THE REGULATION OF STOMATAL APERTURE IN TOBACCO LEAF EPIDERMAL STRIPS

III.* THE EFFECT OF ATP

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Abstract

The addition of ATP in the light and dark and ADP in the light to bathing solutions containing K^+ can stimulate stomatal opening in tobacco leaf epidermal strips. UTP or AMP do not stimulate opening in the light or dark. The presence of ouabain prevents ATP or ADP stimulating stomatal opening. The additions of Ca^{2+} and Mg^{2+}, though they reduce stomatal aperture, do not prevent ATP stimulating stomatal opening. Increasing the concentration of ATP presented to the stomata in the dark increases the aperture. The rate of stomatal opening in the presence of ATP is increased in the light. In bathing solutions which contain Na^+ as the only cation ATP neither initiates nor maintains stomatal opening. The results are discussed in relation to a postulated light-stimulated, ATPase-mediated accumulation of K^+ in the guard cells which is followed by an influx of water, osmotic swelling, and stomatal opening.

I. INTRODUCTION

Experiments using leaf epidermal strips have shown that the opening of stomata in the light is stimulated by the presence of ions, particularly K^+, in the bathing solution (Fujino 1967; Fischer 1968*a*; Fischer and Hsiao 1968; Humble and Hsiao 1969; Willmer and Mansfield 1969; Thomas 1970*a*). This has led to the consideration that a light-stimulated accumulation of K^+ in the guard cells is effective in reducing the cell water potential with respect to the surrounding water and results in a passive influx of water, guard cell swelling, and stomatal opening. Previous work (Thomas 1970*a*) suggested that the light-stimulated accumulation of K^+ in the guard cells could occur either by a process of facilitated diffusion or active transport in which K^+ could be moved against its electrochemical gradient. This type of ion uptake could involve coupling with a "carrier" mechanism which transports ions across the guard cell membranes. The closing of stomata caused by the addition of low concentrations of ouabain to the bathing solution (Thomas 1970*b*) suggested that the carrier mechanism might be a membrane-bound transport ATPase in which the act of transport is brought about by utilizing the free energy released on

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the hydrolysis of ATP. Light could then stimulate stomatal opening by increasing the supply of ATP as a result of photosynthetic phosphorylation and stimulation of other metabolic pathways through which ATP is synthesized.

It was found that exposure to light could cause the closure of stomata which had opened in the dark when Na^+ was the only cation present in the bathing solution (Thomas 1970*a*). This suggested that there was an energy-dependent Na^+ efflux mechanism present in the guard cells.

In human erythrocytes, Whittam and Ager (1965) have shown that in all cases there is a strict linear relationship between the extent of active K^+ influx and the amount of ouabain-sensitive transport ATPase activity present in the erythrocytes. ATPase-dependent cation transport mechanisms have also been identified in nerve, muscle, secretory tissue, leucocytes, and ascite cells (Bonting, Caravaggio, and Hawkins 1962).

In red beet tissue, Jacoby (1965) found that 1 mM ATP added externally inhibited Na^+ and K^+ uptake. This inhibition was considered to be competitive. In a study of ion uptake by cells enzymically isolated from green tobacco leaves, Jyung, Wittwer, and Bukovac (1965) found that the addition of 10^{-4}M ATP increased the uptake of Rb^+ by 13% but reduced phosphate uptake by 13%. In plant organelles such as mitochondria, added ATP can greatly stimulate ion transport (e.g. Elzam and Hodges 1968).

In a study of the effect of ATP on stomata, Fujino (1967) showed that 10 mM ATP added to epidermal strips of *Commelina communis* bathed in phosphate buffer and KCl solution caused increased stomatal opening both in the light and dark. In a study of *Vicia faba* stomata, Fischer (1968*b*) found that 5 mM ATP had no effect.

It seems that the main problem connected with the use of ATP in the study of ion fluxes associated with whole cells is that ATP itself has to be taken up by the cell to reach sites within the cell where it can be effectively used by transport mechanisms. ATP supplied to the outside of a cell could have many effects on the membranes it traverses as could the membranes on the ATP. These effects could confuse the interpretation of the action of the normal endogenous ATP supply on the process of influx and efflux. The effect of ATP on plant ion transport systems has not been as extensively studied as that in animals where ATP has been specifically shown to be the energy substrate used in active transport (e.g. Garrahan and Glynn 1967*a*).

II. Materials and Methods

These were the same as those described by Thomas (1970*a*). The method essentially monitors stomatal aperture by measuring the flow rate through the stomatal pores on isolated epidermal strip material in a solution-flow porometer. Unless otherwise stated the basal bathing solution was 10 mM KCl.

The sources of chemicals used were as follows:

Adenosine triphosphate (ATP):	National Biochemicals Corporation, Cleveland, Ohio, and Sigma Chemical Company, St. Louis, Missouri.
Adenosine diphosphate (ADP):	Calbiochem, Los Angeles, California.
Adenosine monophosphate (AMP):	Pabst Laboratories, Milwaukee, Wisconsin.
Uridine triphosphate (UTP):	Calbiochem, Los Angeles, California.
Phosphoenol pyruvate (PEP):	Calbiochem, Los Angeles, California.

III. Experimental

(*a*) *Effect of Added ATP on Stomatal Aperture*

Figure 1 shows the typical effect obtained when ATP at a concentration of 0·05 mM or greater was added to the bathing solution in which the stomata had opened in the light. Initially the addition of ATP caused a decrease in aperture and

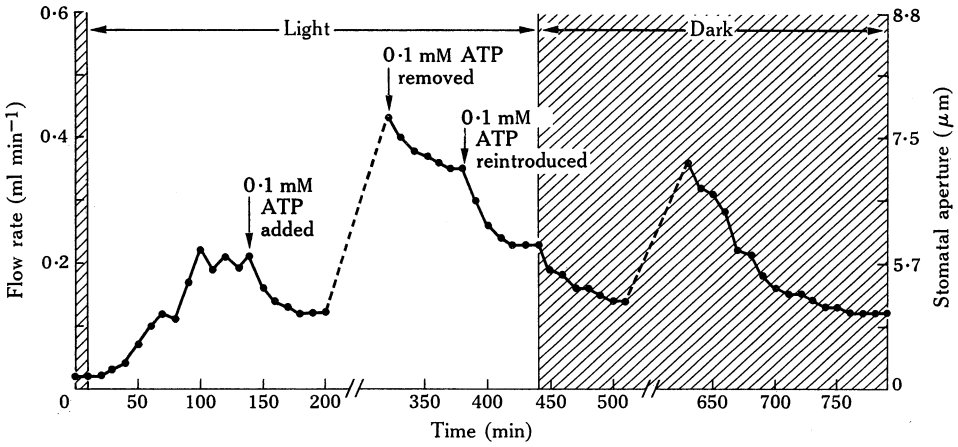


Fig. 1.—Effect of added ATP, together with its subsequent removal and reintroduction, on stomatal aperture in the light and dark. Basal bathing solution 10 mM KCl. In this and subsequent figures, periods during which flow was stopped is shown by dashed lines.

increasing the concentration of ATP presented to the stomata gave progressive decreases in aperture (Table 1). Similar results were obtained when ATP was added to the bathing solution in which the stomata on *Vicia faba* epidermal strip were opened in the light.

