J. Membrane Biol. 12, 145–158 (1973)

ATP-driven Chloride Pumping and ATPase Activity in the *Limonium* Salt Gland

B. S. Hill* and A. E. Hill*

Life Science Institute, The Hebrew University, Jerusalem,
and The Botany School, University of Cambridge, England

Received July 3, 1972

Summary. Using the short-circuit current as a measure of the electrogenic chloride transport in the salt glands of *Limonium*, the effects of various inhibitors, of light-dark changes and of oxygen removal have been studied during steady-state pumping. The results are consistent with the hypothesis that ATP is the energy source for the chloride pump in this system.

When microsomes from salt-loaded tissue are tested for ATPase activity, a substantial fraction of this is found to be chloride-stimulated. In uninduced tissue the Cl-ATPase activity is very much lower, and the induction by salt-loading can be blocked by puromycin. The parallels with Cl-pumping in this tissue are close enough to assume that the Cl-ATPase activity is that of the pump itself; the way is therefore open to study the pump *in vitro*.

The problems and complexities surrounding the demonstration of the precise energy source for chloride pumping in green plant cells has been reviewed in considerable detail by MacRobbie (1970), and although there is some data on higher plant cells, most of the discussion centers around algal systems. The more complicated and sophisticated the experiments become in this field, the less easy it is to draw any clear conclusions from them, and it is perhaps true that the point of diminishing returns has been long passed. In some systems such as *Nitella* (MacRobbie, 1965, 1966), *Hydrodictyon* (Raven, 1967, 1968, 1969), *Tolypella* (Smith, 1968) and *Atriplex* (Lüttge, Pallaghy & Osmond, 1970) it would appear that ATP production is not simply coupled to chloride transport, while in others such as *Chara* (Smith & West, 1969) and *Elodea* (Jeschke, 1967; Jeschke & Simonis, 1969), ATP can be implicated more directly. From none of the work on chloride transport so far described can it be adduced that ATP

* *Present address:* Botany School, University of Cambridge, Downing Street, Cambridge CB2 3EA, England.

is not involved in chloride transport, only that conditions which lead to an increase in the cellular synthesis of ATP and the stimulation of chloride transport do not always go hand in hand. Once one is prepared to invoke a complex control system involving compartmentation and allosteric control, and to admit that the affinities of many of the key enzymes for their substrates are unknown, then the question becomes an open one. In addition to ATP, $NADH_2$ has also been considered as the agent responsible for powering Cl-transport. This could be generated cytoplasmically in the light by some mechanism such as the triose-phosphate shuttle suggested by Latzko and Gibbs (1969), but the direct utilization of $NADH_2$ by the plasma membrane of eucaryotic cells for the performance of osmotic work is very unlikely, although it may occur in bacterial systems. The time is certainly ripe for a shift of emphasis onto the chemistry and energy-transducing properties of plant cell membrane fractions in relation to the various ions of physiological importance. Various microsomal preparations from plant cells have been examined for specific ion-dependent ATPase activity, but little has yet been found. Bonting and Caravaggio (1966) could not detect any Na^+–K^+-ATPase activity in the marine alga *Ulva*, and in the salt-gland-bearing mangrove *Avicennia* Kylin and Gee (1970) found possible evidence for the activity of this enzyme at low salt concentrations, but the data are highly variable and no clear picture emerges whatsoever. In other tissues there seems to be good evidence that ATPase activity can be salt-stimulated (Atkinson & Polya, 1967; Fisher & Hodges, 1969; Lai & Thompson, 1971), but to what extent this is merely due to an increase in the total ionic strength is not clear; chloride is usually the anion in most of these experiments, but its action never seems to have been considered.

With regard to the energetics of chloride transport in *Limonium* we have adopted a combination of the two approaches. Using the short-circuit current as a measure of the electrogenic chloride pumping (Hill & Hill, 1973), the effects of various inhibitors in the light and dark on the current have been recorded to test whether they are consistent with a hypothesis of ATP-linkage. The microsomal fraction has then been tested for chloride-dependent ATPase activity. The *Limonium* gland cell differs from other systems in that the chloride transport takes place from the symplasm of the tissue to the exterior, and during secretory activity the exudation consists of a neutral solution of NaCl when the underlying mesophyll is bathed with this salt; there can be therefore no question of the pump being driven by a coupling with hydrogen ion extrusion (Spear, Barr & Barr, 1969), or by exchange for hydroxyl or bicarbonate ions.

