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Sodium efflux from *Chlorella pyrenoidosa*

Recent electrical and chemical analyses of Chlorella cells under fixed external conditions have suggested that this organism may maintain its low Na^+ concentration by means of an active extrusion mechanism[1]. The work presented in this communication was designed to investigate this process further and to compare its nature with the well established Na^+ pump found in many animal cells[2] and some plant cells[3–5].

The alga, *Chlorella pyrenoidosa*, was an Emerson strain and cultured as described previously[1]. Effluxes were measured by resuspending cells, which had been loaded with ^{22}Na, in fresh, inactive culture medium. This isotope was obtained from the Radiochemical Centre, Amersham, as the aqueous Cl^- with a specific activity of about 2 mC/mg Na^+. Experiments were carried out in a glass water tank at 25 ± 0.5° and illuminated on either side by two 150-W incandescent spotlights giving an intensity of 500 ft candles at the reaction vessels. These vessels were flat-sided glass flasks, some of which were covered with black tape for measuring dark rates. The cells were kept in suspension and continuously aerated by bubbling through moist air. The time course of the efflux was followed by withdrawing 1-ml aliquots which were rapidly filtered through millipore discs and washed twice with 20 ml of distilled water, once in suspension and then after sedimenting out on the membrane filter. The internal radioactivity of the cells was assayed by means of a Nuclear Chicago gas-flow counter. The duration of the experiments and the density of the cell suspensions were such as to minimize the back flow of tracer. In some experiments the cells were suspended in a modified medium which had all the K^+ compounds replaced by the corresponding Na^+ salts and this is referred to as Na^+ culture medium in the text. Internal concentrations were determined either by flame photometry or from isotopic equilibrium as already described[1].

The efflux rates were calculated assuming the cells to be in a steady state and governed by a single rate constant. Thus, in the absence of tracer influx the internal radioactivity of the cells A_i, at any time, t, is given by $A_i = A_i' \exp(-kt)$, where A_i' is the initial internal activity and k is the efflux rate constant. The efflux rate M' in moles/sec·5 μl packed cells is given by $M' = kc'$ where c' is the concentration of Na^+ per 5 μl packed cells. Alternatively, the efflux M in moles/sec·cm² can be calcu-

TABLE I

LIGHT AND DARK Na^+ EFFLUXES

Efflux rates measured in culture medium (pH 6.8) in the absence of net Na^+ movement. Results are quoted as ± S.E. (number of experiments on which mean is based).

Condition	*Efflux rates*	
	μμmoles/sec·5 μl packed cells	*μμmoles/sec·cm²*
Light	2.68 ± 0.74 (8)	0.14 ± 0.04
Dark	0.22 ± 0.04 (8)	0.012 ± 0.002

Abbreviation: CCCP, carbonyl cyanide *m*-chlorophenyl hydrazone.

lated from $M = (kV_1c_1)/a$, where V_1 is the internal volume of a quantity of cells in cm^3, a is the surface area of the same quantity of cells in cm^2 and c_1 is the internal concentration in moles/cm^3. The geometrical parameters required for this latter equation have been measured and reported elsewhere[6].

In accordance with the above assumptions it was found that the ^{22}Na efflux from Chlorella cells gave straight lines when log A_1 was plotted against time and the calculated efflux rates are given in Table I. It can be seen that the light rate is about 12 times greater than the dark.