Table 1

Effect of adding increasing ATP concentrations on the equilibrium aperture of stomata opened in the light in a 10 mM KCl bathing medium

Concn. of ATP added (mM):	0	0·01	0·05	0·10	0·50
Stomatal aperture (μm):	6·0±0·6	6·2±0·8	5·6±0·7	4·5±0·4	3·6±0·4
Percentage of initial aperture:	100	103	93	75	60

The reduction in aperture caused initially by the addition of ATP also occurred when ADP (Figs. 9 and 10), AMP (Table 3), UTP [Fig. 3(*a*)] were added to the bathing solution in which the stomata were opened. Similar results were obtained when these compounds were added to stomata opened in other bathing solutions which support stomatal opening, e.g. 5 mM K_2SO_4 (Thomas 1970*a*), and a solution consisting of 17·6 mM KH_2PO_4–Na_2HPO_4 phosphate buffer, pH 8·0 (Figs. 9 and 10).

As the addition of ATP, ADP, AMP, etc. gives similar results in a buffered solution it does not seem likely that the reduction in aperture could be due to a pH change on the addition of these compounds. Also, calculation shows that any reduction in ion concentration in the porometer by guard cell uptake is negligible.

While ATP was being continually supplied in the solution flowing to the stomata there was little tendency for the aperture to increase except at a concentration of 0·01 mM [Table 1; Fig. 6(*e*)] when the aperture increased slightly. However, it was found that by stopping the flow of the ATP-containing solution to the stomata, e.g. over a period (2 hr as shown in Fig. 1), the ATP remaining in the solution-flow porometer caused marked increases in aperture. This occurred both in the light and dark (Fig. 1 and Table 2). These conditions are similar to those under which Fujino (1967)

TABLE 2

EFFECT OF STOPPING THE FLOW OF BATHING SOLUTION (10 mM KCl) WITHOUT AND WITH ADDED 0·1 mM ATP FOR A PERIOD OF 2 HR

Conditions	Initial Equilibrium Aperture (μm)	Stomatal Aperture (μm)	
		Without ATP	With ATP
Dark	1·7±1·0	1·8±1·2	6·8±1·0
Light	5·7±0·9	6·0±1·1	8·3±0·7

found that ATP stimulated stomatal opening on epidermal strips immersed in unstirred bathing solutions, whereas Fischer (1968*b*) found that the addition of ATP had no effect when added to a stirred bathing solution. Of the compounds tested only ATP and PEP in the light and dark and ADP in the light caused stimulations in aperture when they remained in contact with the stomata while the continued flow of solution through the stomata was stopped.

At first it was thought that the stomatal aperture might have increased due to the cessation of the flow of solution through them. But it was found that stopping the flow, e.g. for 2 hr, to stomata that had reached an equilibrium opening in the light or dark in the absence of ATP did not result in large increases in aperture (Table 2), but following the addition of ATP (0·1 mM) the effect of stopping the flow was to cause increases in aperture, particularly in the dark (Table 2), whereas the addition of other compounds, e.g. UTP, AMP, caused reductions in aperture on their addition to the bathing solution but no subsequent increase in aperture. Hence the presence of ATP in the bathing solution could open stomata in the absence of light, and amplify the extent of opening in the light.

After stopping the flow of the ATP-containing solution had stimulated the opening of the stomata, restarting the flow caused a decrease in aperture [e.g. Figs. 3(*a*), 3(*b*), 6(*a*), 6(*d*)]. It was thought that this decrease might be in response to the flow of solution through the stomata. In the experiment shown in Figure 1 stomatal opening was stimulated by the addition of ATP and subsequently stopping the continuous flow of ATP to the stomata. After the stomata had opened as a result of this treatment the solution was changed for an ATP-free solution. This resulted

in a small reduction of aperture. Reintroduction of ATP resulted in an increased rate and extent of stomatal closure. Therefore it seems that while a continuous supply of ATP is being presented to the stomata there is a reduction in aperture. Also, some compound might be formed and released into the solution when ATP was allowed to remain in prolonged contact with epidermal strips, and that this compound was effective in causing the observed stomatal closing or opening while ATP in itself was not. To determine whether this might be so, a solution of 0·1 mM ATP was incubated with epidermal strips so that the total strip area to the volume of solution approximated that in the solution-flow porometer. The incubation period was 3 hr. After the epidermal strips had been removed this solution was used to replace the 10 mM KCl solution in which the stomata were opened after the stomata had reached an equilibrium opening. The result is shown in Figure 2. There

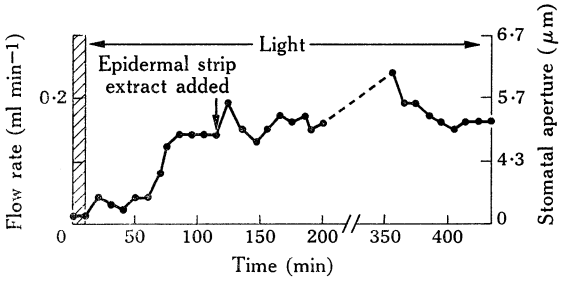


Fig. 2.—Effect of changing to a 0·1 mM ATP–epidermal strip extract solution on stomatal aperture. Basal bathing solution 10 mM KCl.

was no marked change from the pattern shown when ATP itself was added to the solution except that the reduction in aperture and stimulation of opening was less than when ATP itself was added to the solution. This could be explained by the removal of ATP from the solution on incubation with the epidermal strips. There is no indication that any compound which reacts with stomata is released when epidermal strips are incubated with ATP.

