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STIMULATION OF ATP SYNTHESIS IN *HALOBACTERIUM HALOBIUM* R_1 BY LIGHT-INDUCED OR ARTIFICIALLY CREATED PROTON ELECTROCHEMICAL POTENTIAL GRADIENTS ACROSS THE CELL MEMBRANE

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SUMMARY

The relationship between proton movement and phosphorylation in *Halobacterium halobium* R_1 has been investigated under anaerobic conditions. The light-induced changes in the bacteriorhodopsin are accompanied by proton movements across the cell membrane which result in pH changes in the suspending medium. The initial alkaline shift is shown to be closely paralleled by (and hence correlated with) ATP synthesis. Acidification of the medium in the presence of valinomycin, under conditions of low external potassium, brings about ATP synthesis in the dark.

INTRODUCTION

The light-driven proton pump in the membrane of the extreme halophile *Halobacterium halobium* R_1 has provided a valuable model for the study of energy conversion, especially in view of the relative simplicity of the system [1]. Evidence reported from several laboratories shows that the oriented bacteriorhodopsin complex present in the cell membrane undergoes light-induced cyclic conformational changes associated with proton movement across the cell membrane [1–4]. This proton movement is outwardly directed in whole cells. The outward flux of protons gives rise to an inwardly-directed proton electrochemical potential gradient which is used by the ATP-synthesizing system to drive phosphorylation, since the "return" proton flux is presumably coupled to the phosphorylation process [5, 6]. The light energy absorbed by the bacteriorhodopsin is thus directly transduced into chemical energy without the usual photosynthetic requirement of the electron transport chain [5].

The correlation between proton movement in *H. halobium* cells and ATP synthesis has previously been demonstrated [5, 6]. These results will be confirmed and extended here. Furthermore, if the immediate driving force for ATP synthesis is

Abbreviations: CCCP, carbonylcyanide 3-chlorophenylhydrazone; DCCD, *N,N'*-dicyclohexylcarbodiimide; 1799, α,α' bis (hexafluoroacetonyl) acetone.

indeed the electrochemical potential gradient brought about by light-induced proton transport, it should be possible to activate phosphorylation by artificially creating a gradient as is the case in chloroplasts, chromatophores and mitochondria [7–14]. Results of such experiments will be reported.

MATERIALS AND METHODS

Halobacterium halobium R_1 was used throughout this study. The growth conditions and medium have been described elsewhere as well as the procedure used for the harvesting, washing and suspension of the cells [5]. All suspensions were between 1.0 and 1.2 mg protein/ml. Experiments in which pH changes and ATP formation were followed at the same time were performed in the following way: the cell suspension was maintained anaerobic in the dark by bubbling with CO_2-free, O_2-free nitrogen and aliquots were transferred to a glass chamber maintained at 37 °C by means of a water jacket. The pH was monitored by a glass electrode (Radiometer GK2301C) connected to a Beckman pH meter pHasar I, the changes being automatically recorded with a Varian model A 25 recorder. Anaerobicity was maintained in the chamber by sweeping the surface with nitrogen, purified by successive bubbling through alkaline pyrogallol, water, acid and again water. At intervals, 0.1-ml aliquots were taken out and rapidly diluted into 1.9 ml boiling water; ATP was determined in these aliquots by the lucerifin/luciferase method [15]. Illumination was provided by a 500 W lamp slide projector, at a light intensity of 10^6 erg $\cdot$ cm^{-2} $\cdot$ s^{-1} and measured by a Yellow Springs light meter (YSI model 65). The initial pH was adjusted to the desired value by adding either NaOH or HCl to the cell suspensions in small aliquots, and waiting until the pH stabilized.

Base-acid transition and valinomycin-induced potassium movement experiments were also performed in anaerobic ATP-depleted cell suspensions. The cells were transferred to the reaction vessel where they were kept in the dark and nitrogen or argon was bubbled through the suspension. Before any addition, samples were taken to determine the initial ATP level. The experiment was started either by the addition of HCl (in an amount such that the final pH of the suspension would reach the desired level as determined in a separate aliquot of cell suspension) or by the addition of valinomycin. When both valinomycin and acid were to be used, they were mixed together in the predetermined proportions just prior to addition. Samples for ATP determination were taken 5, 10, and 20 s after addition of the inducers. The amount of ATP synthesized in mg protein/min was computed from the first two points, which were usually in the linear range. Duplicate or triplicate runs were made for each experiment. Valinomycin, CCCP, and Fire-Fly extract were obtained from Sigma, DCCD from Fluka and phloridzin from BDH. Dio 9 was kindly provided by Professor M. Avron.