Because the magnitude of the Na^+-efflux rates in Table I are somewhat lower than the corresponding K^+ influxes reported previously[7], it seemed unlikely that these two fluxes are completely coupled. However, the possibility of a small component of the K^+ influx being linked to the Na^+-extrusion mechanism was investigated. Cells previously loaded with ^{22}Na were suspended in the Na^+ culture medium. To overcome the problem of K^+ leakage from the cell interior satisfying any K^+-requiring Na^+ efflux, some initial experiments were conducted on cells containing low concentrations of internal K^+. These were specially grown by inoculating Na^+ culture media with normal high K^+ cells and allowing them to grow for 2 or 3 days. Under these conditions the normal internal K^+ concentration falls from 110 mM to about 10 mM while the Na^+ level rises from 1 mM to approx. 40 mM. It can be seen in Fig. 1a that the Na^+ efflux from these cells could be stimulated by injecting 1 mM KCl while the addition of 1 mM NaCl, to the control suspension, had no effect. In later experiments it was possible to observe a similar stimulation of the Na^+ efflux from normal high K^+ cells when K^+ was added to the suspending medium. Such a result is shown in Fig. 1b where the addition of 2.5 mM K^+ caused an increase of the rate constant from 1.7 h^{-1} to 2.5 h^{-1}. The latter rate constant is of a similar magnitude to that found for cells suspended in normal culture medium.

The sensitivity of the Na^+ efflux to external K^+ suggested that this process may be inhibited by the cardiac glycoside, ouabain. Experiments using 10^{-4} M and $5 \cdot 10^{-4}$ M ouabain did not show any inhibition of the Na^+ efflux. The negative effect of $5 \cdot 10^{-4}$ M ouabain is shown in Fig. 2a which does, however, clearly demonstrate

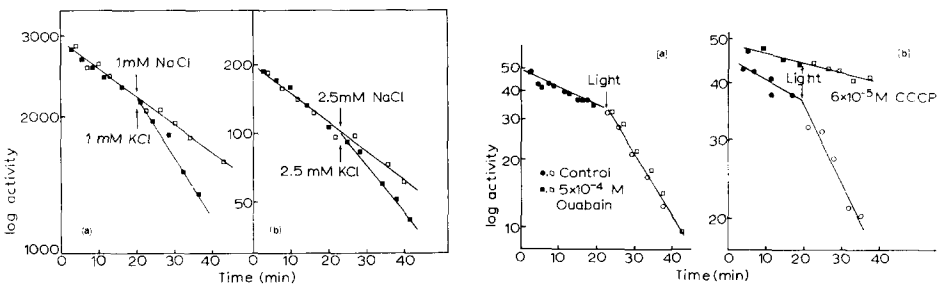

Fig. 1. a. The efflux of ^{22}Na from illuminated cells grown and suspended in Na^+ culture medium. The arrows show the time of addition of 1 mM NaCl to one suspension (□) and 1 mM KCl to the other (■). b. The efflux of ^{22}Na from illuminated cells grown and loaded with isotope in normal culture medium and suspended in Na^+ culture medium. The arrows show the time of addition of 2.5 mM NaCl to one suspension (□) and 2.5 mM KCl to the other (■).

Fig. 2. a. The dark and light efflux of ^{22}Na from cells suspended in culture medium, one suspension containing $5 \cdot 10^{-4}$ M ouabain. b. The dark and light efflux of ^{22}Na showing the effect of $6 \cdot 10^{-5}$ M CCCP on cells suspended in culture medium.

the light sensitivity of this efflux mechanism. To ensure that this compound had sufficient time to interact with the transport site it can be seen that the resuspended cells were initially placed in the dark. This enabled a reasonable pretreatment time in the absence of a large decrease of the internal radioactivity. This method also proved a rather convenient way of testing the effect of other inhibitors as shown in Fig. 2b. In this plot it can be seen that $6 \cdot 10^{-5}$ M carbonyl cyanide *m*-chlorophenyl hydrazone (CCCP) considerably inhibits the dark efflux and completely inhibits the light-stimulated rate. At 10^{-5} M this uncoupler partially inhibited the light rate, reducing it to about 50 % of the control. Similar experiments with the herbicide, 3-(3,4-dichlorophenyl 1)-1,1-dimethyl urea (DCMU) at 10^{-5} M did not cause any inhibition of the light-dependent efflux although at this concentration O_2 evolution was completely inhibited[7].