(*b*) *Specificity for ATP*

Figure 3(*a*) shows the effect of adding UTP to the bathing solution in which the stomata had opened in the light. The addition of UTP has the same initial effect as ATP in causing a partial reduction in stomatal aperture but there is no increase in aperture while UTP remains in contact with the stomata even when the flow to the stomata is stopped. Replacing UTP with ATP results in the characteristic increase in aperture found when ATP remains in contact with the epidermal strip. Figure 3(*b*) shows that UTP does not stimulate stomatal opening in the dark and that replacing UTP by ATP can cause opening. These results suggest that the postulated stomatal opening mechanism, i.e. via a K^+ transport ATPase mediated influx, may be specifically fuelled by ATP.

(*c*) *Effect of ATP and Distilled Water on Stomatal Opening*

Figure 4 shows that the addition of ATP to a bathing medium of distilled water can cause a small stomatal opening and that the opening could be increased by the subsequent addition of KCl. The results shown in Figures 12–15 indicate that the

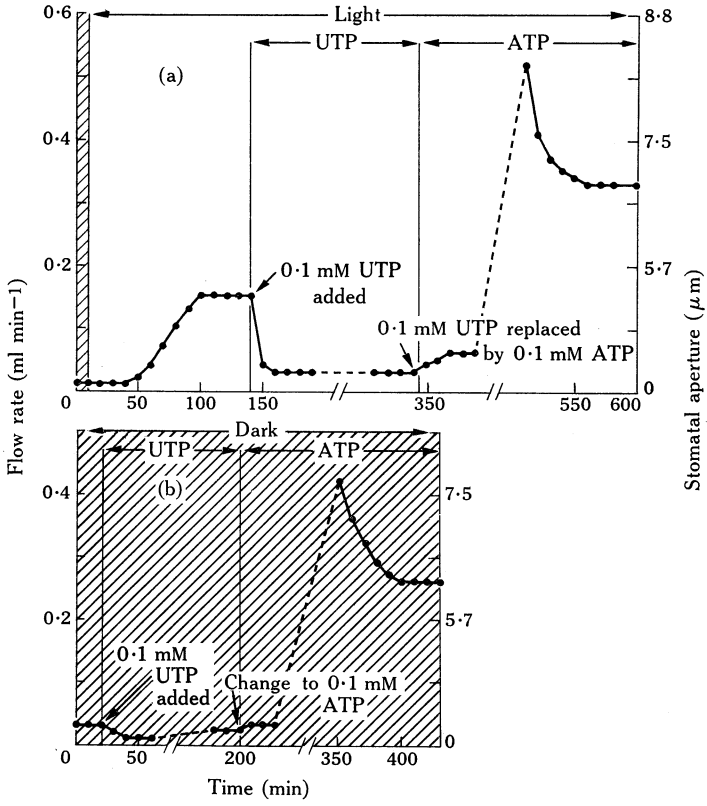


Fig. 3.—Effect of 0·1 mM UTP and its replacement by 0·1 mM ATP on stomatal opening (*a*) in the light, and (*b*) in the dark. Basal bathing solution 10 mM KCl.

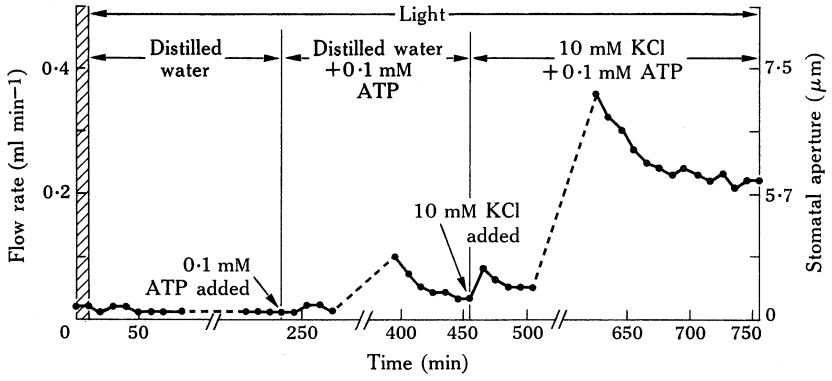


Fig. 4.—Effect of 0·1 mM ATP in distilled water and the subsequent addition of 10 mM KCl on stomatal aperture.

stimulation shown by the presence of ATP is not due to the Na^+ ions added together with ATP. Similar results have been obtained by Fujino (1967). The results suggest that ATP may have an effect in increasing stomatal aperture in its own right as well as stimulating K^+ influx into the guard cells.

(*d*) *Effect of ATP Concentration on Stomatal Opening in the Dark*

In the previous experiments the time course of stomatal opening in the presence of ATP, when the flow to the stomata was stopped, was not followed. It was found that this could be done by stopping the flow through the stomata for periods of 30 min and then timing the flow of small volumes, e.g. 0·3 ml of solution, through the stomata and then stopping the flow for another 30 min before the next flow rate was measured. Figure 5 shows the effect of ATP concentration on stomatal opening in the

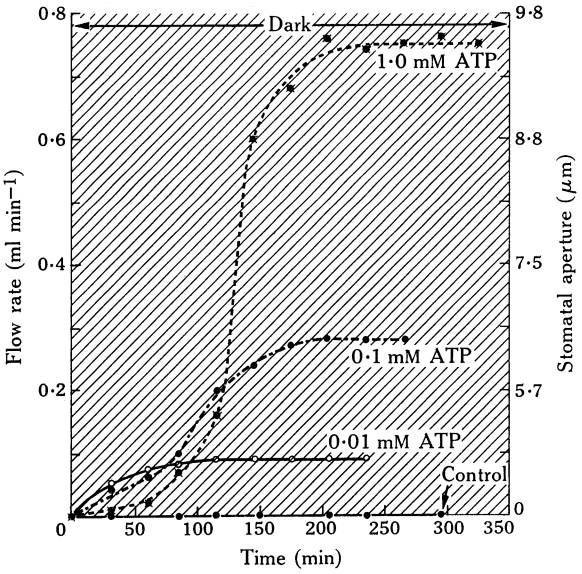


Fig. 5.—Effect of ATP concentration on stomatal opening in the dark in a 10 mM KCl bathing solution.

dark. The epidermal strips for these determinations were obtained from adjacent areas of the same leaf. The leaf was left on the plant and the plant kept in the dark during the period of the experiment. Figure 5 shows that the pattern of stomatal opening in the dark in the presence of ATP is similar to that shown by stomata on epidermal strips bathed in basal solutions of 10 mM KCl or phosphate buffer when exposed to light (Thomas 1970*a*). The pattern is sigmoidal in shape showing a lag, rapid, and equilibrium phase. The lag phase is more noticeable at the highest concentration tested, i.e. 1 mM. Once the rapid phase of opening has started, the rate at which the stomata open and the extent of stomatal opening attained rises with the increase in ATP concentration presented to the stomata, e.g. the rates of opening over the rapid phase are 0·12, 0·05, and 0·02 μm min^{-1} at ATP concentrations of 1·0, 0·1, and 0·01 mM respectively.