Materials and Methods

Metabolic Studies

All the effects of inhibitors on the secretory process were studied by noting the changes in the short-circuit current drawn from suitably prepared leaf discs (Hill, 1967), for this current can be shown to be a direct measure of the electrogenic chloride transport in the glands (Hill & Hill, 1973). With the current in a steady condition, the inhibitors in 0.1 M NaCl solution were added to both chambers of the short-circuiting apparatus. The current was recorded, and the changes are described in this paper along with representative tracings (Figs. 1, 2 and 3). The inhibitors used were: cyanide (10^{-3} M and 10^{-2} M), DNP (dinitrophenol, 10^{-5} M and 10^{-4} M), CCCP (carbonyl cyanide-*m*-phenylhydrazone, 10^{-5} M) and DCMU (dichlorophenyl-dimethylurea, 10^{-5} M).

Light-dark changes and gassing with nitrogen were performed inside a glass desiccator, through the top of which all the electrical connections to the short-circuit apparatus could be made. The chambers were set up inside and voltage-clamped, the system sealed, and then either air or nitrogen scrubbed of all CO_2 and O_2 could be flushed through the enclosure. The leaf disc was either illuminated with focussed tungsten light of high intensity, or the whole apparatus was blacked out. Where inhibitor solutions were to be used under nitrogen these were first cleared of dissolved oxygen by bubbling O_2-free nitrogen through them overnight.

ATPase Studies

Limonium leaf tissue was collected from plants of very low salt status, washed in water, and cut into fine pieces. It was then salt-loaded with 0.1 M NaCl solution for several hours, in some cases overnight. After loading, it was washed again to remove the bathing solution (*see* Results). Controls to this treatment were soaked in distilled water for a similar period. In some experiments where changes in ATPase levels due to induction of the enzyme by salt-loading were studied, portions of tissue were preloaded in puromycin at a concentration which blocks the development of electrical and secretory activity in the gland (125 mg/liter, *see* Shachar-Hill & Hill, 1970). The tissue was then washed and homogenized at 4 °C in $\times 7$ volumes of medium (Tris ascorbate 0.025 M, sucrose 0.5 M, brought to pH 7 with a small amount of NaOH). The tissue was homogenized for a full minute since the glands are encased in a tough cuticular envelope which must be broken. The homogenate was filtered through muslin and spun at $6{,}000 \times g$ for 10 min to remove all cell debris and most of the organelles; a trial spinning at $10{,}000 \times g$ for 20 min showed little difference in the size or protein content of the final microsomal pellet, and so it was assumed that the initial spin removed most if not all of the mitochondria. The centrifugate was then spun at $100{,}000 \times g$ for 1 hr and the resulting microsomal pellet was suspended in 0.025 M Tris sulphate at pH 7. The resulting suspension was assayed immediately in an assay medium (Tris sulphate buffer pH 7, 0.025 M, Mg^{++} 0.004 M, Tris ATP 0.003 M) containing either sodium chloride (0.1 M) or sodium sulphate (0.033 M). These two media have the same ionic strength (0.1 M), to eliminate any effects due to a general salt-stimulation of the enzymatic activities as opposed to a specific ion-stimulation. There is no specific effect due to sodium ions, and the ATPase activity measured in sodium chloride is no different from that in choline chloride. The assay was run for 1 hr at a temperature of 25 °C after which the reaction was stopped with ice-cold trichloroacetic acid. Phosphate liberated was determined by the method of Fiske and Subbarow (1925).

Results

Energetics

When a short-circuited *Limonium* leaf disc is transferred from dark to light in air, or vice versa, the current continues unchanged for a few seconds after which there is a minor perturbation leading to a new steady-state level. This new level is very near the previous one, sometimes higher, sometimes lower, depending on the preparation. The chloride pumping rates are therefore more or less identical in the light and dark. The removal of O_2 in the dark leads to a decline in the pumping rate and after about 3 hr no electrical activity is detectable (Fig. 1). In this condition the tissue will respond to illumination by giving an electrical signal with very little delay; its size is between 50 and 100% that of tissue kept constantly in the light in air, and it is reversible with a very small time constant (Fig. 2).

In the dark or in low light intensity, cyanide at 10^{-3} M will completely abolish the current over about an hour, but at 10^{-2} M the inhibition is complete in half this time. The uncouplers DNP and CCCP were both effective in suppressing the current, the former acting slowly at 10^{-5} M but quite fast at 10^{-4} M, and the latter being very effective at 10^{-5} M. In the dark, however, CCCP was only partially effective as an inhibitor as shown in Fig. 3.

