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The Energetics of Cl^- Active Transport in *Chara*

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Summary. The rate of Cl^- influx in intact *Chara* was inhibited whenever the ATP concentration was reduced by application of metabolic inhibitors. In perfused cells, however, a net influx of Cl^- against its electrochemical gradient could be observed in the absence of ATP. Addition of ATP to the perfusion medium slightly stimulated Cl^- influx in one experiment but had no effect in another. Addition of ADP, NADH or metabolic inhibitors did not alter the influx rate. Consideration of the potential energy gradients across the *Chara* plasmalemma in the perfused state leads to the conclusion that Cl^- influx occurs by cotransport with H^+ or OH^-.

Key Words *Chara* · intracellular perfusion · chloride uptake · proton cotransport

Introduction

There are now a wide range of factors known to affect transport reactions. These factors include turgor pressure (Kamiya & Kuroda, 1956; Tazawa, 1961; Cram, 1976) and pH (Smith & Raven, 1979). The rate of a reaction will, in general, vary with the driving force – including, for active transport, values of the components of ΔG for the driving reaction. Given the low energy consumption of most membrane transport reactions (Hope & Walker, 1975) there is no *a priori* reason why ΔG for the driving reaction must be a limitation of the rate except under conditions of extreme energy stress. There are, however, many observations of reduction in ion transport rates when phosphorylation is inhibited. In many cases the inhibition of transport can be quite large for a relatively slight inhibition of energy transduction (Raven, 1969, 1971; Lin & Hanson, 1974; Wildes, Pitman & Schaefer, 1976). This could mean that the rates of active transport processes are acutely sensitive to the values of ΔG for the driving reactions; but it could equally mean that sensitive regulatory systems reduce the demand for ATP when its production is upset. Many things may be affected if ATP production is slowed: it will be difficult to say which one has reduced an ion transport rate. Similarly, in cases where the ATP concentration remains constant but the rate of active ion transport changes (*see*, for example, Cram, 1983) it is often difficult to distinguish between an effect of transport itself on the ΔG for the reaction and an effect of a regulatory mechanism which is not directly related to the driving force. Some of these difficulties can be overcome using perfused plasma membranes. Intracellular perfusion has been used to study the electrical properties of the plasmalemma in *Chara* (Tazawa, Kikuyama & Shimmen, 1976; Smith & Walker, 1981). It is now possible to obtain perfused cells with plasmalemmas whose electrical characteristics are similar to those of intact cells (Smith & Walker, 1981). This technique has mostly been used to study electrogenic H^+ pumping. Although Sanders (1978, 1980*a*) used intracellularly perfused *Chara* to study regulation of Cl^- influx by the internal Cl^- concentration and pH, he was not able to demonstrate that the integrity of the plasmalemma was preserved after perfusion. Consequently, there is uncertainty as to the degree to which his results relate to the plasmalemma of intact cells.

In the current work we have investigated the nature of the driving force for Cl^- active transport in *Chara* using both intact and perfused cells. Of the sources of energy for Cl^- uptake in *Chara*, influx driven by $\Delta\mu_H{}^+$ (Smith, 1970) or directly by a Cl^--ATPase appeared to be the most probable. Evidence is presented here to show that ATP is not directly required for Cl^- active transport and that the probable mechanism is Cl^- cotransport with one or more protons as suggested by Sanders (1980*a*) and Beilby and Walker (1981).

