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LIGHT-INDUCED PROTON UPTAKE IN WHOLE CELLS OF *DUNALIELLA PARVA*

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SUMMARY

Light-induced H^+ uptake ("proton pump") has been observed with whole cells of the halophilic alga *Dunaliella parva*. The rate of H^+ uptake is in the range of 2–6 μequiv H^+/min per mg chlorophyll. The extent of the reaction is about 9–10 μequiv H^+/mg chlorophyll at an initial pH of 6.2. The extent increases linearly with increase of initial pH. The rate of O_2 evolution decreases parallel to the decrease in rate of H^+ uptake. Tris and other buffers prevent the decrease in rate of O_2 evolution, decreases parallel to the decrease in rate of H^+ uptake. Tris and other buffers prevent the decrease in rate of O_2 evolution, and increase the extent of H^+ uptake.

3(3,4-Dichlorophenyl)-1,1-dimethylurea inhibits H^+ uptake, but does not affect the decay in the dark. Atebrin and, to a lesser extent, NH_4Cl increase the rate of decay. Carbonyl cyanide *m*-chlorophenylhydrazone does not affect the rate of decay at all.

INTRODUCTION

Dunaliella parva, a unicellular halophilic alga, has some unusual properties which make it suitable as a tool for the kind of studies usually carried out on chloroplast preparations. Like those of other Volvocales, each cell has one large cup-shaped chloroplast which occupies about half of the cell volume. A unique property of this organism, however, is its very highly permeable membrane[1]. From measurements of the trapped volume of cell pellets, it was concluded that the outer cytoplasmic membrane is almost freely permeable to sucrose and even inulin (mol. wt., 5000–6000); inulin equilibrated with a fraction of the internal volume within less than 1 min (ref. 1). About half of the cell volume was unavailable to inulin. It was suggested, therefore, that the outer cytoplasmic membrane is very permeable, whereas the chloroplast or lamellar membrane is impermeable, at least to inulin. It can be argued that a suspension of cells of this alga is equivalent to a suspension of chloroplasts immersed within a thin cytoplasmic layer in free communication with small molecules in the outer medium. In other words, we have a suspension of intact chloroplasts ready for experimentation, without the need for preparative procedures.

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

On illumination, chloroplasts in suspension have been shown to take up protons from unbuffered media[3–7]. It is assumed that H^+ is very likely taken up into the intralamellar space. This light-induced proton uptake has been observed even with chloroplast fragments. The results suggest that a proton gradient, and perhaps even an electrical potential gradient, is built up as a result of this apparent H^+ gradient across the lamellar membrane. It has been postulated that these proton and electrical gradients are the driving forces of ATP formation[8]. It is suggested that these gradients are observable intermediates in the series of reactions which convert light energy to chemical energy.

In this paper we would like to show that it is possible to observe a similar phenomenon in intact cells of *D. parva*.

METHODS

D. parva was isolated from the Dead Sea. It was cultured in a growth medium adapted from McLACHLAN[9] by addition of 1.5 M NaCl, 5 mM $NaNO_3$ and 20 mM Tris–HCl at pH 7.4 ± 0.2. Cultures were kept at 32 ± 1° in 2-l containers and were stirred continuously by means of a magnetic bar. They were aerated by means of an air current which bubbled through the solution. The cultures were sterile. Illumination was continuous. Under these conditions, the mean generation time was 15–18 h.

Algae to be used for experiments were taken from cultures at the mid-logarithmic phase of growth, when the cell density was $1 \cdot 10^6$–$2 \cdot 10^6$ cells/ml. Samples of the suspension were centrifuged at 3000 × *g* for 5 min; the cells were rinsed once and resuspended in 1.5 M NaCl at a concentration of $0.8 \cdot 10^7$–$1.2 \cdot 10^7$ cells/ml (8–12 μg chlorophyll/ml suspension).

The pH of the suspension was brought to 6.1 by addition of NaOH or HCl.

pH changes in the suspension were measured with a Radiometer pH Meter, Model 22, with a combined electrode, GK 2026. The pH meter was connected to a Varian recorder, Model G-14-A-2.

In order to convert pH changes to amounts of proton, the algal suspension was titrated with 0.01 M NaOH in a series of small steps corresponding to given changes of pH.

