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INTRACELLULAR SODIUM AND POTASSIUM CONCENTRATIONS AND NET CATION MOVEMENTS IN *CHLORELLA PYRENOIDOSA*

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SUMMARY

1. Chlorella cells will accumulate K^+ to a high internal level if this cation is present in the bathing medium.

2. In the absence of external K^+ these cells will accumulate Na^+.

3. When K^+ was added to a suspension of these Na^+-rich cells a relatively rapid exchange of internal Na^+ for external K^+ occurred with a half time of less than 10 min.

4. During this change of internal Na^+ for K^+ there was no obvious change in the rates of respiration or photosynthesis.

5. The Na^+–K^+ exchange was sensitive to light and temperature and seemed to be under metabolic control.

6. Inhibition of the net fluxes with carbonyl cyanide *m*-chlorophenylhydrazone and *N,N'*-dicyclohexylcarbodiimide implicates phosphorylation and a membrane-bound ATPase with the exchange.

7. The action of 3-(3,4-dichlorophenyl)-1,1-dimethylurea and anaerobic conditions on illuminated cells suggested that the Na^+–K^+ exchange can be supported by cyclic phosphorylation.

8. The Na^+–K^+ exchange was not inhibited by the cardiac glycoside, ouabain.

9. In addition to the Na^+–K^+ exchange there also seemed to be a H^+–K^+ exchange which represented about 10% of the total net cation movement.

INTRODUCTION

Like many other microorganisms *Chlorella pyrenoidosa* is able to accumulate K^+ in preference to Na^+. It has been suggested that the distribution of these two ions between the cell interior and environment resulted from active transport mechanisms possibly sited in the plasma membrane[1]. This conclusion was partly based on measurements involving the estimation of the electrical and ionic gradients across the cell membrane[1] and partly from tracer studies under steady-state conditions[2–4]. However, unlike *Escherichia coli*[5] and *Streptococcus faecalis*[6], it has not been possible with Chlorella to observe large net movements of K^+ and Na^+ across the cell membrane simply by disturbing metabolic activity. For example, with the above bacteria it is found that their capacity to retain Na^+ and K^+ is lost under conditions of limited

Abbreviations: CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; DCCD, *N,N'*-dicyclohexylcarbodiimide.

energy supply. On addition of substrate to these non-metabolising cells, Na^+ together with H^+ are pumped out while K^+ is accumulated. In the case of Chlorella[4], however, and possibly other microorganisms which live in dilute media[7], the leakage of Na^+ and K^+ down their electrochemical potential gradients seems to be very much limited by the low permeability of the outer membrane.

In this paper we report experiments designed to study the mechanism of Na^+ and K^+ distribution and transport in Chlorella. We have been able to induce net fluxes of these ions by adding K^+ to cells which have relatively high internal Na^+ concentrations. The method of obtaining these Na^+-rich or K^+-depleted Chlorella cells is described.

MATERIALS AND METHODS

The alga, *Chlorella pyrenoidosa*, was an Emerson strain obtained from the Indiana University Algae Collection. The cells were grown at room temperature in liquid culture medium, gassed with CO_2–air (4:96, v/v), and illuminated at 500 ft-candles by two 100-W incandescent spotlights. The composition of the liquid medium was a modified medium of BARBER[1] which had its Na^+ concentration increased to 4 mM and K^+ concentration reduced to 3 mM and with other ionic levels the same. Procedures employed in the present study have also been fully described elsewhere[1]. For obtaining K^+-depleted cells, cells grown in normal K^+-containing medium were harvested by centrifugation, washed twice with distilled water and resuspended in sodium culture medium in which all the K^+ salts were replaced by the corresponding Na^+ salts. These were then allowed to grow in this medium for 5–6 days under the same gassing and lighting conditions as above.