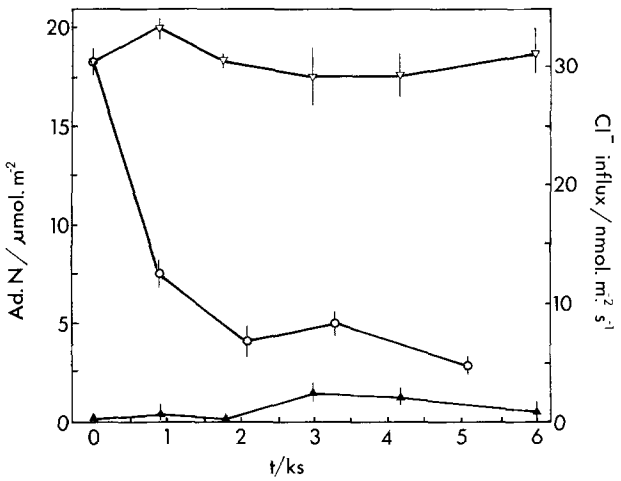

Fig. 1. Effect of 5 μM CCCP on ATP content (▽), ADP content (▲) and Cl^- influx (○) in the light. $pH_o = 7.5$. Means ± SEM of 18 to 20 cells

Material and Methods

Chara corallina Klein ex Will. = australis R.Br.) was either (a) cultured in the laboratory in large glass tanks or a 15 hr/9 hr light/dark cycle or (b) collected from cultures grown outdoors in concrete tubs in a shaded position. Before use, internodal cells were isolated from neighboring cells and kept for 1 to 2 days in artificial pond water (APW: 1 mM NaCl, 0.2 mM KCl, 0.05 mM $CaSO_4$ + 5 mM zwitterionic buffer adjusted to the desired pH with NaOH). Experiments were conducted at 20 to 22° C in darkness or under fluorescent lighting with a photon flux of 60 μmol m^{-2} s^{-1}.

In the study of time courses of effects of inhibitors, Cl^- influxes and adenylate concentrations were measured for each cell and means calculated for batches of cells. Cl^- influx was measured by conventional tracer techniques using ^{36}Cl. Uptake of ^{36}Cl was allowed for 1.2 ksec followed by 0.3 ksec rinse after which the cell was frozen for measurement of adenylate concentrations. This procedure results in a time difference between the average influx time and the time when the adenylate concentrations were estimated. Because of this it was normally necessary in the time courses to extract adenylates from an equivalent batch of cells at the mean time of the first influx period to give an early measure of adenylate concentrations. Except for this case, the same cells were used for influx and adenylate determinations. The methods of extraction and estimation of ATP, ADP and AMP are published elsowhere (Reid & Walker, 1983).

Perfusion Methods

Two perfusion methods were used. The basic perfusion technique was, as far as practicable, the same as that used by Smith and Walker (1981). A full description of the method and the electrical characteristics of the plasmalemma after perfusion are given in that paper. Briefly, an internodal cell was mounted on a perspex block having three compartments. The two end compartments were bathed in perfusion medium (PM: mM approx. 130 K^+, 2.5 EGTA, 1 Mg^{2+}, 140 MES; pH 7.8).

The center compartment which exposed a length of cell 10 mm was irrigated with flowing medium (OM: mM approx. 0.1 K^+, 0 to 0.5 Cl^-, 0.2 Na^+, 0.2 Ca^{2+}, 250 sorbitol, 2 MOPS; pH 7.0) at a rate of approximately 20 μl s^{-1}.

The osmotic potentials of the OM and PM were carefully adjusted for each batch of cells so they barely plasmolyzed the cells. Correct adjustment of these solutions was critical for a successful perfusion. To start perfusion the ends of the cell were removed and the solution surface was raised at one end by 4 to 6 mm so that PM flowed through the cell. Flow continued for 50 to 100 sec after which the cell was left to allow dissolution of the tonoplast. This was detected by the cessation of protoplasmic streaming. Introduction of ATP to the inside of the cell was made by adding it to the pool of PM at one end and inducing a flow through the cell. For determination of Cl^- influx ^{36}Cl was added to the OM for 0.6 ksec followed by a rinse of 0.6 ksec.

The second perfusion method (designated "forced flow") involved sealing a fine glass cone over one of the cut ends of the cell. The narrow end of the cone was connected via plastic tubing to a syringe drive so that PM could be pumped through the cell. There are several advantages of this technique for the study of ion fluxes. Firstly, the inside of the cell can be perfused at a controlled rate. This procedure avoids sudden gushes of solution which might damage the cell and allows the vacuolar contents to be more completely removed. This is important when studying the effect of internal concentrations of ions such as Cl^- whose concentration may be high in the vacuole but low in the cytoplasm. A further advantage of this technique is that one can obtain more than a single influx measurement on a cell because the solution flowing through the cell can be easily changed.