For pH stat experiments, a Radiometer pH stat TTTlc was used.

Oxygen evolution was measured with a Clark-type oxygen electrode, connected to a Varian G-14 recorder. pH and oxygen evolution measurements were made on 2 samples of the same suspension in separate containers, the contents of which were pooled before and after each treatment. It was shown that the same pH changes occurred in the two containers which stood side by side.

The source of light during experiments was a 500-W Braun slide projector. The light was filtered through a solution of $CuSO_4$.

For measurement of reactions in the absence of CO_2, the algal container was enclosed within a second glass vessel containing 40 % KOH. The algal suspension was rendered CO_2-free by first lowering the pH to 4.5 by the addition of HCl, and then bringing the pH back to the desired value[10].

Chlorophyll was determined by the method of ARNON[11].

Carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) was dissolved in methanol and titrated to the desired pH. It was kindly given to us by Dr. HEYTLER, DuPont

(Delaware). A sample of commercial 3(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU) was given by the Abic Co., Tel Aviv.

RESULTS

When a light is shone through an unbuffered suspension of *D. parva*, there is a rise in the pH of the suspension. The rate of pH change decreases with time until a steady pH value is reached (Fig. 1). The total increase in pH can be as much as 2.5 pH units. On switching off the light, there is a spontaneous drop in pH until the initial value is restored. Fig. 1 also shows evolution of O_2 measured at the same time as change in pH. The rate of O_2 evolution falls in a way which is similar to the fall in the rate of change of pH.

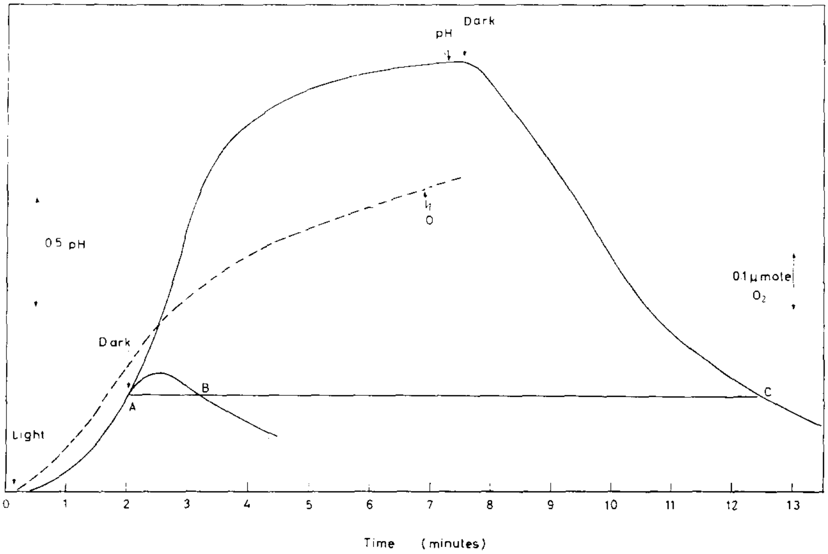

Fig. 1. Recorder tracing of pH changes and O_2 evolution induced by light, in *D. parva* cells. Cells suspended in 1.5 M NaCl; initial pH, 6.2. ———, pH changes; – – – –, O_2 evolution.

In Fig. 2 the maximal rate of net H^+ uptake and the extent of the reaction (the total amount of H^+ taken up until the steady state is reached) have been plotted *vs.* concentration of alga in the suspension, expressed as µg of chlorophyll/ml. It can be seen that both variables are linear with algal concentration up to 12 µg chlorophyll/ml. All the work described in this paper is within the range of these linear relationships.

We found that the three parameters measured remained unchanged whether CO_2 was present in the system or not. (Rate of formation: control, 3.2 ± 0.2; without CO_2, 3.2 ± 0.5. Extent: control, 8.7 ± 0.4; without CO_2, 8.7 ± 0.6. Rate of decay: control, 1.3 ± 0.2; without CO_2, 1.3 ± 0.5.)