For experiments, the K^+-depleted cells were harvested by centrifugation at 2500 $\times$ *g* for 2 min, washed once with distilled water and once with K^+-free culture medium containing 4 mM Na^+ and finally resuspended in this same K^+-free medium. The experimental suspensions of 1 % packed cell volume were contained in a 150 ml pyrex flask placed in a constant temperature tank at 25 $\pm$ 0.5° and illuminated with two 40-W white fluorescent tubes at 800 ft-candles except for dark-treated cells which were placed in similar vessels covered in black tape. The cells were kept in suspension by bubbling with moist air and shaking. The time courses of net K^+ uptake and extrusion of Na^+ induced by the addition of 3 mM KCl were followed by withdrawing 10-ml aliquots into test tubes. These were initially centrifuged at 2500 $\times$ *g* for 2 min and then again after washing once with 15 ml of distilled water. The harvested cell pellets were digested in 1 ml of conc. HNO_3 by heating in a water bath. When the solutions became clear they were diluted to a total volume of 10 ml and analysed for Na^+ and K^+ on a Gallenkamp flame photometer by comparison with standards.

Continuous measurements of pH were made with a Beckman pH electrode using a Beckman expanometer which had its output connected to a Smiths potentiometer chart recorder (Type RE51). These measurements were carried out under CO_2-free nitrogen on 5 ml of 1 % cell suspension in distilled water. The suspensions were continuously stirred in a temperature controlled vessel (25 $\pm$ 0.5°) and could be illuminated with light from a projection lamp at an intensity of 1000 ft-candles.

Approximate determinations of changes of intracellular H^+ content were carried out using a modified method of GEAR *et al.*[8] and HAROLD *et al.*[6]. Essentially 2 ml of

ethanol was added to a sample of washed cell pellet obtained in the same way as for Na^+–K^+ exchange experiments. The suspension was evaporated to dryness in a water bath. The resulting residue was resuspended in 7 ml of distilled water and the pH determined. Changes in H^+ content of the cells were estimated by converting the differences in pH using a calibrated titration curve. The concomitant net movements of Na^+ and K^+ were determined at the same time.

Oxygen measurements were made with a Rank oxygen electrode and carried out under similar stirring, temperature and illumination conditions as employed for continuous recording of extracellular pH changes. Variations in oxygen tensions were detected as potential difference changes registered on a Smiths chart recorder (Type RE 51).

RESULTS

Distribution of Na^+ and K^+

The differences in internal concentrations of Na^+ and K^+ in cells grown either in normal culture or in Na^+ culture medium are shown in Table I. In the normal medium where the external K^+/Na^+ ratio was 0.75 the internal ratio was found to be about 33. In comparison it can be seen that cells grown in only Na^+ containing culture medium have an internal ratio of K^+/Na^+ less than 1. The actual ratio of internal K^+/Na^+ for these Na^+-rich cells varied somewhat from culture to culture being mainly dependent on the length of time between inoculating the Na^+ medium with normal K^+-cells and harvesting. We usually found that cells containing Na^+ levels in the region of 40–60 mM could be obtained after 6 days under our particular culturing conditions.

TABLE I

CATION CONCENTRATIONS IN NORMAL AND Na^+-RICH CHLORELLA CELLS

The values listed are the internal levels of Na^+ and K^+ expressed in terms of cell water. For both culture conditions the cells were grown in vessels illuminated at 500 foot-candles at 25°. The Na^+-rich cells were harvested 5 or 6 days after inoculating the K^+-free culture medium with normal K^+-containing cells. The figures given in the parentheses represent the number of separate cultures analysed and values are quoted as the mean ± S.E. In addition the external Na^+ and K^+ concentrations are given for both culture solutions together with the internal and external K^+/Na^+ ratio.

Ion	*K^+ medium*		*Na^+ medium*	
	External concn. (mM)	*Internal concn. (mM)*	*External concn. (mM)*	*Internal concn. (mM)*
K^+	3.0	142 ± 7 (18)	0.04	28.2 ± 1.3 (21)
Na^+	4.0	4.3 ± 0.8 (17)	7.0	54.0 ± 2.8 (23)
K^+/Na^+	0.75	33	0.006	0.52

In Table II we have presented further data which emphasises the ability of Chlorella to accumulate high levels of K^+ from media containing low concentrations of this cation. The results of Table II also suggest a very pronounced homeostatic mechanism maintaining the internal K^+ independent of external K^+ in the range 1–100 mM K^+.