Results

Effects of Inhibitors on Intact Cells

The time courses of the effects of 5 μM CCCP on Cl^- influx and on the concentrations of ATP and ADP are shown in Fig. 1. The concentration of ATP changed slightly and there were reciprocal changes in the concentration of ADP, but after 6 ksec of treatment the concentrations were not significantly different from those of the control. In contrast, the rate of Cl^- influx in the light was reduced by more than 85%. The pH of the treatment solution was important in determining whether the ATP concentration was affected by a particular concentration of CCCP. At pH 7.5 there was usually little or no effect of 5 μM CCCP on the concentration of ATP in the light (as in Fig. 1) but at pH 6.5 the ATP concentration was significantly reduced (*results not shown*). This is presumably due to the more rapid penetration of neutral CCCP or its greater accumulation in the cytoplasm at low pH_o. In the dark in 5 μM CCCP, the Cl^- influx declined much more rapidly that the ATP concentration (Fig. 2). After only 0.9 ksec the influx was 4% of the control, but the ATP concentration was still 96% of the control. The maximum reduction in ATP concentration was 43% after 1.8 ksec. The changes in ADP concentration were always in the opposite direction to those of ATP, but were smaller.

The effects of 1 μM DCMU are shown in Fig. 3. The ATP concentration after 5.4 ksec (18.0 ± 0.7)

was significantly higher than the dark control (14.4 ± 0.8). The Cl^- influx was reduced to a much greater extent and the final value after 6.6 ksec was lower than the dark control rate. In a separate experiment the Cl^- influx and ATP concentration after 3.6 and 4.5 ksec, respectively, were not significantly different from the dark control values (Table 1). The ADP concentration in this experiment was also similar to that in the dark but the AMP concentration was lower in the light in the presence of DCMU than in the dark (Table 1). Concentrations of DCMU up to 5 μM did not cause a significantly greater reduction in the final ATP concentration than did 1 μM.

The effect of oligomycin on the concentrations of ATP, ADP and AMP and on the Cl^- influx in the light is shown in Fig. 4. At 3 μg ml^{-1} the Cl^- influx was inhibited by 87% but the ATP concentration was reduced by only 14%. In the dark, the Cl^- influx was strongly inhibited by oligomycin at 5 μg ml^{-1} and ATP was reduced to 55% of the control value (Table 1). A feature of the effect of oligomycin on the adenylate pool which was not observed with other inhibitors was the absence of any increase in the concentration of ADP in either light or dark suggesting a mode of inhibition of ATP production which is different to that of the other inhibitors.

L-ethionine has been shown to reduce ATP concentrations in higher plant roots (Atkinson & Polya, 1968; Lin & Hanson, 1974) by trapping adenosine as s-adenosyl ethionine. It had no effect in *Chara* on the concentrations of adenylates in either light or dark (Table 1). The Cl^- influx was slightly reduced in the light after 6 ksec but not in the dark. Cyanide at 1 mM reduced the ATP