The rate of net disappearance of H^+ from the outer solution is plotted *vs.* time in Fig. 3. It took 2.5–3 min after switching on the light to approach the maximum rate. After 6–7 min the rate decreased to zero. It is postulated that in this steady state, the rate of H^+ uptake equaled the rate of the leakage of H^+ back into the medium. This backflow is usually referred to as a decay. Since it is assumed to be the same in the dark and in the light, it should be possible to estimate it by switching off the light at any point on the time curve. It is not possible to do this directly in our system, as the pH continues to shoot up for a moment after the light is extinguished (Fig. 1). This "overshoot" has been reported by NEUMANN AND JAGENDORF[2,3] with isolated chloroplasts. Due to this difficulty we have tried 2 ways to calculate the rate of decay at any given point, as shown in Fig. 1. It is assumed that the rate of decay depends only on the extent of the reaction, no matter how it is arrived at. To know the rate of decay at A, either the light was switched off at this point, and the decay rate measured at point B, or the rate of decay was measured at point C, with the same extent of reaction as point A. In some cases good agreement was found between these 2 calculated rates; in other cases the rate at point C was higher than at B by 10–20%.

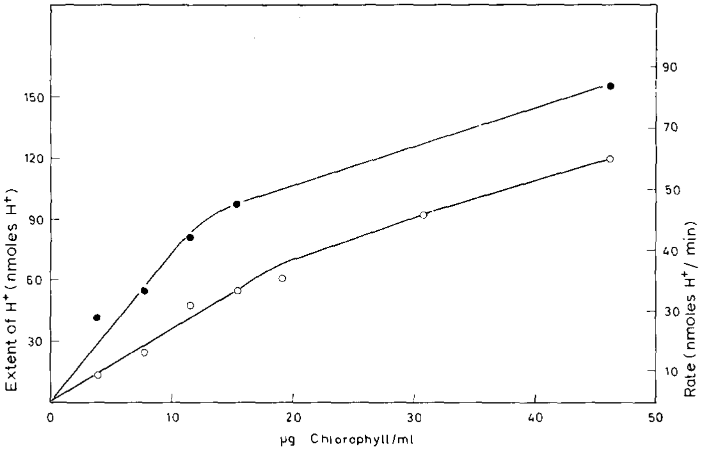

Fig. 2. Light-induced H$^+$ uptake as a function of chlorophyll concentration. Conditions as in Fig. 1. ●—●, extent of reaction; ○—○, maximal net uptake rate.

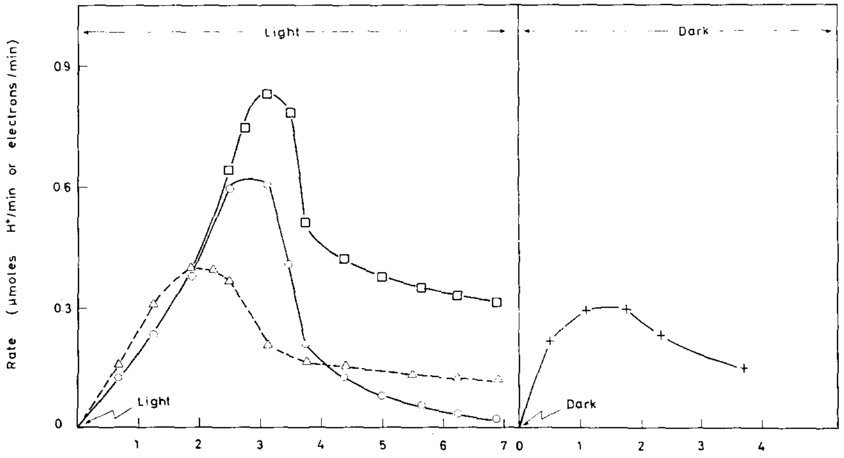

Fig. 3. Rates of light-induced H$^+$ uptake, O_2 evolution and dark H$^+$ release as functions of time. Conditions as in Fig. 1. △----△, rate of O_2 evolution; ○—○, net H$^+$ uptake rate (measured); □—□, H$^+$ uptake rate (calculated); +—+, H$^+$ release rate (decay).