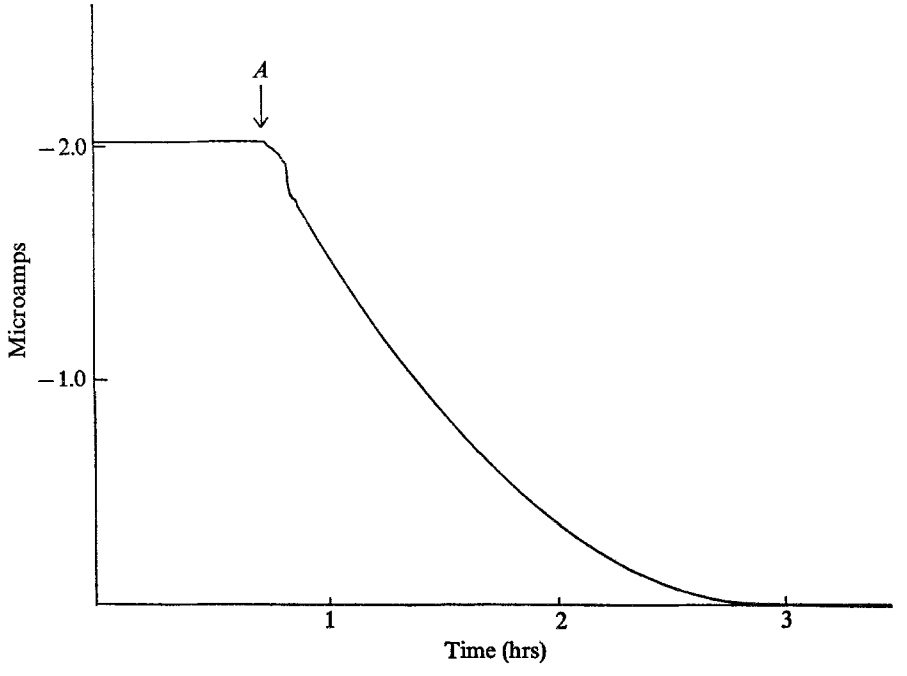

Fig. 1. Decline of the dark short-circuit current due to O_2 removal (*A*)

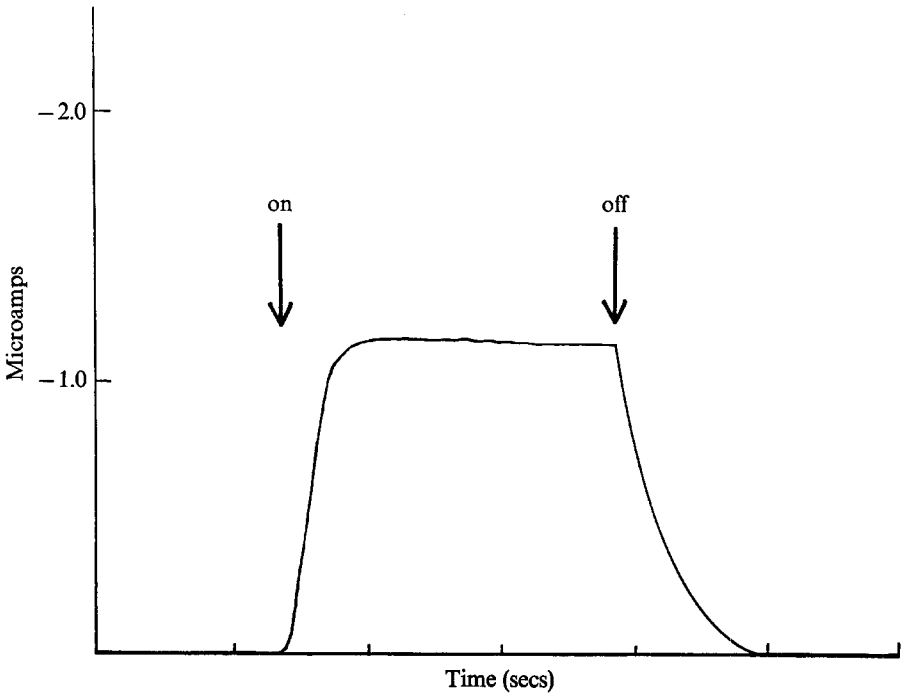

Fig. 2. Response of the short-circuit current to bursts of white light under nitrogen. This response occurs with similar magnitude after pretreatment with 10^{-5} DCMU