(e) Stomatal Opening in the Light and Dark in the Presence of ATP

Measurement of the rate of stomatal opening in the presence of ATP by the method described in Section III(*d*) showed that the rate of stomatal opening was more rapid and the extent of opening greater in the light than in the dark. Figure 6(*a*) shows the effect of the change from light to dark and Figure 6(*b*) the change from dark to light.

Following the initial rapid increase in aperture on exposure to light, when the flow of solution was maintained through the stomata, two types of behaviour were observed. Figure 6(*c*) shows one type of reaction. In this case the stomata were opened in the dark by the addition of ATP; following the opening came the characteristic reduction in aperture when the flow of solution was maintained through the stomata in the presence of ATP. On exposure to light there is a rapid increase in

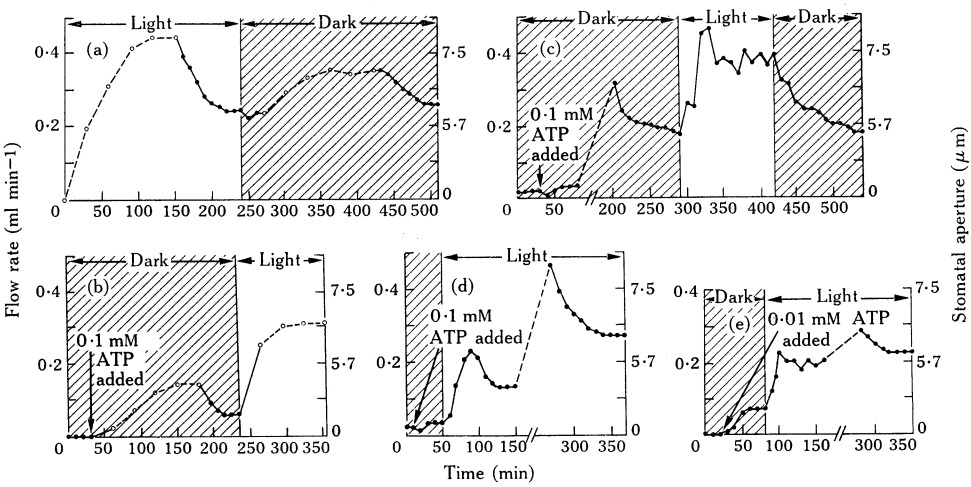


Fig. 6.—Effect of light and dark on stomatal opening in the presence of ATP. Basal bathing solution 10 mM KCl. (*a*) Effect of light followed by dark in the presence of 0·1 mM ATP. (*b*) Effect of dark followed by light in presence of 0·1 mM ATP. (*c*) Effect of exposure to light on stomata opened in the dark by the addition of 0·1 mM ATP, showing the oscillations that can be obtained on exposure to light. (*d*) Effect of exposure to light of stomata supplied with 0·1 mM ATP in the dark, showing an initial rapid increase in aperture followed by a decrease and the effect of stopping the continued flow of ATP to the stomata. (*e*) Effect of 0·01 mM ATP in the dark and light on stomatal aperture.

stomatal aperture which is followed by fluctuations in the aperture. On returning to the dark there is a slow reduction in aperture and the fluctuations in aperture are dampened out. The other type of reaction found is shown in Figure 6(*d*). In this case ATP was only in contact with the stomata for 40 min in the dark before exposure to the light. On such exposure there is an initial rapid increase in aperture followed by a decrease. After the decrease a steady level of stomatal opening is reached and maintained. The decrease in aperture which follows the stimulation in opening is again a feature of the presence of ATP in the solution flowing through the stomata. Stopping the continued flow of ATP to the stomata again results in the characteristic

stimulation of stomatal opening that results when ATP is left in contact with the stomata and is not replenished by ATP continually flowing through the stomata.

Figure 6(*e*) shows a similar experiment in which the concentration of ATP was reduced to 0·01 mM. Following the addition of ATP there is a stimulation of stomatal opening in the dark. On exposure to light there is a rapid increase in aperture which reaches a peak and then decreases slightly. Again when the flow of solution to the stomata is stopped there is a stimulation of opening.

(*f*) *Effect of Ouabain*

Figure 7 shows that the presence of ouabain in the bathing solution stops the stimulation of stomatal opening by ATP. Rapid opening can be obtained on the removal of ouabain. Similar results were obtained by Thomas (1970*b*) when ouabain was added to the light-stimulated stomatal opening supported in bathing solutions

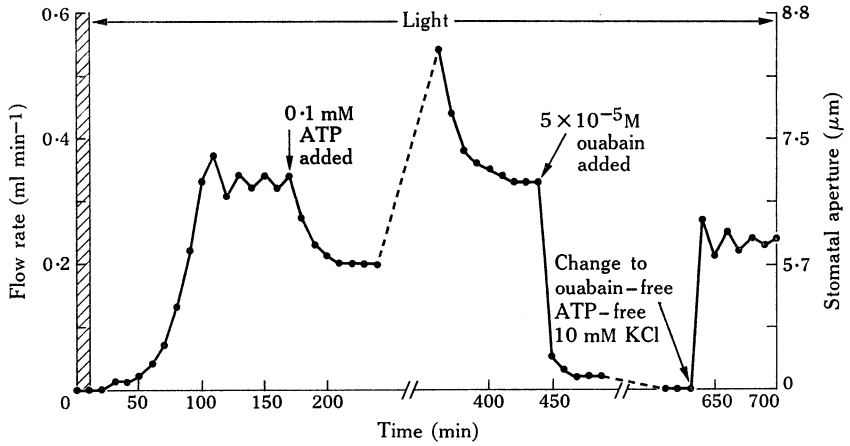


Fig. 7.—Effect of the addition of 5×10^{-5}M ouabain on stomatal opening in the presence of 0·1 mM ATP. Basal bathing solution 10 mM KCl.

of 10 mM KCl or KH_2PO_4–Na_2HPO_4 buffer. This seems to be consistent with the idea that ouabain inhibits the working of an ATPase-dependent K^+ transport system because it is found in the red blood cell that the presence of ouabain inhibits K^+ influx even in the presence of ATP (Garrahan and Glynn 1967b; Whittam and Wiley 1967). What effect ouabain has on the uptake of ATP is unknown.