The true forward reaction at any given time has been calculated. These calculated rates of decay and of the forward reaction are plotted in Fig. 3. The time courses of rates of oxygen evolution and light-induced H^+ influx, corrected for backflow, are compared. The two curves have a similar convex shape. The maximum of O_2 evolution rate precedes the maximum of the H^+ uptake rate by about 2 min. The shape of the curve is not likely to be due to sluggish instrumental response, as has been suggested for chloroplasts[4]. Maximum response could be achieved within seconds, with both O_2 and H^+ electrodes. It should be recalled that the site where the reaction takes place is in the photosynthetic membrane, which is separated by 2 membranes (cytoplasmic and chloroplastic) from the measuring probes. We therefore should expect to have a "transport step" in a kinetic description of the events described. If we add this "transport step" to a model system such as that described by KARLISH AND AVRON[5], we may expect to get a curve resembling the one seen in Fig. 3. As it is not yet possible to estimate this transport step quantitatively for either O_2 or H^+, it is impossible to know whether the time difference between the 2 maxima is real or apparent. This information will be very useful for an understanding of the mechanism controlling O_2 evolution and H^+ uptake.

It is interesting to note that the rates of O_2 evolution and H^+ uptake decrease to a steady level which is about 30% of their maximum rate. This is conceivably not a coincidence. A possible explanation is as follows: The uptake of H^+ into the internal space of the grana may result in a decrease in pH of the intragrana space which may affect the rate of O_2 evolution in the light. As the H^+ uptake is the result of electron flow, or is coupled to it, then the rates of these two parameters should decrease, too. This explanation is consistent with the results shown in Fig. 4: the extent of H^+ uptake is plotted against the initial pH of the algal suspension. There is an almost linear relation between extent and initial pH from pH 8 to pH 4.5 at which point the extent is almost zero. If the assumption is made that the initial pH of the intragrana space is equal or near to that of the medium, it can be concluded that the pH of the grana space is at or below pH 4.5 at the steady state. By decreasing the outer pH

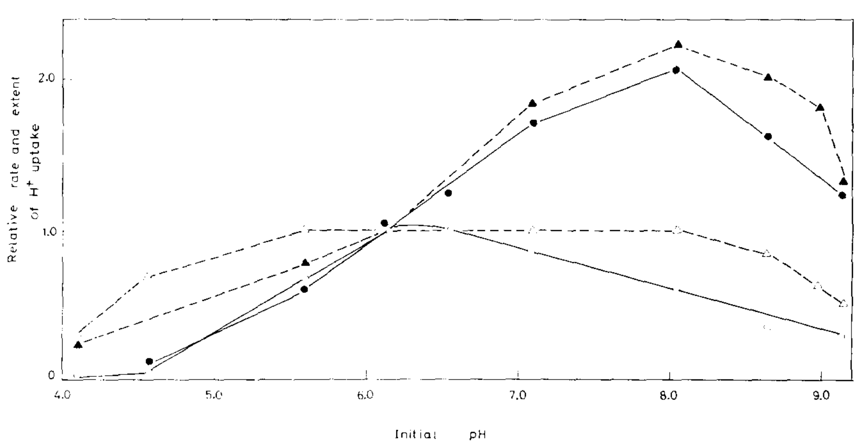

Fig. 4. Effect of initial pH on light-induced H^+ uptake and O_2 evolution. Cells were suspended in 1.5 M NaCl and brought to the given pH by HCl or NaOH. ○—○, maximal net H^+ uptake rate; ●—●, extent of H^+ uptake; △----△, maximal rate of O_2 evolution; ▲----▲, extent of O_2 evolution before the steady state.

to 4.1, the rate of O_2 evolution is approx. 30 % of the maximum that can be achieved when conditions are optimum. This result suggests that the decrease in rate of O_2 evolution is due to acidification of the inner space of the grana; consequently the light-induced H^+ uptake decreases.