TABLE II

THE EFFECT OF VARYING THE EXTERNAL K^+/Na^+ RATIO ON THE INTERNAL CONCENTRATIONS OF THESE CATIONS

The cells were grown by giving an inoculation of normal K^+ cells to culture medium containing various ratios of Na^+ and K^+. For external K^+ concentrations of 3.0 mM and below the Na^+/K^+ ratios were varied while keeping the anion concentrations identical. Above the external concentration of 3.0 mM K^+ the additional K^+ levels were achieved by addition of KCl. The values are quoted in terms of cell water.

Ion	*External concn. (mM)*						
K^+	0.0	0.1	1.0	3.0	10	50	100
Na^+	7.0	6.9	6.0	4.0	4.0	4.0	4.0
	Internal concn. (mM)						
K^+	28	76	163	173	162	174	172
Na^+	98	51	4.1	3.2	3.2	4.2	2.4

K^+-induced net Na^+ efflux

We were interested to test whether the Na^+-rich cells described above were able to pump out Na^+ and accumulate K^+ when K^+ was added to the external medium. In fact we were able to detect a relatively fast net exchange of Na^+ for K^+ as shown in Fig. 1. As the internal K^+ level rose there was a corresponding net efflux of Na^+. The kinetics for the K^+ transport seemed to consist of a rapid initial uptake followed by a slower phase while the Na^+ content decreased to a low level during the first stage of the K^+ uptake.

Because of the relatively slow sampling times there was some difficulty in obtaining the precise values of the initial rates but for illuminated cells the rates of

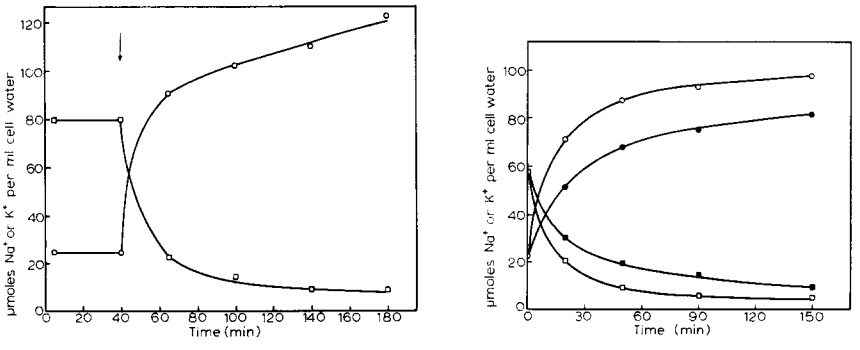


Fig. 1. The time course of net Na^+ extrusion (□) and K^+ accumulation (○) induced by injecting 3 mM KCl, as indicated by the arrow, into a suspension of Na^+-rich cells. The composition of the suspension medium before the KCl added was: 2 mM $NaNO_3$, 1 mM NaCl, 0.5 mM NaH_2PO_4, 0.5 mM Na_2HPO_4, 2 mM $MgSO_4 \cdot 7H_2O$, 0.25 mM $Ca(NO_3)_2 \cdot 4H_2O$ and 2 ml/l of trace elements[1] at pH 7.0. The cells were illuminated and had been suspended for 1 h in the above medium before adding the K^+.

Fig. 2. The effect of light (○, □) and dark (●, ■) on the Na^+ extrusion (□, ■) and K^+ accumulation (○, ●) induced by adding 3 mM KCl at time zero. The other conditions were the same as given for Fig. 1.

exchange were about 20 pmoles·cm^{-2}·sec^{-1} which represent values higher than reported for steady-state fluxes of K^+ and Na^+ in this organism[2-4].