RESULTS AND DISCUSSION

Fig. 1 shows the pH and ATP changes in an anaerobic cell suspension having a pH value in the dark of 6.5. Switching on the light induced a transient pH rise [1] of about 0.1 pH unit followed by a fall to a level 0.15 pH units lower than the maximum reached during the alkaline phase. The ATP level in the cells rose linearly with time

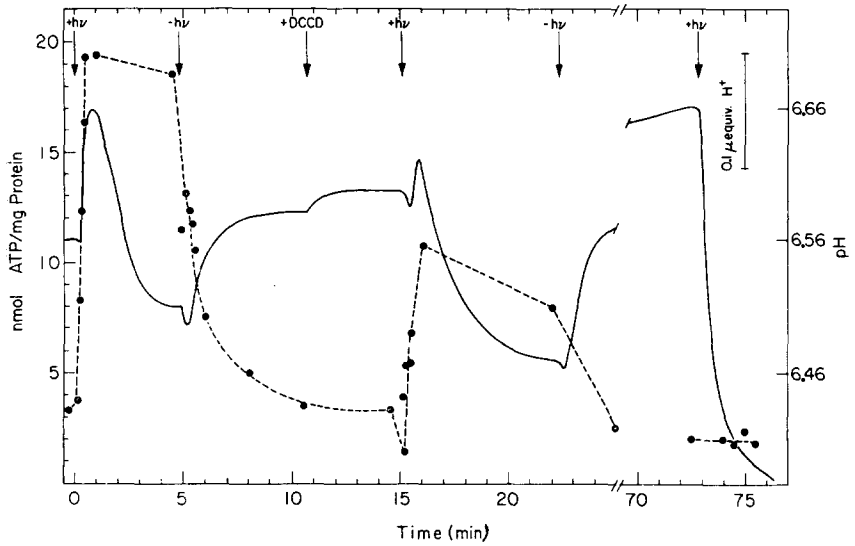

Fig. 1. Observed pH changes and ATP synthesis in a cell suspension in basal salt. Initial pH of the anaerobic suspension in the dark was 6.56. The suspension contained 1.2 mg protein/ml. Experimental details as described under Methods. Solid line indicates pH, dashed line ATP production.

during the first 30 s of illumination, closely following the pH rise and reached a plateau just as the pH of the medium started to fall. Switching off the light caused the pH to return to its initial level, while the ATP decreased. This pattern is typical of the cell behaviour and could be repeated for at least two to three cycles without loss in rate or amplitude.

When the ATPase inhibitor DCCD was added in the dark, a time-dependent effect was observed which is also shown in Fig. 1. About 5 min after addition of the drug, the light was still able to bring about a rise in pH, but this was preceded by a preliminary net extrusion of protons and was smaller and briefer than without the drug. The amount of ATP synthesized was also lower. Increasing the length of time allowed to elapse after addition of DCCD, before switching the light on, led to a gradual disappearance of both the pH rise and ATP synthesis. Fig. 1 shows that 1 h after addition of DCCD only a large proton extrusion occurred, and no ATP was synthesized at all. Similar results were obtained with phloridzin ($5 \cdot 10^{-4}$M) and Dio 9 (20 μg/ml cell suspension), both of which are known to be energy-transfer inhibitors.

If the initial pH of the cell suspension was raised to about 8.4 or lowered to about 5.4, no qualitative differences were noticeable; only the magnitude of the pH change was lower, as was the final amount of ATP synthesized. On adding known amounts of acid at the end of an experiment for calibration purposes, unexpectedly large differences were obtained in the magnitudes of the resultant pH changes depending on the pH range of the cell suspension. At pH 6.5 an addition of 0.1 μequiv. H^+ produced a change of 0.1 pH unit, while at pH values of 8.4 and 5.4 the changes produced were 0.003 and 0.01 pH units respectively. This suggested that the buffering

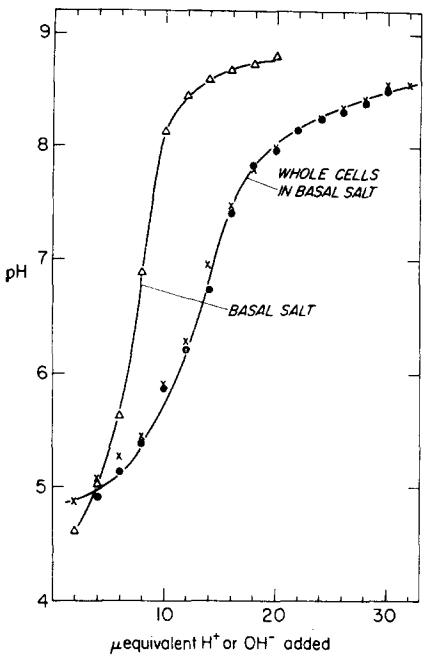

Fig. 2. Complete titration of a cell suspension with HCl or NaOH. The anaerobic cell suspension was equilibrated for 15 min in the light. 20-μl aliquots of 0.1 M HCl were added and the successive pH changes recorded after reaching equilibrium (● — ●). The back titration was performed similarly with equimolar amounts of NaOH (× — ×). The same procedure was followed with a solution of basal salt (△ — △).