(*g*) *Effect of ATP on Stomatal Aperture in the Presence of Ca^{2+} and Mg^{2+} Ions*

Figure 8 shows that though the addition of 0·5 mM $CaCl_2$ causes a marked reduction in stomatal aperture similar to that shown by Thomas (1970*a*) its presence does not prevent the increase in aperture which can occur when ATP is present in the bathing solution. A lower concentration of ATP was used in this experiment as it was thought that this might accentuate differences in stomatal behaviour due to the presence of Ca^{2+}. The addition of 0·5 mM $MgCl_2$ also reduces stomatal aperture, but its presence does not prevent ATP stimulating stomatal opening. These results suggest that the effect of Ca^{2+} and Mg^{2+} in reducing stomatal aperture is due to their effect

on some other aspect connected to the uptake of K^+ into the guard cells (Thomas 1970*a*), rather than an effect on a postulated transport ATPase.

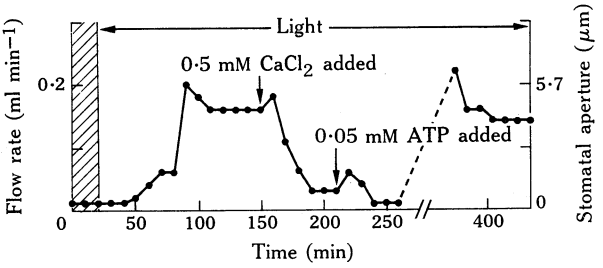


Fig. 8.—Effect of 0·5 mM Ca^{2+} on ATP-stimulated stomatal opening. Basal bathing solution 10 mM KCl.

(h) Effects of ADP and AMP Addition

Figure 9 shows the effect of adding ADP on stomatal opening in the light and dark in a 17·6 mM K_2HPO_4–NaH_2PO_4 buffer (pH 8·0) bathing medium. A bathing

Fig. 9

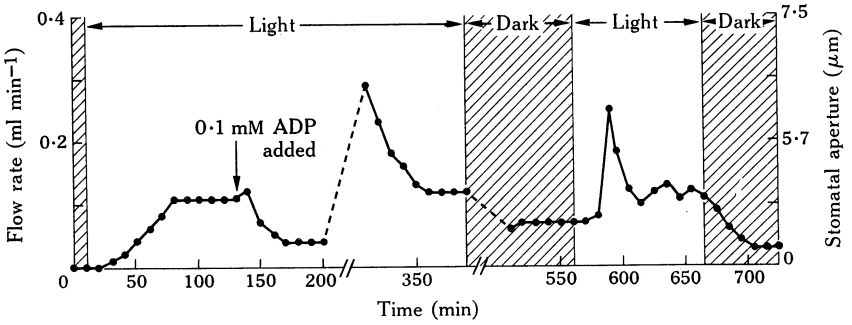


Fig. 10

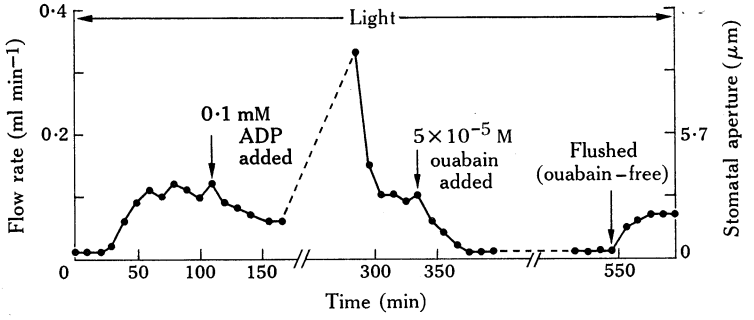


Fig. 9.—Effect of 0·1 mM ADP on stomatal opening in the light and dark. Basal bathing solution 17·6 mM phosphate buffer, pH 8·0.

Fig. 10.—Effect of 0·1 mM ADP on stomatal opening in the absence and presence of 5×10^{-5}M ouabain. Basal bathing solution 17·6 mM phosphate buffer, pH 8·0.

medium of phosphate buffer was used in these experiments to ensure that there would be adequate inorganic phosphate available for phosphorylation. As with ATP

the initial effect of adding ADP is to cause the characteristic partial closure. Also stopping the flow of ADP in the light results in a stimulation of opening similar to that caused by ATP, but unlike ATP it does not stimulate opening in the dark. Exposure to light after the dark period, after a delay of 20 min, results in a stimulation of opening. Except for the delay period this is similar to the results obtained with ATP. These results suggest that in the light ATP is synthesized in adequate amounts from the added ADP to stimulate stomatal opening, but in the dark it is not.

Figure 10 shows that the presence of ouabain prevents the stimulation of stomatal opening by ADP. This is similar to the effect of ouabain on the ATP-stimulated stomatal opening (Fig. 7) and again suggests the involvement of a transport ATPase system in stomatal opening.

The effect of adding increasing concentrations of AMP to stomata opened in the light is shown in Table 3. Each increase in concentration results in a decrease in

TABLE 3

EFFECT OF INCREASING AMP CONCENTRATION ON STOMATAL APERTURE

Basal solution 17·6 mM KH_2PO_4–Na_2HPO_4 phosphate buffer, pH 8·0

AMP concn. (mM):	0	0·05	0·10	0·50
Equilibrium stomatal aperture (μm):	8·6	6·6	5·0	3·2
Percentage of initial aperture:	100	77	58	37

aperture. AMP does not stimulate stomatal opening in the light or dark when its continued supply is restricted. From this it is considered that there is little or no synthesis of ATP from AMP in guard cells, at least over a 2-hr period.

(*i*) *Effect of PEP and ADP*

The effect of adding PEP and subsequently ADP in the dark on stomatal aperture is shown in Figure 11.