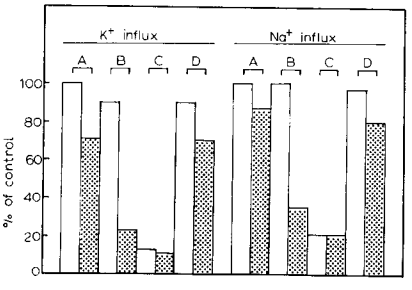
The action of light

In Fig. 2 it can be seen that the net exchange was stimulated by light. However, this photosensitivity did not seem to be as great as detected for the fluxes of K^+ and Na^+ in normal cells under conditions of no net movement.

The effect of nitrogen, low temperature and 3-(3,4-dichlorophenyl)-1,2-dimethylurea (DCMU)

The ability of Chlorella to pump out Na^+ and accumulate K^+ is characteristic of similar exchange systems found in animal cells, microorganism and plant cells. Whether exactly the same mechanism is operating in these different systems is doubtful but they almost certainly require an energy source. The above sensitivity to light suggests that photosynthesis can supply some of the required energy. The data shown in Fig. 3 clearly demonstrate the energy requirement for net K^+ uptake and Na^+ extrusion. Both the net fluxes of these cations were inhibited by low temperature whether they were illuminated or not.

DCMU, a specific inhibitor of Photosystem II, added at a concentration which totally blocks oxygen evolution[9] was found to slightly reduce the exchange in the light but had no effect in the dark. Under N_2 the dark rates of exchange were considerably reduced suggesting a dependence on respiration. However, N_2, like DCMU, had little or no effect on the light rates indicating that the exchange mechanism is probably independent of CO_2 fixation and probably more closely associated with primary electron transport in photosynthesis. In fact under anaerobic conditions green photosynthetic organisms can still maintain System I activity and are thought to carry out cyclic phosphorylation.



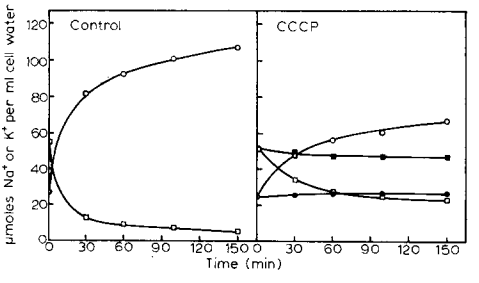


Fig. 3. The effect of various treatments on the initial rates of Na^+–K^+ exchange induced from Na^+-rich cells by adding 3 mM KCl. The cells were suspended in the K^+-free medium given in the legend of Fig. 1 and were either illuminated (unshaded) or dark treated (shaded). The results are presented as the percentage of the light control. The various treatments were: (A) light and dark controls; (B) the magnitude of the light and dark fluxes measured under N_2. The suspensions were pretreated with N_2 by bubbling for 1 h before injecting the K^+; (C) the net flux rates of Na^+ and K^+ at 1°. The cells were pretreated for 30 min at this temperature before adding K^+; (D) the effect of $5 \cdot 10^{-5}$ M DCMU on the light and dark Na^+–K^+ exchanges. Pretreatment time of 1 h.

Fig. 4. The effect of 10^{-5} M (○, □) and $5 \cdot 10^{-5}$ M CCCP (●, ■) on the net Na^+ extrusion (□, ■) and K^+ uptake (○, ●). The suspensions were pretreated for 30 min with CCCP. The figure also shows the control fluxes for Na^+ (□) and K^+ (○) obtained with untreated cells. The cells were illuminated and suspended in the same medium as given in the legend of Fig. 1.

The effect of carbonyl cyanide m-chlorophenylhydrazone (CCCP)

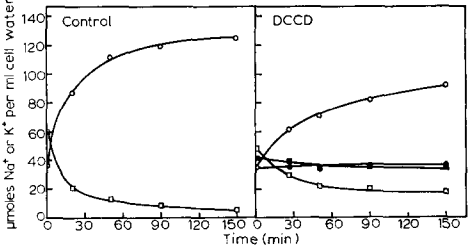
The net Na^+–K^+ exchange was sensitive to CCCP as shown in Fig. 4. An inhibition of about 50 % was found with a concentration of 10^{-5} M while $5 \cdot 10^{-5}$ M completely inhibited the exchange mechanism. This compound acts as an uncoupler of both oxidative and photosynthetic phosphorylation[10] and has already been shown to inhibit the transport of Na^+ and K^+ in normal K^+-containing Chlorella cells.