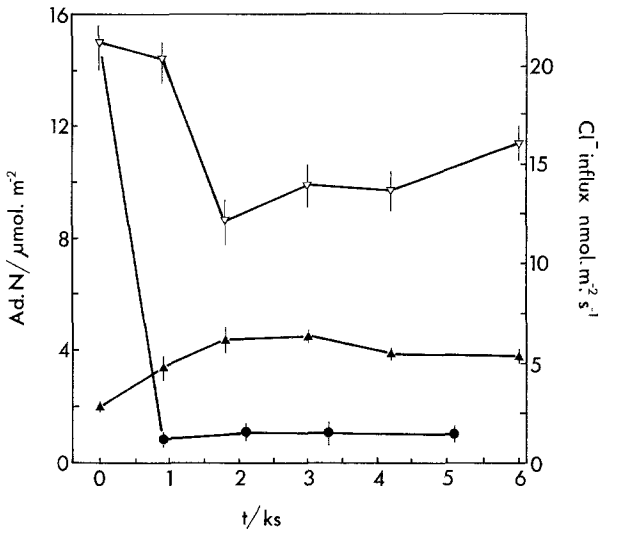

Fig. 2. Effect of 5 μM CCCP on ATP content (▽), ADP content (▲), and Cl^- influx (●) in darkness. $pH_o = 7.5$. Means ± SEM of 18 to 20 cells

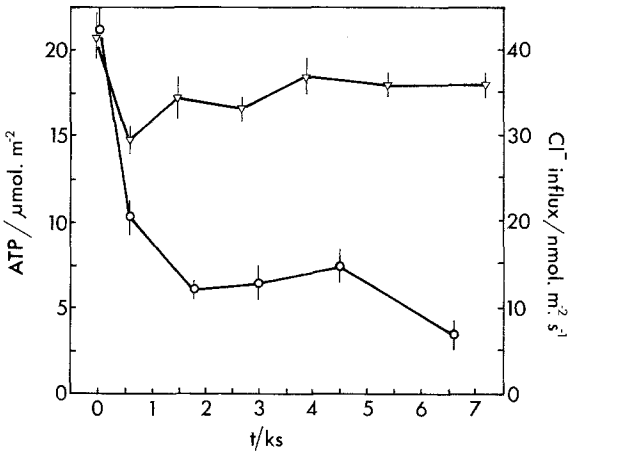

Fig. 3. Effect of 1 μM DCMU on ATP content (▽) and Cl^- influx (○) in the light. $pH_o = 7.5$. Mean ± SEM of 18 to 20 cells

Table 1. Effects of inhibitors on ATP, ADP and AMP concentrations and Cl^- influx

	Cl^- influx (nmol m^{-2} sec^{-1})	[ATP] (μmol m^{-2})	[ADP] (μmol m^{-2})	[AMP] (μmol m^{-2})
Expt. 1 Light:				
Control	14.4±2.0	18.6±0.5	1.0±0.3	0.3±0.3
Ethionine, 1 mM	10.9±1.4	20.3±1.2	1.0±0.1	0.6±0.2
DCMU, 1 μM	2.8±0.6	16.9±0.6	2.0±0.2	0.5±0.2
Dark, control	3.2±0.7	16.2±0.8	2.1±0.2	1.3±0.2
Expt. 2 Light:				
Control	26.0±1.7	20.0±1.0	2.1±0.3	0 ±0.3
Cyanide, 1 mM	14.9±1.5	18.8±0.9	1.8±0.2	0.6±0.4
Expt. 2 Dark:				
Control	8.8±1.2	18.2±0.8	1.7±0.3	1.1±0.6
Oligomycin				
1 μg/ml	8.0±0.9	14.9±0.9	1.6±0.2	1.0±0.4
5 μg/ml	1.2±0.3	8.3±0.7	1.3±0.2	4.7±0.5
Cyanide, 1 mM	10.0±1.0	17.7±1.2	1.5±0.5	0.6±0.5
Ethionine, 1 mM	11.7±1.5	18.6±1.4	1.6±1.4	1.6±0.3

All pretreatments with inhibitors were 3.6 ksec, ^{36}Cl influx 1.2 ksec. Adenylates measured after a total of 4.1 ksec treatment with inhibitor. Solution: APW-HEPES, pH 7.5.