These experiments suggest that Chlorella cells behave essentially as single compartments for Na^+ extrusion. The light rate of 0.14 $\mu\mu$moles Na^+ per sec·cm^2 correlates well with the influx rate of 0.11 $\mu\mu$moles Na^+ per sec·cm^2 also measured on similarly treated cells[6] suggesting that no net movement of Na^+ was occurring under the experimental conditions. The sensitivity of the efflux to light is in agreement with the process being active and the inhibitory effect of CCCP suggests that ATP could be supplying the required energy. The lack of effect of the specific inhibitor, ouabain, does not necessarily mean that an ATPase system similar to that found in animal cells[8] is not operating. There may be some restriction imposed by the cell wall for this compound to reach the transport site. Indeed, it is not uncommon to find that established cation pumps of other plant cells have a similar insensitivity to this glycoside[9,10] and, where an inhibitory effect has been observed, rather high concentrations are sometimes required[5]. Certainly the sensitivity of the Na^+ efflux to K^+ suggests that Chlorella may possess a coupled exchange pump. However, care must be taken in coming to this conclusion as it has been shown that increasing the external K^+ concentration depolarizes the membrane potential of this organism[1]. This change in membrane potential is such as to reduce the energy gradient up which the Na^+ pump operates.

Thus, although it seems most likely that Chlorella cells contain an ATP-dependent Na^+-efflux mechanism, it would be impossible to say with certainty that this efflux is linked to a component of the K^+ influx without detailed electrical measurements.

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Insulin stimulation of [^{14}C]leucine incorporation into protein

It has been suggested that the primary effect of insulin is exerted at the extracellular–intracellular barrier of the cell, *i.e.*, the plasma membrane. In this way insulin initiates a sequence of intermolecular rearrangements which accounts for its effects on metabolism[1,2]. In regard to the incorporation of ^{14}C-labelled amino acids into protein, MANCHESTER[3] found that when isolated diaphragm was cut into successively smaller fragments prior to incubation *in vitro*, the influence of insulin progressively diminished. Furthermore, the repeated inability to demonstrate either a clear-cut or consistent effect of insulin added *in vitro* to a subcellular preparation is well known[4,5]. In this report we will present data consonant with the hypothesis that insulin requires an intact cell membrane in order to stimulate [^{14}C]leucine incorporation into protein and that this enhancement may be related to regulation of amino acid transport across the cell membrane.

Materials and methods were the same as described in earlier studies[6,7]. Additional details are given in the text and legends.

In Fig. 1 are shown the results of experiments wherein tissues obtained from untreated animals are incubated with and without insulin. It can be seen that stripping the tissue into halves optimizes both baseline incorporation of [^{14}C]leucine and insulin stimulation of this process. Stripping into quarters leads to reduction in both baseline and insulin-stimulated incorporation of [^{14}C]leucine. Stripping into eighths produces no further reduction in baseline incorporation, but a further reduction of insulin's effect occurs. The maintenance of baseline incorporation in going from quarters to eighths implies that the synthetic machinery remains relatively unharmed, while the concomitant vanishing of insulin's effect implies that some other essential cellular component, perhaps cell membrane, has been damaged.

It can be seen in Table I that tissues taken from rats which received insulin intraperitoneally incorporate more [^{14}C]leucine into 0.20 ionic strength extractable protein than controls. Here insulin was given *in vivo*, and the muscles were subsequently incubated *in vitro*. It should be noted in the case of stripped halves that both incorporation of [^{14}C]leucine into protein and insulin stimulation of this process are approximately the same whether insulin was first given *in vivo* (1 h before tissue sample preparation) 183 *vs.* 124 counts/min per mg (Table I), or whether it was added directly to the incubating flask 181 *vs.* 131 counts/min per mg (Fig. 1). Apart from the route of introducing insulin, both incubations were identical and of 1-h duration. This suggests that the observed insulin effect is "relatively primary", *e.g.*, at the cell