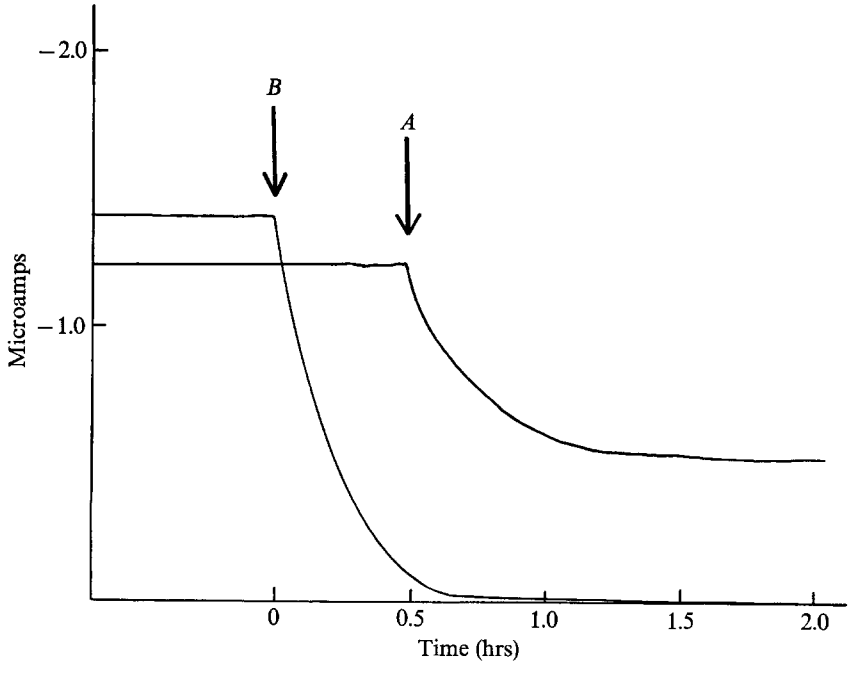

Fig. 3. Effects of the uncoupler CCCP on the short-circuit current. (*A*) In the dark in air. (*B*) In the light under nitrogen

Under CO_2-free nitrogen in the dark, DCMU at 10^{-5} M, was unable to prevent the current rise upon illumination as shown in Fig. 2, even after the leaf discs had been pretreated in this inhibitor overnight in air. A light-stimulated current of at least -1.0 μamp was repeatedly observed, although this sometimes declined a little with time; the chloride pump was certainly capable of being driven by the photosynthetic system operating without photosystem II. CCCP at 10^{-5} M when added to the preparation under nitrogen completely abolished the DCMU-insensitive light current, the tissue becoming electrically dead.

ATPase Activities

When microsomes from salt-loaded tissue were tested for their ability to promote ATP hydrolysis, the rate was four times as high in chloride media as in sulphate media. In Fig. 4 the percentage stimulation is shown, and in Fig. 5 the absolute values of the activities, which were somewhat lower. This is due to the fact that the scatter is quite appreciable, and the

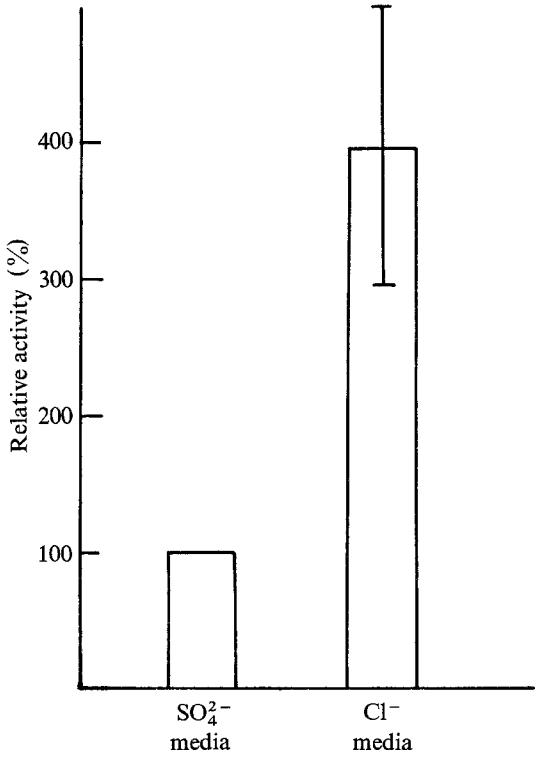

Fig. 4. Percentage stimulation of the microsomal ATPase activity by chloride ions at an ionic strength of 0.1 M. Sulphate ions were used to determine the "basal" ATPase activity

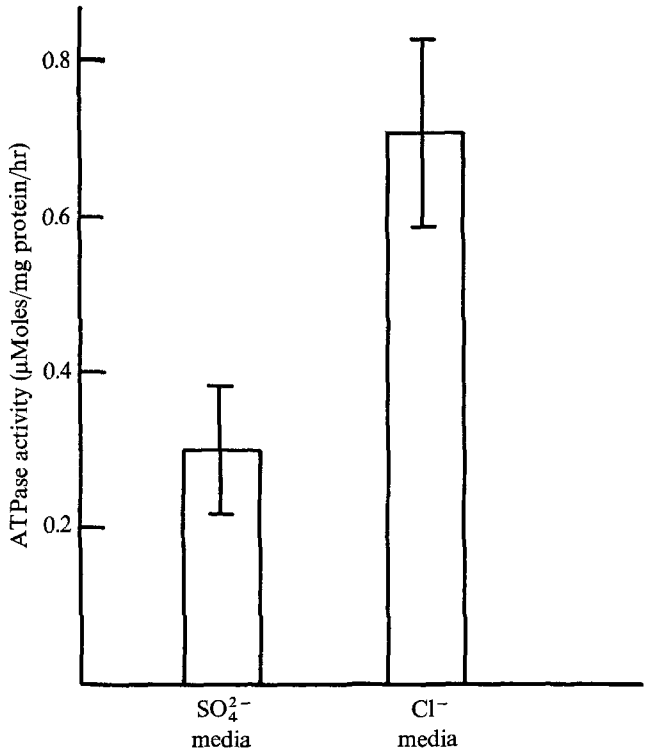

Fig. 5. Absolute values of the microsomal ATPase activity in Cl and Cl-free media