Fig. 5 shows that on addition of Tris or phosphate buffer to the medium, no decrease in O_2 evolution occurs at all. Also, addition of Tris buffer at pH 7.5, at the point where the rate of O_2 evolution is at its minimum, brings the rate back to its maximal value. This is again consistent with the explanation that the decrease in O_2 evolution is due to acidification of the internal grana space. It also indicates that this space has access to external supplies of the buffer. This conclusion is borne out by the next set of experiments. It is possible to measure the extent of the H^+ uptake by

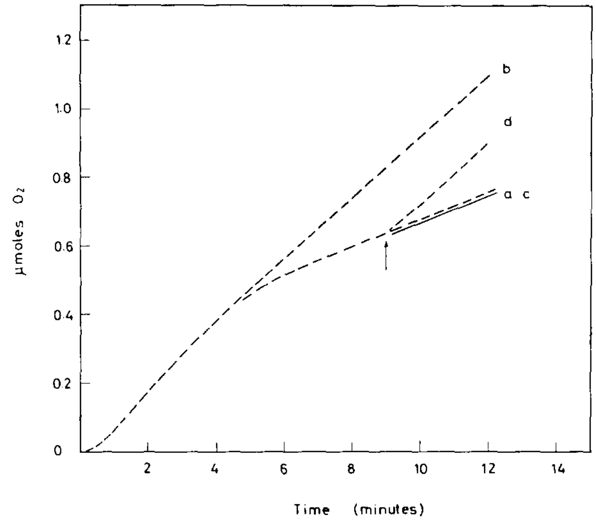

Fig. 5. Effect of Tris buffer on O_2 evolution. Cells were supended in 1.5 M NaCl and brought to pH 6.9. a, cells in 1.5 M NaCl; b, Tris–HCl (20 mM) added before switching on the light; c, 1.5 M NaCl added at the time shown on the curve; d, Tris–HCl 20 mM added as in c.

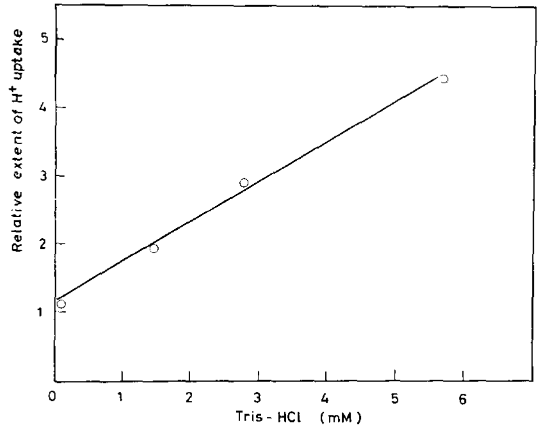

Fig. 6. Effect of Tris–HCl on the extent of H^+ uptake. The extent was measured by means of a pH-stat. For details see text. Cells were suspended in 1.5 M NaCl and brought to pH 6.95.

use of a pH stat, by means of which the pH is kept constant by compensation for disappearance of protons from the medium by an external source of H^+. In unbuffered medium the extent observed in the pH stat was exactly the same as observed beforehand by direct recording of the change of pH. Addition of Tris–HCl increased the extent. It can be seen that there is a linear relationship between the amount of buffer added and the increase in extent (Fig. 6).

We have measured the effect of inhibitors on the light-induced H^+ uptake and on O_2 evolution in *D. parva*. DCMU, known to be an inhibitor of electron transport, inhibited both reactions. A concentration of $2 \cdot 10^{-7}$ M caused 50 % inhibition in O_2 evolution; $1.4 \cdot 10^{-7}$ M DCMU inhibited net H^+ uptake by 50 %. The effective concentrations are in the same range as those which affect Chlorella[12] and isolated chloroplasts[3,5]. There was no effect on the rate of decay which was exactly the same as in the dark, no matter which concentration of DCMU was used.

Fig. 7 shows the time course of the two reactions at $2 \cdot 10^{-7}$ M DCMU which reduced the O_2 evolution by 50 %. It can be seen that the initial lag of the two rates is about the same as the controls. The duration of the maximal rate is much longer than in the control. In both cases there is a decrease of the maximal rate. The interesting point is that the rate starts to decrease when the same net amount of H^+ has been taken up, which is at different times due to the different rates of these reactions. This is again consistent with the idea that the internal pH controls O_2 evolution and hence H^+ uptake.

The next group of inhibitors is considered to be uncouplers of photophosphorylation in isolated chloroplasts[13]. Fig. 8 shows the effects of atebrin. In agreement with[5], atebrin at the concentrations used did not affect the rate of H^+ uptake at all. At 40 μM atebrin the rate of O_2 evolution increased by about 10 % and the rate of decay by 30–50 %, consequently the extent of the reaction was decreased.