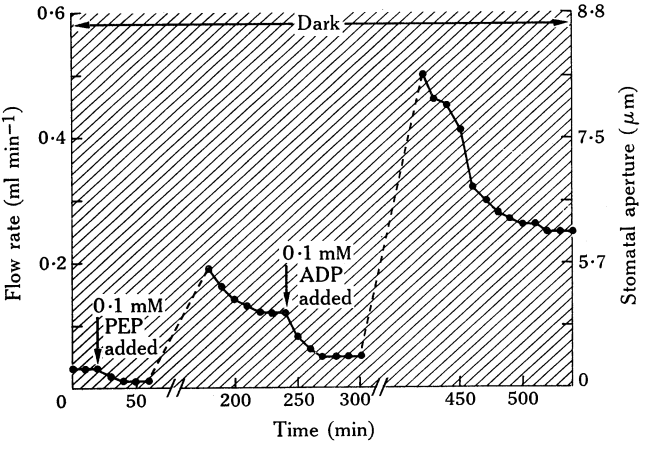
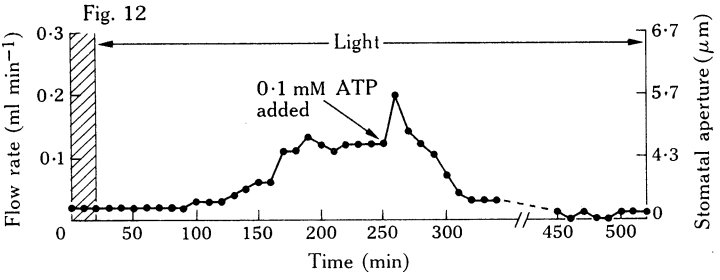


Fig. 11.—Effect of 0·1 mM PEP and the subsequent addition of 0·1 mM ADP on stomatal opening in the dark. Basal bathing solution 10 mM KCl.

As with ATP, the addition of PEP can cause stomata to open in the dark and the subsequent addition of ADP can stimulate further opening, which it cannot do on

its own in the dark (Fig. 9). The result suggests that adding substrates that could synthesize ATP in the guard cells, e.g. the effect of ADP in the light (Fig. 9), can stimulate stomatal opening. The "energy-rich" bond of PEP could be transferred to ADP to form ATP in the guard cells by enzymes such as pyruvic kinase and phosphoenolpyruvic carboxykinase.



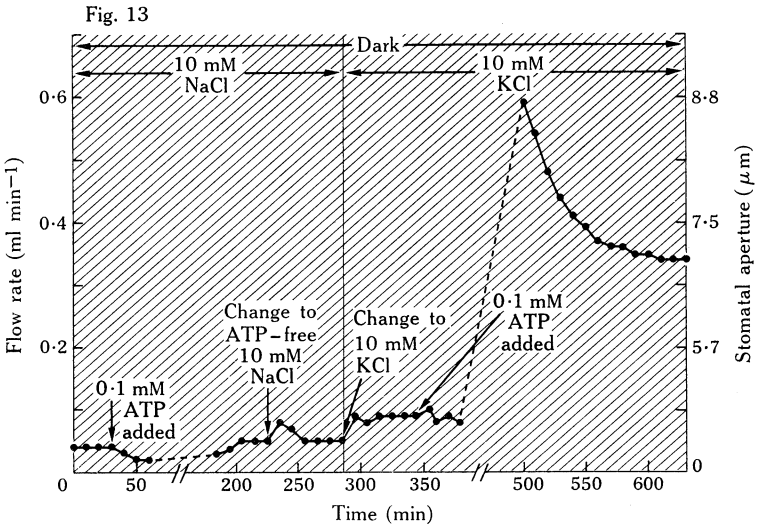


Fig. 12.—Effect of adding 0·1 mM ATP on the stomata opened in the light in 10 mM NaCl solution.

Fig. 13.—Effect of 0·1 mM ATP added in 10 mM NaCl and subsequent change to 10 mM KCl on stomatal opening in the dark.

(*j*) *Effect of ATP on Na⁺-supported Stomatal Opening*

Previous work (Thomas 1970*a*, 1970*b*) has shown that the response of stomata on tobacco epidermal strips was different in bathing solutions containing only NaCl than in those which included K^+. In a NaCl bathing solution, stomata would open in the light and particularly in the dark, but this opening was slower and showed a different pattern. Stomata opened in the dark in a NaCl bathing solution could show closure

when exposed to light provided that the stomata had not opened to too great an extent. It was considered that there might be an energy-dependent Na^+ efflux mechanism present in tobacco guard cells. Figure 12 shows the effect of adding ATP to the 10 mM NaCl solution in which the stomata had opened in the light. Initially the addition of ATP causes a transient increase which is then followed by a decrease in aperture. Similar results have been observed when stomata which have opened in the dark in a 10 mM NaCl solution are exposed to light (Thomas 1970*a*). Unlike the effect of ATP when the bathing solution contains K^+, the decrease in aperture is maintained in the presence of ATP even when the continued flow of the 10 mM NaCl + 0·1 mM ATP solution to the stomata is stopped. The stomatal opening which occurs in the dark when stomata are bathed in 10 mM NaCl is also markedly reduced when ATP is added to the solution and this reduction is maintained in the presence of ATP (e.g. Fig. 15). The result suggests that ATP can only stimulate stomatal opening in the presence of K^+. Also, it seems that light alone, at least under the experimental conditions, cannot supply the energy required to fuel a Na^+ efflux mechanism or maintain a discrimination of the guard cell membranes against an influx of Na^+. Figure 13 shows the effect of ATP in solutions of 10 mM NaCl and 10 mM KCl on stomatal opening in the dark. In the NaCl bathing solution, ATP did not stimulate opening. In the absence of ATP it could be expected that over the same time interval stomata would open in a 10 mM NaCl bathing solution (Thomas 1970*a*, 1970*b*). When the bathing solution is changed to 10 mM KCl the addition of ATP stimulates opening.

These results suggest that the presence of ATP prevents stomatal opening when the bathing solution contains NaCl alone and ATP can only support stomatal opening in the presence of K^+. Figure 14 compares the effect of adding ATP in the dark when the bathing solution consists of 10 mM KCl, 1 mM KCl + 9 mM NaCl, and 10 mM NaCl. It shows that while K^+ is present in the bathing solution, the addition of ATP can stimulate opening. When the solution is changed to one containing only 10 mM NaCl there is a sudden increase in aperture. This is similar to the rapid increase in aperture observed by Thomas (1970*a*), when partially open stomata were exposed to 10 mM NaCl.