Effect of N,N'-dicyclohexylcarbodiimide (DCCD)

The above experiments using CCCP strongly suggest that the exchange of Na^+ for K^+ is utilizing ATP as its energy source. If this is so, the mechanism would probably involve a membrane ATPase. We, therefore, chose to test the sensitivity of the exchange to DCCD, a compound which has been reported to be a potent inhibitor of membrane-bound ATPase but not an inhibitor of the solubilized form of the enzyme[11]. Indeed this compound has already been shown to inhibit energy dependent transport processes in the microorganism *S. faecalis*[12]. We had some problem with its low water solubility and found it necessary to treat the K^+-depleted cells with DCCD overnight in the darkness at 5°. As shown in Fig. 5 after such treatment the control cells having no DCCD were still able to maintain the capacity to pump out Na^+ on addition of K^+ while those treated with DCCD were either partially or completely unable to carry out the Na^+–K^+ exchange.

Lack of effect of ouabain

The Na^+–K^+ exchange induced by adding K^+ to Na^+-rich Chlorella cells seems to require a supply of ATP, either from oxidative or photosynthetic phosphorylation, and the presence of a membrane-bound ATPase system. However, there is some doubt whether this exchange system is similar to that found in animal tissues since it was found earlier that both the K^+ influx and Na^+ efflux from normal K^+-containing cells were not inhibited by ouabain. MITCHELL[13] has recently suggested that certain eukaryotic organisms like Chlorella, *Saccharomyces cerevisiae*, Neurospora and also plant cells are more analogous to prokaryotic cells and mitochondria in that they do



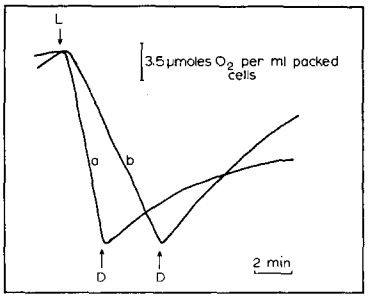


Fig. 5. The effect of 10^{-4} M (○, □) and $5 \cdot 10^{-4}$ M DCCD (●, ■) on the net Na^+ extrusion (□, ■) and K^+ influx (○, ●). The suspensions were pretreated with DCCD for about 20 h at 5° in the darkness. The figure also shows the control fluxes for Na^+ (□) and K^+ (○) obtained from cells treated in a similar way but without DCCD present. Other conditions were the same as for Fig. 4.

Fig. 6. A recording of the rate of oxygen exchange under light (L) and dark (D) conditions recorded with cells suspended in K^+-free medium containing 1 mM $NaHCO_3$. Trace a was recorded with normal K^+-containing cells while Trace b was obtained with Na^+-rich Chlorella cells.

not have a (Na^+-K^+)-ATPase system similar to that found in animal cells. Certainly the action of ouabain has been reported to have little effect on these systems, there being only a few exceptions[14,15].

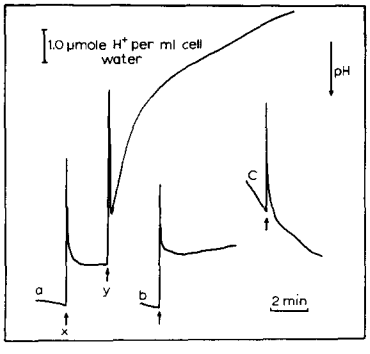
Since we apparently had a clear case of a K^+-stimulated Na^+ efflux from Chlorella we were interested to establish whether or not ouabain would influence these net cation fluxes. To be sure that our ouabain preparation was satisfactory we tested its activity by conducting experiments on frog skin similar to those of MACROBBIE AND USSING[16]. This involved checking the sensitivity of the electrical potential across isolated frog skin bathed on both sides with chloride ringer solution. Although 10^{-5} M ouabain was able to induce a rapid drop of potential difference across the frog skin we were unable with the same preparation to inhibit the K^+–Na^+ exchange in Chlorella even at concentration as high as $5 \cdot 10^{-4}$ M.