The effect of NH_4Cl is not very clear. In contrast to its effects on isolated chloroplasts[3,5] and Chlamydomonas[10] it has almost no effect on *D. parva* when used at a

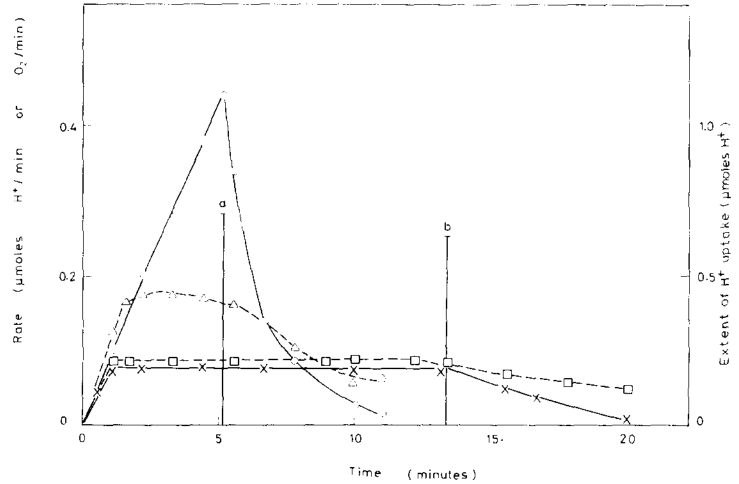

Fig. 7. Effect of DCMU on rates of H^+ uptake and O_2 evolution, as functions of time. ○—○, net H^+ uptake rate; ×—×, net H^+ uptake with $2 \cdot 10^{-7}$ M DCMU; △----△, rate of O_2 evolution; □-----□, O_2 evolution rate with $2 \cdot 10^{-7}$ M DCMU. (a) Extent of reaction when net H^+ uptake rate begins to decrease; (b) as in (a), but with DCMU.

concentration of 1 mM. At higher concentrations it somewhat increased the rate of decay. From its effect on O_2 evolution, it appears to function as an inefficient buffer. It could be that the uncoupling effect is masked by its buffering capacity.

The third uncoupler used was CCCP. Two difficulties were encountered in following its effects. First, its effectiveness is strongly dependent on pH; as changes of pH are large in our system, care was taken to compare its various effects within the same pH range. Secondly, there is a time lag in the onset of its effectiveness; it is therefore difficult to test the inhibitor directly on the decay rate by applying it at the time the light is switched off. It was found that it takes about a minute to get the maximum effect of the CCCP on the rate of H^+ uptake. Assuming the same time lag for the decay, the effect of the CCCP was tested on the decay only when the extent was small, so that the duration of decay was long at about the same pH. Fig. 10 shows that there was a 10 % increase of O_2 evolution at $1.25 \cdot 10^{-7}$ M CCCP. At concentrations causing no inhibition of O_2 evolution, there was already considerable inhibition of the net H^+ uptake. As will be shown later, since CCCP did not affect the rate of decay, it follows that it had directly affected H^+ uptake. The ratio H^+/e had therefore decreased. The effect of CCCP on the decay rate was tested in one of two ways. Either

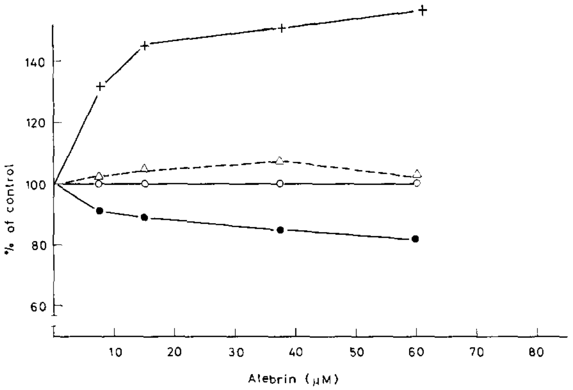

Fig. 8. H^+ uptake, O_2 evolution and H^+ release as functions of atebrin concentration. ○—○, net H^+ uptake rate; ●—●, extent of H^+ uptake; +—+, decay rate; △----△, rate of O_2 evolution. The reaction mixture consists of: Dunaliella cells suspended in 1.5 M NaCl. Initial pH, 6.2. The inhibitor was added at the specified concentration.