The addition of ATP to the NaCl solution in which the stomata have opened causes rapid stomatal closure and while ATP remains in the solution no stomatal opening occurs. This result again shows that stomatal opening is not supported by Na^+ when ATP is present in the bathing medium. Figure 15 shows a similar result. Stomata bathed in 9 mM NaCl + 1 mM KCl solution were opened in the dark by the addition of 0·1 mM ATP. When the stomata had reached an equilibrium opening, the 9 mM NaCl + 1 mM KCl + 0·1 mM ATP solution was replaced by one containing only 10 mM NaCl. This again resulted in a rapid increase in aperture. Exposure to light caused a further increase in aperture and returning to the dark only caused a very small transitory decrease. It is only when ATP is added that the aperture is decreased and this reduction maintained. The stimulation of opening seen on exposure to light may mean that under these conditions, i.e. large stomatal opening, light may increase the permeability of the guard cell membranes, independent of ATP synthesis and metabolism, because at smaller stomatal openings, which result when stomata

are bathed in 10 mM NaCl in the dark, exposure to light reduces the stomatal aperture (Thomas 1970*a*).

Fig. 14

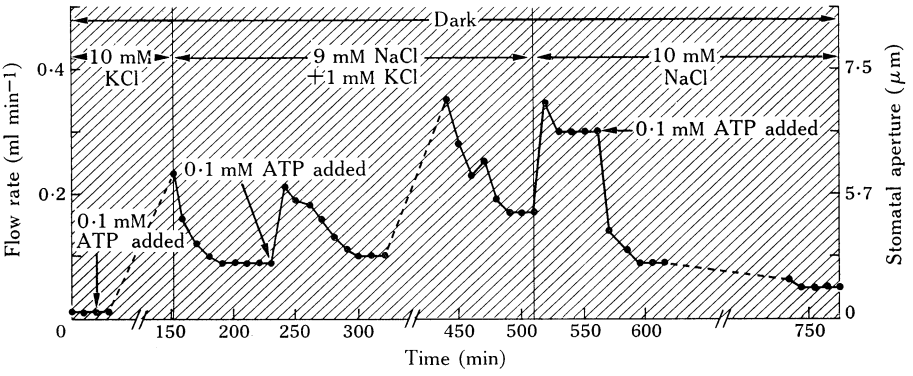


Fig. 15

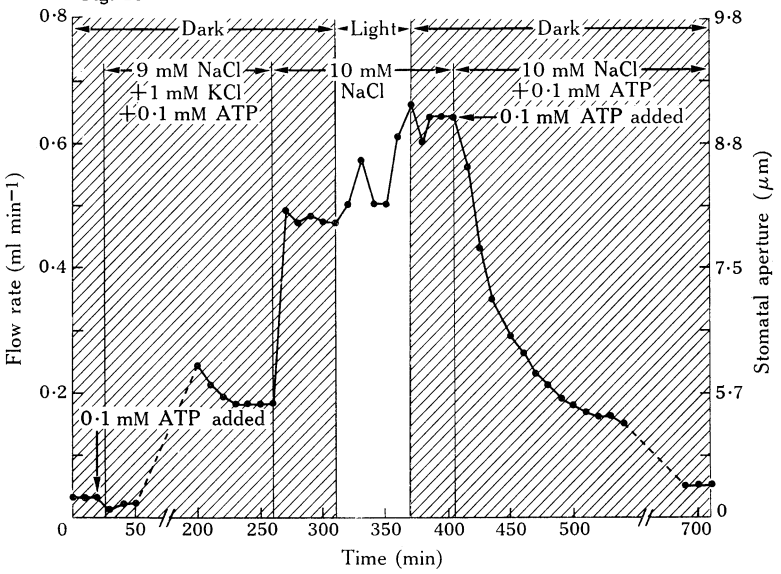


Fig. 14.—Effect of 0·1 mM ATP in 10 mM KCl, 1 mM KCl+9 mM NaCl, and 10 mM NaCl on stomatal opening in the dark.

Fig. 15.—Effect of 0·1 mM ATP in bathing solutions of 1 mM KCl+9 mM NaCl and 10 mM NaCl.

IV. Discussion

It is puzzling why the addition of compounds such as ATP, ADP, UTP, AMP, and PEP to the bathing solution in which the stomata have opened results in a reduction of aperture and why the continued flow of ATP or PEP in the light and dark and ADP in the light has to stop in order to stimulate stomatal opening. *In vivo* it seems that the normal substrates presented to the guard cells for uptake would be ions and water. As the guard cells in tobacco are photosynthetic and the surrounding

epidermal cells are not it seems more likely that the guard cells will act as sources rather than sinks for organic molecules, although some organic molecules may be translocated to the guard cells from the mesophyll via the epidermal cells. Compounds such as ATP, UTP, ADP, and AMP do not seem to be normal substrates in intercellular transport, nor does the nature of their uptake into whole cells appear to have been studied. The effect of added ATP on the response of whole cells of other excitable tissues is not fully understood. Its addition gives only a transitory stimulation of ion transport in squid axon (Keynes 1961) and in the potential difference across the wall of the jejunum, ileum, and colon of the rat (Kohn, Newey, and Smyth 1970). Initially when ATP and related compounds are presented to smooth muscle there is a hyperpolarization of the membrane and the muscle relaxes (Poskonova and Mal'chikova 1970). They consider that ATP and related compounds are capable of interfering with normal processes occurring on the muscle cell membrane.

If stomatal opening is considered to be the result of a net influx of ions and water into the guard cells, some of the ways in which the net influx could be affected by these compounds are:

(1) by causing a non-specific blockage of some section of the path of entry into the guard cells;

(2) increasing the efflux from the guard cells;

(3) uptake of all compounds is an active process and the energy supplied to uptake processes is limited, hence the competition for energy may result in the reduction of uptake of K^+ which seems intimately concerned with the stomatal opening process;

(4) initially increasing the metabolic production of CO_2 in the guard cells. Increasing the concentration of CO_2 is known to reduce stomatal aperture (Linsbauer 1916).