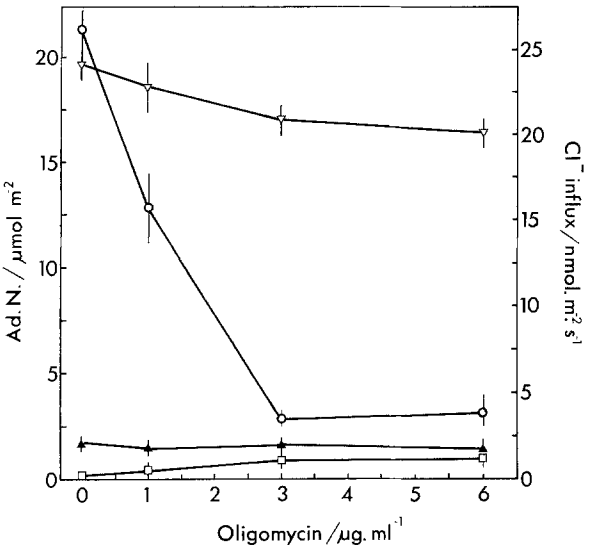

Fig. 4. Effect of oligomycin on ATP (▽), ADP (▲), AMP (□) and Cl^- influx (○). Light, $pH_o = 7.5$. Mean ± SEM of 18 to 20 cells. All parameters measured on the same cell

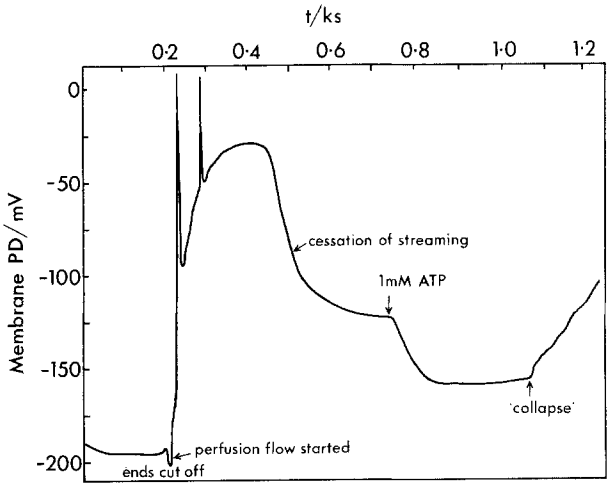

Fig. 5. PD recording of a cell during perfusion

concentration by 10% and the Cl^- influx by 43%, but no effect was observed in darkness (Table 1).

Characterization of Perfused Cells

The normal sequence of events during perfusion is shown in Fig. 5. Cutting the ends of the cell commonly caused one or more action potentials superimposed on a general depolarization to between −40 and −90 mV. There was then a hyperpolarization to a plateau of −115 to −130 mV; this was thought to represent the plasmalemma PD since the hyperpolarization coincided with the loss of the tonoplast. This plateau potential was close to the diffusion potential for K^+ (E_k) and was sensitive to changes in the external concentration of

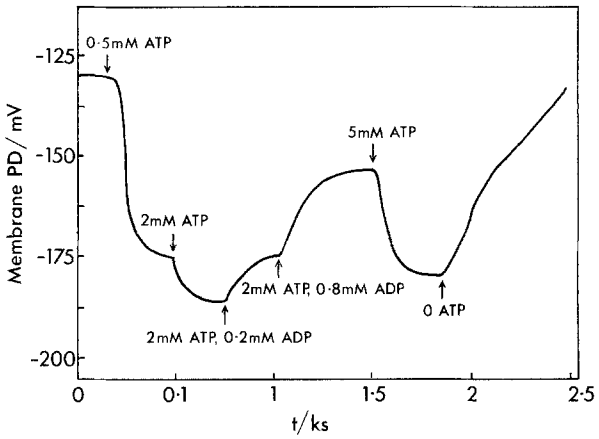

Fig. 6. Effects of added ATP and ADP on the PD of a perfused cell

K^+. If the perfusion was successful the PD would remain stable at this value for at least 1 to 2 ksec during which time Cl^- influx could be determined. However, if the perfusion was too rapid or the osmotic potentials of the solutions not correctly adjusted then the PD was liable to depolarize slowly. This occurrence was termed "collapse" and probably represents irreversible damage to the plasmalemma. Because of the possibility of this collapse it was necessary to monitor the PD during the entire period of exposure of the cell to ^{36}Cl. Cells which collapsed were discarded.