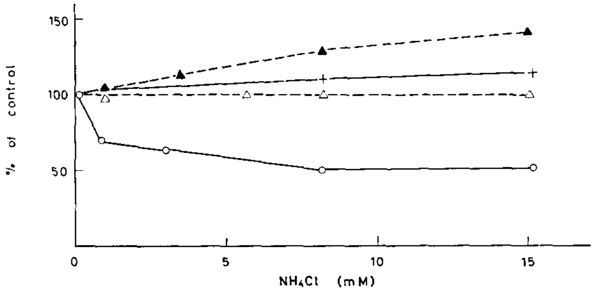

Fig. 9. H^+ uptake, O_2 evolution and H^+ release as functions of NH_4Cl concentration. ○—○, net H^+ uptake rate; +—+, decay rate; △----△, rate of O_2 evolution; ▲-----▲, extent of O_2 evolution. The reaction mixture consists of Dunaliella cells suspended in 1.5 M NaCl. Initial pH, 6.2. The inhibitor was added at the specified concentration.

it was added to the suspension before the light was turned on, and the rates of decay were measured at various extents and compared to the control rates. Alternatively, the light was switched off after changes in pH of the order of 0.2–0.4 pH unit, and the inhibitor was then added to the suspension. In both cases, no effect of CCCP on the rate of decay was observed, even at concentrations of CCCP high enough to inhibit O_2 evolution completely. This is quite different from what had been found in other systems, such as isolated chloroplasts[3,5,7]. The lack of any observed effect on the decay of H^+ gradient must be due either to the fact that the CCCP did not affect the gradient of H^+ assumed to exist across the grana membrane, or to some change being masked by the transport step at the cytoplasmic membrane. The second possibility is unlikely, because a strong effect of atebrin on the rate of decay was indeed observed. It is possible that the CCCP caused a considerable decrease in the permeability to H^+ of the cytoplasmic or chloroplast membrane; this is not usually observed.

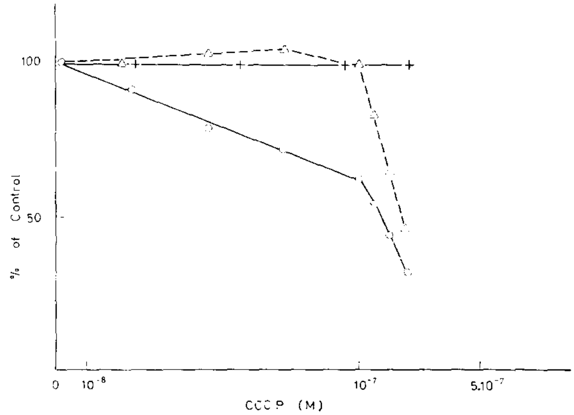

Fig. 10. H^+ uptake, O_2 evolution and H^+ release as functions of CCCP concentration. ○—○, net H^+ uptake rate; +—+, decay rate; △----△, rate of O_2 evolution. The reaction mixture consists of Dunaliella cells suspended in 1.5 M NaCl. Initial pH, 6.2. The inhibitor was added at the specified concentration.

DISCUSSION

There are common features between the light-induced proton uptake observed by us on whole cells of the halophile *D. parva* and that observed with isolated chloroplasts from higher plants[2–7]. Very similar results were also reported recently by SHULDINER AND OHAD[10] for whole cells of Chlamydomonas.

In all of the systems studied, there appears to be a big light-induced uptake of H^+, which is reversed completely when the light is switched off. Inhibitors like DCMU and atebrin have the same effects in all the systems studied.