It has been considered by Hodgkin and Keynes (1955) and Hope and Walker (1960) that compounds pass through pores on their entry into cells. Thomas (1970*a*) suggested that pores may open up in guard cell membranes in association with the swelling of the guard cells. The diameter of these pores is considered to be only slightly greater than that of molecules. A transport ATPase which forms a pore through a membrane has been envisaged by Skou (1964). In erythrocyte ghosts it has been found by Hoffman and Ryan (cited by Hoffman 1962) that the (Na^+–K^+)-ATPase considered responsible for the influx of K^+ and the efflux of Na^+ is situated on the cytoplasmic side of the plasma membrane. If the transport ATPase in the guard cells is similarly situated it would mean that K^+ would have to traverse the cell wall–plasmalemma complex before it could be attached to the ATPase uptake mechanism. If molecules, ions, and water share pores as a common path of entry into guard cells the addition of compounds such as ATP, UTP, ADP, AMP, and PEP might result in the adsorption or congregation of these compounds along the pore and increase the resistance to the influx of K^+ or water or both of these while the supply of these compounds was maintained. In the case of ATP, when the continuous supply is stopped the number of ATP molecules available for uptake will be reduced as the

concentration of ATP outside the cell decreases, e.g. the addition of ATP at a concentration of 0·01 mM can cause small increases in aperture [Table 1 and Fig. 6(*e*)]. This could decrease the resistance to the influx of K^+ or water or both, and the ATP that has been taken up can then be effective in stimulating the influx of K^+ and hence increase the stomatal aperture.

Three main considerations come out of this work:

(1) Stomatal opening in the presence of K^+ can be stimulated by ATP, or by the addition of compounds from which ATP can be synthesized.

(2) In the presence of ATP the stomatal opening that occurs in the presence of Na^+ alone is neither maintained nor supported.

(3) ATP may influence processes other than K^+ accumulation connected with the stomatal opening, e.g. guard cell organization.

In the dark, the extent of stomatal opening can be quantitatively related to the concentration of ATP presented to the stomata. This indicates that the increase in stomatal aperture which occurs with increase in light intensity may be brought about by the amount of ATP synthesized by the process of photosynthetic phosphorylation. The addition of substrates that could lead to the synthesis of ATP, e.g. ADP in the light and PEP and ADP in the dark, also stimulates stomatal opening in the presence of K^+. Consideration of these results together with the inhibition of stomatal opening caused by the addition of ouabain [Sections III(*f*) and III(*h*); see also Thomas 1970*b*] suggests that the mechanism of stomatal opening is related to a K^+ transport ATPase located in the guard cell membranes which, when ATP is supplied, hydrolyses ATP and utilizes at least part of the energy released to transport K^+ into the guard cell. This influx of K^+ lowers the water potential in the guard cells and results in a water potential gradient between the guard cell and the surrounding water, which could then move passively down this gradient and cause swelling of the guard cell and stomatal opening (Thomas 1970*a*).

Estimates of the osmotic work (W) done in taking up an equivalent of K^+ can be made from the relation,

$$W = \boldsymbol{R}T\ln([K_i]/[K_o]),$$

where $\boldsymbol{R}$ = gas constant, T = absolute temperature, $[K_i]$ = concentration of K^+ inside the cell, and $[K_o]$ = concentration of K^+ outside the cell. Using the estimates of Fischer and Hsiao (1968) of $[K_i]$ = 300 mM for maximum stomatal opening, when $[K_o]$ = 10 mM the above relation gives W a value of 2000 cal. Estimates of the free energy of hydrolysis of ATP, depending on the conditions within the cell, are in the range 7000–13,000 cal. These estimates suggest that the energy released on the hydrolysis of ATP could account for the uptake of 3·5–6·5 equivalents of K^+ even when the stomata have reached maximum opening, i.e. when the guard cell membranes are fully stretched. During the opening of the stomata the $[K_i]$ would be less but an unknown quantity of energy may be needed in mechanically stretching the guard cell walls and membranes and in overcoming the resistance of these structures to stretching. It has been considered by Cockrell, Harris, and Pressman (1966) that the stoicheiometry between the number of ion equivalents translocated and the

energy equivalents expended on the hydrolysis of ATP may not be constant and could vary so as to adjust for the variable thermodynamic load imposed by a variable ion gradient.

Over the concentration range tested [Section III(*d*)], added ATP gives half maximal stimulation of stomatal opening at approximately 0·014 mM.

The effect that light has in increasing stomatal opening in the presence of ATP [Section III(*e*)] is interesting in that it might be the result of a stimulation in the activity of a transport ATPase. However, the stimulation might also occur due to increases in the permeability of the guard cell membranes or by increases in the metabolic turnover of ATP.

In a bathing medium containing Na^+ as the only cation, the addition of ATP neither initiates, supports, nor maintains stomatal opening. This suggests that the transport ATPase specifically catalyzes the influx of K^+. The closing of Na^+-induced stomatal opening on the addition of ATP suggests that ATP supports a net efflux of Na^+ and that in the presence of an adequate supply of readily available energy substrate, guard cells, in common with many other cells, can maintain a discrimination against Na^+ reaching high concentrations within the cell.

Fujino (1967) considered that stomatal closure was an active process because, in the presence of 10 mM NaF, stomata did not close. The above considerations suggest another explanation. F^- could prevent the utilization of ATP and under these conditions Na^+ could irreversibly flood into the guard cells and prevent stomatal closure.

It seems likely that the addition of ATP to cells could affect the whole spectrum of metabolism. However, the effect that ATP has in causing small stomatal openings when added to a bathing medium of distilled water [Section III(*c*) and Fujino 1967], indicates that the energy released on the hydrolysis of ATP may bring about initial changes in the guard cell wall, membranes, and cytoplasm that are required to adjust these structures to the stretching and change of shape that they undergo when the guard cells swell. The initial configurational changes brought about by the action of ATP are then amplified by ion uptake and accumulation, followed by water influx which leads to osmotic swelling. The transport ATPase, or other ATPases, may form part of a "mechano-enzyme" system which is able to convert the chemical energy released on the hydrolysis of ATP into mechanical work and bring about configurational changes in the cell. Direct effects of ATP on the configuration of chloroplasts have been observed (Packer and Marchant 1964).

The addition of ATP has been observed to cause changes in the colloidal state of proteins extracted from the vascular bundles of tobacco and pumpkin and these proteins have the enzymic activity of an ATPase (Yen and Shih 1965). Similar contractile proteins have been isolated from chloroplasts (Ohnishi 1964). The coiling of tendrils has been associated with a contractile ATPase which causes contraction or expansion of the ventral and dorsal cells of the tendrils (Jaffe and Galston 1967). Similar ATPase-mediated changes may take place in the guard cells.

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VI. References

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