Successfully perfused cells hyperpolarized further on addition of ATP but the change in PD was highly variable, ranging from 2 to 60 mV. Figure 6 shows the trace of a perfused cell which gave a large hyperpolarization on addition of ATP. This degree of hyperpolarization was rarely encountered; 5 to 10 mV was generally found. However, Fig. 6 does show clearly the effects of different concentrations of adenylates. The cell was perfused using "forced flow" to give more reliable internal adenylate concentrations. The PD was a function of both the ADP and ATP concentrations.

As far as could be determined the cells perfused in this way retained the membrane intact. After perfusion, the cells could maintain a stable diffusion potential and were sensitive to ATP and ADP. Because of the similarity of the perfusion technique and the PDs to those of Smith and Walker (1981) the conductance of the membrane is expected to be like that of the intact cell, as shown by them.

Chloride Influx in Perfused Cells

Table 2 summarizes the Cl^- influx data for perfused cells. In the absence of added ATP the Cl^- influx was equivalent to that of intact cells. That

Table 2. Effects of ATP, ADP and NADH on Cl^- influx in perfused cells

Expt.1. "Forced-flow" perfusion		
Control	23.5 ± 4.3	(5 pairs)
ATP (1 mM)	26.1 ± 3.7	
Expt. 2. Normal perfusion		
Control	21.4 ± 4.3	(12)
ATP (0.2 to 2 mM)	24.4 ± 2.4	(14)
2 mM ATP, 0.5 mM ADP	19.1, 20.3	
ADP (0.5 mM)	19.6	
NADH (0.1 mM)	18.4	
1 mM ATP + 1 μM DCMU	21.2, 22.6	
1 mM ATP + 5 μM CCCP	18.7, 29.0	

Number of determinations shown in brackets. OM and PM as in Table 2 except that $Cl_i^- = 0$ (Expt. 1), $Cl_i^- = 0.5$ mM (Expt. 2). Control and +ATP significantly different at $p = 0.05$ by Wilcoxon Signed Rank Test in Expt. 1. There was no significant difference between control and any treatment in Expt. 2.

Table 3. Cl^- efflux from perfused cells

	Cl^- efflux (nmol m^{-2} s^{-1})
Control	1.36 ± 0.4 (0.51, 1.83, 1.73)
+1 mM ATP	1.38 ± 0.3 (1.81, 0.85, 1.47)

$Cl_i = 1$ mM.

this was not due to exchange diffusion is indicated by the low Cl^- efflux from these cells (Table 3). There is therefore a substantial uptake of Cl^- in the absence of ATP.

Addition of 1 mM ATP during "forced flow" perfusion resulted in a slight (11%) stimulation of influx. The results of this experiment are in the form of pairs of values since influxes with and without ATP were measured on each cell. The stimulation is statistically significant. No such stimulation of influx was observed using the normal perfusion method with a much larger number of cells. The possibility that chloroplast or mitochondrial activity in the perfused cell was producing ATP was tested by adding 1 μM DCMU or 5 μM CCCP to the perfusion stream. No effect was observed with either inhibitor. ADP, either alone or in the presence of ATP did not affect the influx rate, nor did 0.1 mM NADH.

There was no correlation between the degree of ATP-induced hyperpolarization of the membrane and Cl^- influx.