Oxygen measurements

To check whether photosynthetic or respiration rates were different in Na^+-rich and K^+-rich cells we looked at oxygen exchange. Essentially it appeared that the high Na^+-containing cells had a lower rate of photosynthesis than the normal K^+-containing cells while the reverse was true for respiration (see Fig. 6). In addition we also checked on the oxygen exchange rates both for light and dark conditions after adding K^+ to Na^+-rich cells. We were unable to detect any significant change in photosynthesis or respiration during the course of net ionic movements.

Release of H^+

In all the experiments it was found that the net uptake of K^+ was not completely balanced by Na^+ extrusion. This suggested that other ions are balancing the additional K^+ influx. One obvious possibility is that a net H^+ extrusion is occurring in the same way as reported for other microorganisms. Fig. 7 clearly demonstrates



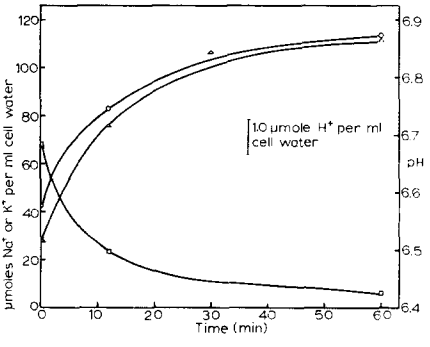


Fig. 7. Traces of extracellular pH changes with time. Trace a was recorded with Na^+-rich cells suspended in distilled water bubbled with CO_2-free N_2. At point X 3 mM NaCl was injected followed by a 3 mM KCl injection at Y. Trace b was obtained by adding 3 mM KCl, as indicated by the arrow, to Na^+-rich cells which had previously been pretreated with 3 mM KCl for 4 h. These K^+ pretreated cells were resuspended in distilled water for this measurement. Trace c shows the effect of injecting 3 mM KCl into a suspension of normal K^+-containing cells.

Fig. 8. The time course for intracellular pH changes (△) induced in illuminated Na^+-rich cells by the addition of 3 mM KCl at zero time. The net movements of Na^+ (□) and K^+ (○) are also shown for the same suspension. The other conditions were the same as given for Fig. 1.

a decrease in the external pH immediately after the addition of 3 mM KCl to Na^+-rich cells while the addition of 3 mM NaCl to the same cells had very little effect. This figure also shows that the injection of KCl did not induce a similar pH change with normal K^+ cells or after treating Na^+-rich cells with K^+ for several hours. The apparent H^+ efflux induced by K^+ addition to Na^+ cells was reduced by dark conditions. In the light the initial rates of the K^+ induced H^+ efflux were normally below 5 pmoles$\cdot$cm$^{-2}\cdot$sec^{-1}.

We were also able to monitor an increase in the internal pH of the cells while following the net movement of Na^+ and K^+ as shown in Fig. 8. The rise of internal pH had kinetics similar to the net K^+ influx. It was usually estimated that less than 10 % of the total K^+ transported was balanced by H^+ extrusion.

DISCUSSION

The experiments reported in this paper clearly demonstrate the ability of Chlorella cells to reduce their internal Na^+ concentration to a low value in the presence of external K^+. The net Na^+ extrusion induced from Na^+-rich cells by adding K^+ to the suspension is relatively rapid having a half time of less than 10 min. Electrical neutrality seems to be maintained by a net uptake of K^+. From these studies we are unable to decide whether the K^+ accumulation is secondary to the Na^+ extrusion or that the exchange is brought about by a tightly coupled mechanism. Alternatively the K^+ uptake could itself be the primary process inducing the efflux of Na^+. Whichever mechanism is operating there is the further observation that an additional K^+ uptake occurs during these ionic exchanges which is possibly associated with a net H^+ efflux.