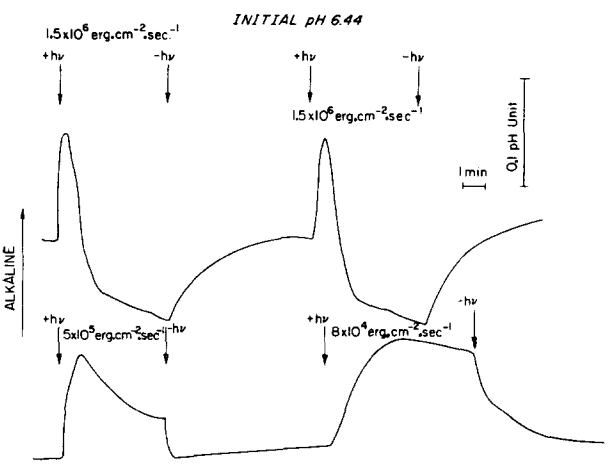

Fig. 3. Influence of light intensity on the pH changes of a cell suspension. The initial pH in the dark, under anaerobiosis, was 6.44.

capacity of the cells might be considerable. Titration of a suspension of the cells in basal salt showed a strong buffering effect in the regions below pH 6 and above pH 7.4 (Fig. 2).

The nature of the light-induced pH change (at any given initial pH) is also dependent upon the light intensity, as is shown in Fig. 3. The pH changes occurring in a cell suspension adjusted initially to pH 6.4 were followed at different light intensities. At $1.5 \cdot 10^6$ erg $\cdot$ cm$^{-2} \cdot$ s^{-1}, the highest intensity used, the usual fast proton uptake followed by a release was observed. The rate and amplitude of the proton movement was reproducible for several cycles, two cycles being shown in Fig. 3. At lower light intensities the kinetics are slowed down considerably, the acidification gradually disappears, and at $8.4 \cdot 10^4$ erg $\cdot$ cm$^{-2} \cdot$ s^{-1} only the alkaline effect remains. When the initial pH of the cells was below 6 or above 7.5, lower light intensity always suppressed the acid effect and only a net proton uptake was observed.

It has previously been shown that inhibition of photophosphorylation by uncouplers like CCCP or 1799 can be overcome by increasing the light intensity, which presumably increases the rate of the proton pump [6]. Since proton pumping is most probably the consequence of light-induced conformational changes in the bacteriorhodopsin [1–3], which promote a vectorial translocation of protons from the intracellular environment to the outside medium, one might have expected the initial fast pH change to be an acidification of the medium. However, it is only when ATP synthesis is blocked by DCCD that this indeed is the case. In general, illumination produces an alkalinization of the medium correlated with the rise in ATP level, and acidification occurs only when the photophosphorylation reaches a plateau. These observations suggest that a translocation of electrical charge accompanied by a rise in internal pH takes place, sufficient to create an electrochemical potential gradient large enough so that protons flow back into the cell driving the ATP synthesizing system. Although the net pH change observed in the medium is in the direction of increasing alkalinity, one must keep in mind that during the illumination the bacteriorhodopsin continuously pumps protons outward. This mechanism does not exclude the possibility that the large primary alkaline shift is brought about by the phosphorylation per se, since the chemical reaction ADP+$P_i \rightarrow$ ATP has a stoichiometric requirement for protons (Bakker, E. P., in preparation).

An earlier report [6] also indicated that at low light intensity the potassium-specific ionophore valinomycin inhibited photophosphorylation in the presence of 30 mM K^+ (or higher). Evidently valinomycin, in increasing the permeability of K^+, tends to abolish the electrical potential across the membrane and so inhibits synthesis unless sufficient protons are continuously extruded by the pump to compensate adequately for the leak of positive charge into the cell. It was also shown that under certain conditions the inhibitory effect of increasing the cell permeability for protons with CCCP could be at least partially counteracted with valinomycin, apparently by increasing the outflow of potassium.

All these data strongly indicate that the driving force for the phosphorylation is largely an electrical potential gradient. This conclusion has recently been substantiated by Bakker et al. [16] using suitable probe molecules. If such a gradient could be artificially imposed ATP synthesis should proceed, as was shown in the case of chloroplasts, chromatophores and mitochondria [7–14].