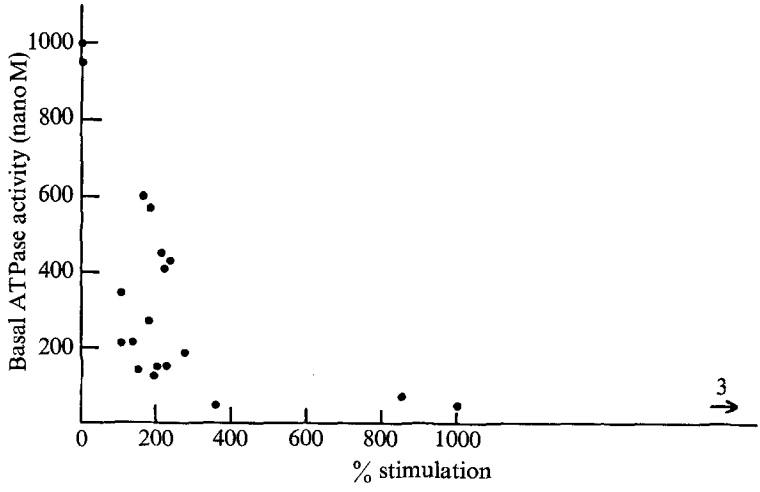

Fig. 6. A plot of the percentage Cl-stimulation of the microsomal ATPase activity against basal ATPase activity for different experiments. There seems to be a hollow relationship with virtually no points showing high stimulation and high activity simultaneously. At bottom right, three points are indicated which were off the scale; these points show no basal activity but enormous percentage Cl stimulations

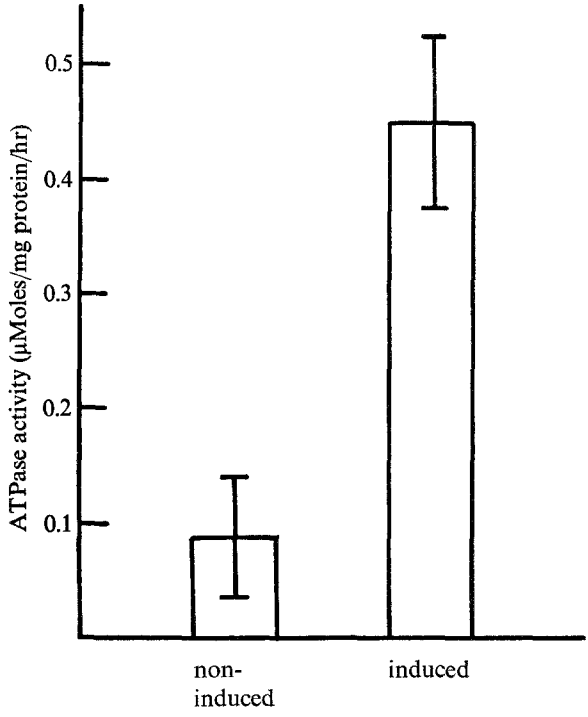

Fig. 7. The increase in Cl-ATPase activity of the microsomal fraction after loading tissue with 0.1 M NaCl for several hours prior to extraction

experiments in which the highest stimulations by chloride ions were demonstrated tended to have smaller absolute ATPase activities, and this is shown in Fig. 6 (*see also* Discussion).

The Cl-stimulated ATPase activity of tissue that had been salt-loaded for some time was much higher than that of tissue treated with water, and as shown in Fig. 7 amounted to about 45% of the control. In a later series of experiments *Limonium* tissue was first preloaded with puromycin overnight and then salt-loaded for 4 to 10 hr in 0.1 M NaCl. Controls run at the same time were aliquots of the total tissue sample salt-loaded without puromycin, or soaked in water. The microsomal fraction was then prepared as usual, and the results are shown in Fig. 8, where *A* is uninduced

Fig. 8. The effect of puromycin in blocking the inductive response to salt-loading. (*A*) Cl-ATPase activity in the microsomes of uninduced tissue. (*B*) Cl-ATPase activity after prior salt-loading. (*C*) Cl-ATPase activity in salt-loaded tissue pretreated with puromycin

Fig. 9. Two experiments in which puromycin was applied to salt-loaded tissue overnight prior to assay. The Cl-ATPase activity is not inhibited but even somewhat enhanced; hatched = puromycin-treated; unhatched = salt-loaded control. Puromycin does not seem to affect the induced activity if applied at this late stage