With normal high K^+ cells bathed in culture medium it was previously suggested[1] from thermodynamic arguments that both Na^+ and K^+ are actively transported by this alga. During this earlier study it was shown that the cell interior of Chlorella was electrically negative with respect to the bathing medium and that K^+ was closer to equilibrium than Na^+. Thus it is feasible that the net extrusion of Na^+ and H^+ from Chlorella reported in this paper can generate a membrane potential which is negative on the cytoplasmic side. In this case K^+ would tend to be accumulated in response to the electrical gradient. A critical test, which has yet to be carried out, would be to record the membrane potential directly before and after the addition of K^+ to Na^+-rich cells. Such measurements, however, are difficult because of the dimensions of Chlorella cells.

The initial flux rates for net Na^+ and K^+ transport are ten or more times higher than the unidirectional fluxes under the conditions of no net ion movement. Under steady-state conditions the K^+ influx is about 1 pmole$\cdot$cm$^{-2}\cdot$sec^{-1} (ref. 3) and most of this seems to be closely coupled to the efflux of this cation. Only a small portion of the K^+ influx is likely to be associated with the Na^+ extrusion which effluxes at about 0.14 pmole$\cdot$cm$^{-2}\cdot$sec^{-1} (ref. 2) from normal illuminated K^+-containing cells. This low pump rate for Na^+ extrusion is probably due to a relatively impermeable cell membrane reducing the back leakage of this cation down its electrochemical potential gradient. [An estimate of the permeability coefficient (P_{Na}) for Na^+ movement across the Chlorella cell membrane based on the Goldman theory gives a value of about $2\cdot10^{-9}$ cm$\cdot$sec^{-1} (see refs. 1 and 3).] It would seem from the experiments in

this paper that under conditions where the internal Na^+ level has become high the pump is able to operate considerably faster.

The net uptake of K^+ and corresponding extrusion of Na^+ from Chlorella cells represent an energy requiring process as indicated by the sensitivity of these net fluxes to changes in metabolism. Our experiments implicate the hydrolysis of ATP, derived either from photosynthetic or oxidative phosphorylation, as the energy source. The action of DCCD would seem to indicate that a membrane-bound ATPase is intimately involved in the exchange mechanism. As yet we would be cautious to identify this enzyme with the well established (Na^+-K^+)-ATPase system of animal cells. We have been unable to obtain an inhibition of the net fluxes of Na^+ and K^+ with ouabain. This observation may give further evidence for a more loosely coupled ion exchange system possibly involving an electrogenic pump as discussed above. Alternatively one is always left with the possibility that this cardiac glycoside has not been able to reach the active sites in this alga responsible for the specific exchange mechanism.

Overall our results are strikingly similar to studies on non-photosynthetic microorganisms. HAROLD *et al.*[6] have obtained considerable evidence that *Streptococcus faecalis* accumulates K^+ in response to an active extrusion of Na^+ and H^+. SLAYMAN AND SLAYMAN[17] were able to induce a net Na^+–K^+ movement with *Neurospora crassa* by conducting similar experiments to those reported in this paper. These workers emphasised several common characteristics of the microbiol cell membrane including the presence of not only the Na^+–K^+ exchange mechanism but also a K^+–H^+ system. These workers thought it unlikely that an electrogenic mechanism is responsible for these net fluxes in Neurospora and suggested that a single carrier system may be operating for all the cationic fluxes. In Chlorella we have found that most treatments which reduced the K^+–K^+ system in steady-state conditions also inhibited the Na^+–K^+ exchange. However, at this stage we would hesitate to suggest a single carrier mechanism since we have detected some differences in the response of the two processes to treatment with low concentrations of $HgCl_2$ (J. BARBER AND Y. J. SHIEH, unpublished observations). In addition we have generally found the steady-state K^+–K^+ mechanism to be more light sensitive than the Na^+–K^+ exchange. This could, however, be explained by the apparent difference in the relative rates of respiration and photosynthesis in the Na^+- and K^+-grown cells.

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