Discussion

The results for perfused cells show that normal Cl^- influx can occur in the absence of ATP. Because the efflux was low there was in these experiments a substantial net uptake of Cl^-. Under these conditions net Cl^- uptake is an uphill process requiring an energy input of more than 12.5 kJ mol^{-1} if the internal Cl^- concentration was greater than 0.5 mM. This energy is not provided by metabolism as was indicated by the failure of inhibitors to inhibit uptake, nor was it provided by ATP, ADP or NADH. Presumably the energy is derived from the electrochemical potential difference of another ion. The electrochemical potential difference for K^+ ($\Delta\mu_K{}^+$) is low in perfused cells without ATP because the membrane PD is then close to E_K. Symport of Cl^- with Na^+ would be energetically feasible since $[Na^+]_i$ was nearly zero in the perfused cell. However Sanders (1978, 1980*a*) demonstrated with both intact and perfused cells that Cl^- influx continued when $[Na^+]_o$ was zero and Beilby and Walker (1981) showed Cl^- to depolarize the membrane in the absence of both Na^+ and K^+. Ca^{2+} does not show a sufficiently large flux in intact cells (Spanswick & Williams, 1964) to be responsible for uptake of Cl^- by cotransport. The remaining candidate is H^+ whose electrochemical potential difference in the perfused cells was equivalent to 17.0 kJ mol^{-1} (PD = 0.13 V, $pH_o = 7.0$, $pH_i = 7.8$). Cl^- cotransport with 1 H^+ as suggested by Smith (1970) could support uptake from an external Cl^- concentration of 0.5 mM to an internal concentration of 3.1 mM, while cotransport of Cl^- with 2 H^+ as suggested by Smith and Walker (1976) could occur against an internal concentration up to 3.3 M. Sanders (1978, 1980*a*) also considered that Cl^- influx would operate by cotransport with H^+ but he also postulated an additional requirement for ATP to explain the fourfold stimulation of influx that he found in perfused cells. The effect of ATP seen by Sanders might be a stimulation of influx caused either by a change in internal pH, to which the influx was highly sensitive in his perfused cells, or in the electrochemical potential difference for H^+ ($\Delta\mu_H{}^+$) brought about by the perfusion method. The technique used by Sanders involved ligating the cell after perfusion which in effect left the interior stagnant. Under these conditions operation of an H^+-ATPase, which almost certainly occurs in perfused cells (Shimmen & Tazawa, 1977; Smith & Walker, 1981) could lead to changes in $\Delta\mu_H{}^+$ especially in unstirred layers adjacent to the plasmalemma.

The same explanation can be applied to the slight stimulation of influx by ATP (Table 2) using forced-flow perfusion but not using the basic perfusion method. In the latter, the solution inside

the membrane was continually agitated by a stirrer that alternately sucked and expelled a small volume of solution from one of the end reservoirs thus reducing any effect of unstirred layers. In the former, however, after introduction of ATP into the cell there was no further pumping of perfusion solution and the internal contents therefore remained stagnant during the influx period. Despite the open ends of the cell and the high internal buffering, localized variations in pH brought about by H^+ pumping might lead to the slight stimulation of influx by ATP which was observed using this method.

While there is circumstantial evidence that $\Delta\mu_H{}^+$ is generated by an H^+-ATPase, it has not been established that changes in ATP concentration affect $\Delta\mu_H{}^+$ or that changes in $\Delta\mu_H{}^+$ control the Cl^- influx. The inhibitor studies with intact cells provide some information on the nature of the link between Cl^- influx and ATP concentration. The results showed that the Cl^- influx was much more sensitive to metabolic inhibition than was the concentration of ATP. Although it was possible to inhibit influx without measurably affecting ATP, the Cl^- influx was always inhibited when the ATP concentration was reduced. In those instances where the ATP concentration was not affected but the influx inhibited (CCCP and ethionine – light) it seems likely that the inhibitors were acting on the influx mechanism directly or perturbing a quantity which regulates the rate. There is some evidence which supports this proposition. Firstly, CCCP is known to alter the electrical characteristics of the plasma membrane of *Riccia* at concentrations which are too low to inhibit ATP production (Felle & Bentrup, 1977). Secondly, the effect of ethionine is not consistent with its proposed mode of action. It clearly did not act effectively as a trap for adenosine because the ATP concentration in both light and dark remained unchanged. In the absence of an effect on ATP concentration, its inhibition of Cl^- influx is difficult to interpret.