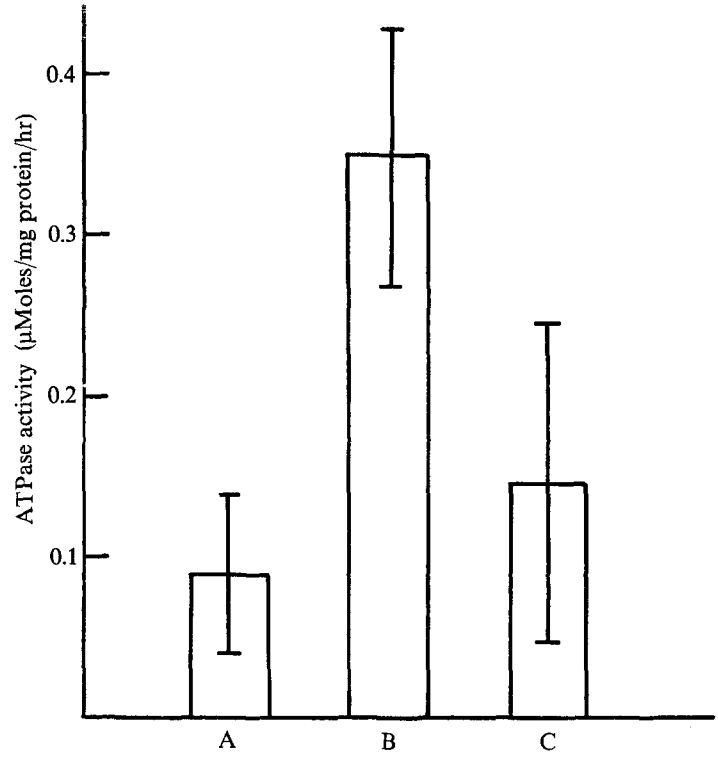
ATPase activity (μMoles/mg protein/hr)
0.4
0.3
0.2
0.1
A
B
C

Fig. 8

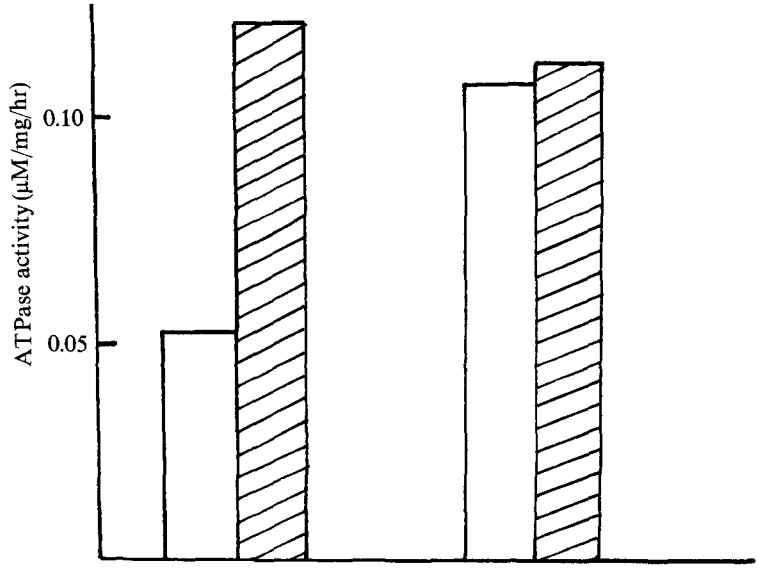
ATPase activity (μM/mg/hr)
0.10
0.05

Fig. 9

material, *B* is induced, and *C* is induced but puromycin-preloaded. The effect of the puromycin is clearly seen in that the level of Cl-ATPase activity (*C*) was not significantly different from that in the uninduced tissue.

Finally, in Fig. 9 are shown the results of two experiments in which the puromycin was applied after several hours of salt-load and left on overnight. The Cl-ATPase activity was then assayed, and it appears that the level of this enzyme seems to be even higher after puromycin treatment. Puromycin does not therefore seem to be causing any inhibition to transport once the system is induced, i.e. to chloride pumping in the steady state.

Discussion

The ability of the *Limonium* gland system to maintain the short-circuit current or secretory potential constant to within quite narrow limits seems to indicate that the control of chloride extrusion is a homeostatic mechanism operating at a constant rate in light or dark. The energy transferring compound must be a diffusible molecule reaching the plasma membranes of the gland cells from either the chloroplasts of the surrounding mesophyll cells, or from the mitochondria of the gland cells themselves. As has been noted already, the secretion is a neutral salt solution excreted to the exterior, and this means that the energy for transport cannot be represented by the concentration gradient of another molecular or ionic species to which the chloride transport is coupled, but must be chemical energy in the form of "high-energy" bonds. This immediately narrows the field down to high energy phosphate esters or reduced pyridine nucleotides of which ATP and $NADH_2$ are the main candidates. We interpret the experiments concerned with the energetics of chloride transport in *Limonium* in the following way: the inhibition of transport in the dark by cyanide and O_2-removal indicates that $NADH_2$ is not involved as this compound accumulates under these conditions unless the membrane itself possesses an electron transport system coupled to chloride transport with a terminal oxidase that is cyanide sensitive. As both O_2-removal and cyanide take some time to act, this is very unlikely; this is also consistent with a slow decline in the cytoplasmic ATP pool. Uncouplers of photophosphorylation such as CCCP are only partially effective in the dark perhaps because they leave glycolytic ATP-production unaffected, and may even stimulate it. In the light, under CO_2-free nitrogen when ATP synthesis can be completely uncoupled from electron transport, CCCP is a very effective inhibitor, although glycolysis can also proceed under these conditions.

