ion-transport mechanisms play an important role in nerve impulse transmission and muscular contraction and relaxation. Membrane-bound ATPase has been closely associated with the proteins actin and myosin.

A membrane-bound ATPase system might also be intimately connected with the preparation of cell wall and membrane proteins for the conformational changes that occur in the guard cell during the swelling which leads to opening of the stomatal pore.

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