If the results using these inhibitors can be set aside then the picture that emerges is of small reductions in ATP concentrations associated with large inhibitions of Cl^- influx. The effects of oligomycin, DCMU and cyanide all support this view. This situation also exists under normal conditions where, between light and dark the influx rates can change by a factor of 5 and the ATP concentration by only 5 to 10%. For example, in Table 1 the dark controls for Cl^- influx and ATP concentration were 22 and 87%, respectively, of the light controls in experiment 1, and 34 and 91%

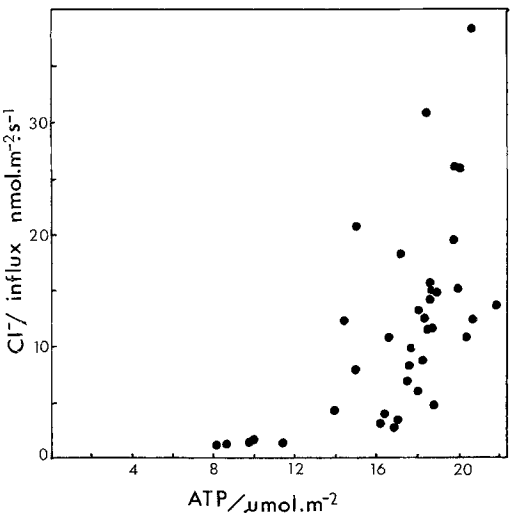

Fig. 7. Distribution of means of Cl^- influx and ATP content of batches of cells from Figs 1–4 and Table 1. Each point is the mean of 10 to 20 cells on which both Cl^- influx rate and ATP content were measured

in experiment 2. The relationship is more clearly seen when the results are shown graphically. Figure 7 is the plot of Cl^- influx rates versus ATP concentrations for all of the batches of cells shown in Figs. 1–4 and Table 1. Each point is the mean of 10 to 20 cells on which both Cl^- influx and ATP concentrations were determined. Statistically there is a significant association between the two quantities. Although the exact nature of this association is unclear there are several aspects of the graph worth noting. Firstly, the Cl^- influx can have any rate from zero to 40 nmol m^{-2} s^{-1} for ATP concentrations in the narrow range of 17 to 21 µmol m^{-2}. Secondly, there are no points in which Cl^- influx is high when ATP is even moderately low. This implies that in intact cells net Cl^- influx may require the ATP concentration to be high. If this reflects an effect of ATP on the driving force then either $\Delta\mu_H{}^+$ is very sensitive to changes in ATP concentration or Cl^- influx is very sensitive to changes in $\Delta\mu_H{}^+$. Figure 6 shows that the electrogenic pump which is probably responsible for maintenance of $\Delta\mu_H{}^+$ is not acutely sensitive to changes in ATP concentration; there is also no obvious teleological reason why the Cl^- influx should be so tightly tied to $\Delta\mu_H{}^+$. An alternative proposition is that the sensitivity to ATP concentration is possessed by a factor which regulates the influx rate such as cytoplasmic pH or cytoplasmic Cl^- concentration. The cytoplasmic pH is probably influenced by the plasmalemma H^+ pump driven by ATP. A difference in pH_c between light and dark of up to 0.2 units has been measured

in some experiments (Walker & Smith, 1975; Reid, 1980). From the data of Sanders (1980*a*) such a change would significantly reduce the influx and might account for the total difference between light and dark.

Feedback regulation of influx by the cytoplasmic Cl^- concentration has also been proposed (Sanders 1978, 1980*b*). The level of cytoplasmic Cl^- is presumably influenced by the rate of Cl^- transport to the vacuole and organelles. Inhibition of influx of Cl^- at the plasmalemma by an increased cytoplasmic concentration as a result of reduced ATP supply would only be feasible if transport of Cl^- from the cytoplasm were to be an active process. If the PD between the cytoplasm and vacuole is +18 mV (Vorobiev, 1967) and the Cl^- concentration is around 123 mM (Bostrom, 1976), transport of Cl^- across the tonoplast would require an energy input unless the cytoplasmic concentration was greater than about 56 mM. This reaction might therefore be the ATP-sensitive process which exerts the strongest effect on the influx rate.

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