The ability of light to power the chloride pump in the presence of DCMU in this system, and the total inhibition of the chloride current by

CCCP under these conditions, are very powerful indications that ATP is the energy source as $NADH_2$ is not synthesized when photosystem II is inoperative. There seems little reason to doubt that DCMU has penetrated after overnight loading at a concentration of 10^{-5} M. ATP thus appears to be the energy transfer compound for chloride pumping in the *Limonium* salt gland.

From an enzymatic standpoint it is quite clear that the ATPase activity of the tissue has to satisfy four criteria if its properties are to complement the transport studies fully. These are (1) there must be a fraction of the ATPase activity of *Limonium* tissue which is Cl-stimulated, in a manner analogous to the Na–K-ATPase of animal cells; (2) this fraction should be inducible, i.e. it should show an increase after salt-loading. (3) The induced rise in Cl-stimulated ATPase activity should be blocked by puromycin at concentrations at which it is effective *in vivo*. (4) The time within which induction occurs should be of the order of a few hours. It seems that all these requirements are fulfilled. The microsomal preparation shows a very high Cl-stimulated fraction (Fig. 4), although in terms of absolute ATPase activity the rate of hydrolysis is low compared with most animal preparations (Fig. 5). The Cl-ATPase activity is rather labile, however, and much of the activity may be lost prior to assay; further work on this system will be directed towards stabilizing the preparation. The scatter in the results of ATPase sensitivity towards chloride are shown in Fig. 6, where percentage stimulations by chloride are plotted against the basal (Cl-free) ATPase activity. The interesting point is that there are virtually no couplings of high Cl-stimulation to high basal activity, and this suggests that the two are mutually incompatible. A reasonable hypothesis would be that the Cl-ATPase loses its control by chloride ions during extraction, leaving an ATPase behind, and this accounts for the high basal activity in preparations that are poorly Cl-stimulated, and vice versa. Whether this is due to a controlling factor lost during the preparative procedure will have to be investigated more fully, but the data as they stand are quite suggestive that this may be so.

In Fig. 7 is shown the appreciable induction of ATPase activity by salt-loading. Although the mean Cl-ATPase activity before loading is not zero, in many individual preparations studied in these experiments it was so: no conclusions can therefore be drawn about the occurrence of this enzyme in other cells. It may be that it is present in the plasma membrane of mesophyll cells, as a constitutive enzyme, and that the induction of it in the gland cells takes place to very high levels; the alternative is that it is absent from the mesophyll but is often present in gland

cells in "uninduced" tissue. This latter proposition is supported by the fact that sometimes glands from uninduced tissue show small short-circuit currents immediately upon salt-loading without any delay, and the transport system seems to be partially induced in these cases.

Puromycin has the effect of suppressing the induction of the Cl-ATPase. In Fig. 8 the Cl-stimulated ATPase levels of microsomes from tissues uninduced, induced, and induced in the presence of puromycin are shown and puromycin is effective in blocking the inductive response; the enzyme levels in tissue preloaded with puromycin are not significantly higher than those in uninduced tissue. This mirrors the effect of this inhibitor of protein synthesis *in vivo*, where it blocks the rise of short-circuit current and secretory potential after salt-loading (Shachar-Hill & Hill, 1970). If puromycin is added to induced material no effect upon secretion is detectable, at least overnight. Similarly, when induced tissue is treated with puromycin overnight and then assayed for Cl-ATPase activity, no inhibition can be recorded; in Fig. 9 are shown the results of two such experiments. It seems evident that puromycin is acting in a classical manner, and that ion-pumping in the gland cells in the steady state is in no way dependent upon protein synthesis or turnover.

Finally, it should be remarked that embedded in the histograms for induced Cl-ATPase activity in Figs. 7 and 8 are the results from several preparations which were salt-loaded for only 4 hr prior to assay; the induced levels in these preparations were well within the standard errors of their respective groups, and this indicates that the enzyme can be fully induced within that time. In previous transport experiments it has been shown that the full secretory capacity is induced within 3 to 4 hr (Hill, 1970; Shachar-Hill & Hill, 1970).