Furthermore, the mean maximal rates of H^+ uptake per unit of chlorophyll are comparable. The rate of H^+ uptake is about 1–2 μmoles H^+/mg chlorophyll per min in Chlamydomonas[10], 2–6 μmoles H^+/mg chlorophyll per min for *D. parva* and 1–6 μmoles H^+/mg chlorophyll per min in chloroplast preparations, depending on the electron acceptor[4]. On the other hand, the extent of the reaction is about 4–6 times higher in the whole-cell system. This last difference must be due either to damage to the prepared chloroplasts, or to the fact that H^+ uptake is usually measured in chloroplasts under nonphosphorylating conditions. When ADP, P, and Mg were added to the chloroplasts[5,14] the extent of H^+ uptake was increased substantially. It may be that photophosphorylation takes place in whole cells while H^+ uptake is being measured. Indirect evidence for this is given by the increase of O_2 evolution caused by atebrin or CCCP. If that is the explanation for the higher extent observed with whole cells in the steady-state phase, it can be taken as evidence against the chemiosmotic hypothesis which states that the H^+ gradient across the membrane is the "intermediate" or driving force of photophosphorylation. As KARLISH AND AVRON have reasoned[5,14], the extent, or steady state of an intermediate should be lower once a further reaction in the reaction chain occurs. As we have not measured H^+ uptake in whole cells under 2 defined conditions, namely presence or absence of phosphorylation, our evidence is not conclusive as yet.

Though, surprisingly, the maximal rate of H^+ uptake is about the same in all the systems studied, there are important differences between the time course of the rate in *D. parva* and in the chloroplast preparations shown in Fig. 3. According to the kinetic model of KARLISH AND AVRON[5], and the experimental data of IZAWA AND HIND[4], the maximal rate of net uptake should be reached instantaneously on switching on the light. We observed a lag of 2–3 min until the maximum rate was reached. This lag is very likely due to the existence of a transport step, such as a passive transfer of ions through 2 membranes (the cytoplasmic and the chloroplastic). If the area of the external membrane is taken into account, the influx of H^+ is more than 1000 pmoles/cm^2·sec. This is a flux which is 2–3 orders of magnitude larger than any known for cations in algal cells[15,16] as well as many other cells. This could be due either to larger driving forces than usually measured, or to higher permeability coefficients to cations in general and to H^+ in particular. As has been shown[1], the outer membrane of the *D. parva* is highly permeable to nonelectrolytes like sucrose and even inulin. In this work, we have presented evidence that the cell membrane is permeable to various buffers like Tris or phosphate. But the fact that Chlamydomonas has shown rates of H^+ uptake almost as high as those in *D. parva* suggests that the H^+ pathway through the membrane is not necessarily that through which the large nonelectrolyte molecules passed. It has been shown in mitochondria and chloroplasts that 2,4-dinitrophenol, for example, increases specifically the movement of H^+ (refs. 17–19). That would fit the possibility of a natural high specific permeability to H^+. The decay of the H^+ gradient, which is assumed to be passive and to take place across the grana membrane, is not evidence for high permeability to H^+ of the grana membranes as their area is much larger than that of the cellular membrane.

Two aspects are to be discussed regarding the connection between O_2 evolution and H^+ uptake: the stoichiometric ratio and regulative relations. As was mentioned before (Fig. 3), there is a considerable time difference in the build-up of the rates of O_2 evolution and H^+ uptake, respectively. Whether this difference is real, or due only to a transport step, it renders impossible the calculation of the ratio of electron transport (as measured by O_2 evolution) to H^+ uptake at points on the time curve. The ratio may, however, be calculated during the steady state. We have obtained the steady-state H^+ uptake rate by calculating the decay rate, which is also subject to some uncertainty. Our measurements yield ratios of 2.2–2.6 H^+/e during the steady state. This number also applies to cells inhibited by any inhibitor or for which the steady-state rate was changed by pH. This is our maximal ratio. The chemiosmotic hypothesis[8] requires a ratio of 2. Should there be cyclic electron flow as well, our ratio is not inconsistent with the above hypothesis.

Next, it is worthwhile to mention that we have seen a very strict correlation between the declines of H^+ uptake and O_2 evolution. We have several indications that the decrease of the pH inside some chloroplast space is the cause of reduction in rate of O_2 evolution and consequently in that of H^+ uptake.

To conclude, even though there are analogies between light-induced H^+ uptake in the chloroplasts and in whole cells, there are some qualitative and quantitative differences, which make it doubtful as to whether the same phenomenon is observed in the two systems.

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