We assume, therefore, that the Cl-ATPase activity demonstrated here is due to the electrogenic chloride pump, and that this is a confirmation that the pump is ATP-driven. Although it is difficult to establish a firm connection between the two processes, the fact that they behave in such a closely parallel way seems to put the matter beyond doubt. The microsomal fraction obviously holds the key to all future work on this transport system, and its vesicular nature offers a prospect of being able to study chloride uptake in a cell-free system.

A. E. H. wishes to thank the Royal Society for full research support, and we should both like to thank Professor W. D. Stein for his kind hospitality and support during the early stages of this work, and E. A. C. MacRobbie for her support during the later stages at Cambridge.

References

Atkinson, M. R., Polya, G. M. 1967. Salt-stimulated adenosine triphosphatases from carrot, beet and *Chara australis*. *Aust. J. Biol. Sci.* **20**:1069.

Bonting, S. L., Caravaggio, L. L. 1966. Studies on Na^+–K^+-activated adenosine triphosphatase. XVI. Its absence from the cation transport system of *Ulva lactuca*. *Biochim. Biophys. Acta* **112**:519.

Fisher, J., Hodges, T. K. 1969. Monovalent ion stimulated adenosine triphosphatase from oat roots. *Plant Physiol.* **44**:385.

Fiske, C. H., Subbarow, Y. 1925. The colorimetric determination of phosphorus. *J. Biol. Chem.* **66**:375.

Hill, A. E. 1967. Ion and water transport in *Limonium*. II. Short-circuit analysis. *Biochim. Biophys. Acta* **135**:461.

Hill, A. E. 1970. Ion and water transport in *Limonium*. IV. Delay effects in the transport system. *Biochim. Biophys. Acta* **196**:73.

Hill, A. E., Hill, B. S. 1973. The electrogenic chloride pump of the *Limonium* salt gland. *J. Membrane Biol.* **12**:129.

Jeschke, W. D. 1967. Die cyclische und die nichtcyclische Photophosphorylierung als Energiequellen der lichtabhängigen Chloridionaufnahme bei *Elodea*. *Planta* **73**:161.

Jeschke, W. D., Simonis, W. 1969. Über die Wirkung von CO_2 auf die lichtabhängige Cl^--Aufnahme bei *Elodea densa*; Regulation zwischen nichtcyclischer und cyclischer Photophosphorylierung. *Planta* **88**:157.

Kylin, A., Gee, R. 1970. Adenosine triphosphatase in leaves of the mangrove, *Avicennia nitida* Jacq. Influence of sodium to potassium ratios and salt concentrations. *Plant Physiol.* **45**:169.

Lai, Y. F., Thompson, J. E. 1971. The preparation and properties of an isolated plant membrane fraction enriched in (Na^+–K^+)-stimulated ATPase. *Biochim. Biophys. Acta* **233**:84.

Latzko, E., Gibbs, M. 1969. Levels of photosynthetic intermediates in isolated spinach chloroplasts. *Plant Physiol.* **43**:396.

Lüttge, U., Pallaghy, C. K., Osmond, C. B. 1970. Coupling of ion transport in green cells of *Atriplex spongiosa* leaves to energy sources in the light and in the dark. *J. Membrane Biol.* **2**:17.

MacRobbie, E. A. C. 1965. The nature of the coupling between light energy and active ion transport in *Nitella translucens*. *Biochim. Biophys. Acta* **94**:64.

MacRobbie, E. A. C. 1966. Metabolic effects on ion transport in *Nitella translucens*. I. Active influxes. *Aust. J. Biol. Sci.* **19**:363.

MacRobbie, E. A. C. 1970. The active transport of ions in plant cells. *Quart. Rev. Biophys.* **3**:251.

Raven, J. A. 1967. Light-stimulation of active ion transport in *Hydrodictyon africanum*. *J. Gen. Physiol.* **50**:1627.

Raven, J. A. 1968. Photosynthesis and light-stimulated ion transport in *Hydrodictyon africanum*. *Abh. dt. Akad. Wiss. Berl.* **4***a*:145.

Raven, J. A. 1969. Effects of inhibitors on photosynthesis and the active influxes of K and Cl in *Hydrodictyon africanum*. *New Phytol.* **69**:1089.

Shachar-Hill, B., Hill, A. E. 1970. Ion and water transport in *Limonium*. VI. The induction of chloride pumping. *Biochim. Biophys. Acta* **211**:313.

Smith, F. A. 1968. Metabolic effects on ion fluxes in *Tolypella intricata*. *J. Exp. Bot.* **19**:442.

Smith, F. A., West, K. R. 1969. A comparison of the effects of metabolic inhibitors on chloride uptake and photosynthesis in *Chara corallina*. *Aust. J. Biol. Sci.* **22**:351.

Spear, D. G., Barr, J. K., Barr, C. E. 1969. Localisation of hydrogen ion and chloride ion fluxes in *Nitella*. *J. Gen. Physiol.* **54**:397.