Figs 4–6 describe the results obtained in such experiments. Valinomycin, in

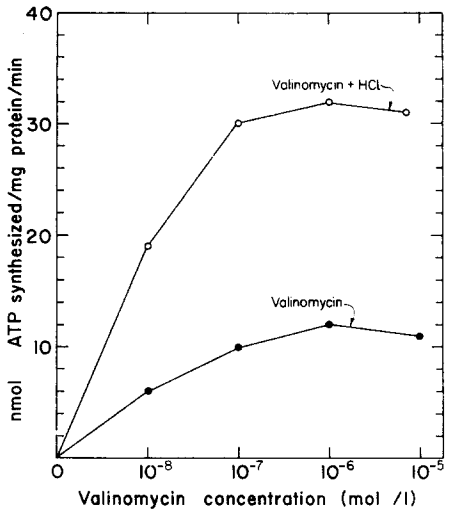

Fig. 4. Stimulation of ATP synthesis in *H. halobium* by an induced proton electrochemical potential difference across the membrane. An electrical potential difference is presumably imposed by adding valinomycin in the presence of 10 mM K^+, and a pH difference is presumably imposed by lowering the pH with HCl. To anaerobic cells in the dark were rapidly added either valinomycin alone at the indicated concentrations, or valinomycin and HCl so that the final pH was 3.5. The initial level of ATP was the amount found in the dark without any addition at pH 7.5.

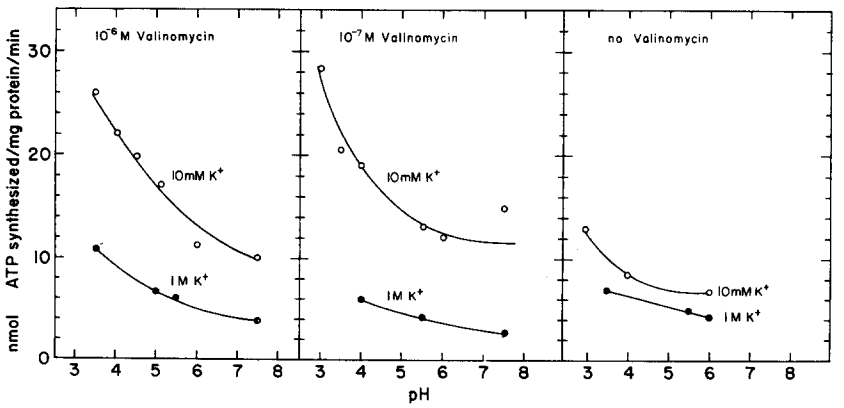

Fig. 5. Influence of a high K^+ concentration on ATP synthesis driven by an induced proton electrochemical potential difference. The same procedure was used as described earlier under Fig. 4: a cell suspension was made either in basal salt containing 10 mM K^+ or in an isosmotic salt solution containing 1 M K^+. As a comparison the amount of light-induced ATP was determined at pH 7.5 at different K^+ concentrations, with the following results. 10 mM K^+: 9.6 nmol ATP/mg protein/min at $1.2 \cdot 10^5$ erg $\cdot$ cm^{-2} $\cdot$ s^{-1}, 24 nmol ATP/mg protein/min at $1 \cdot 10^6$ erg $\cdot$ cm^{-2} $\cdot$ s^{-1}. 1 M K^+: 3.6 and 9.6 respectively at the same light intensities as above. Note that at pH 7.5 the ATP synthesized in the absence of valinomycin is zero.

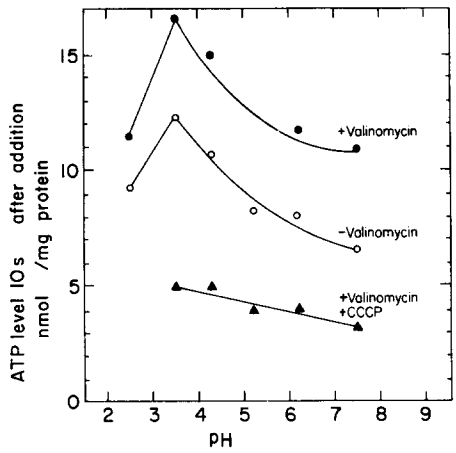

Fig. 6. Inhibition by 10^{-6} M CCCP of the ATP synthesis driven by an induced proton electrochemical potential difference. The same procedure was used as in Figs 4 and 5. ATP is given as the absolute amount reached 10 s after addition of valinomycin or valinomycin+HCl. At the initial pH 7.5, a light intensity of $5 \cdot 10^5$ erg $\cdot$ cm$^{-2} \cdot$ s^{-1} yields 15.4 nmol ATP in 10 s.

the presence of 10 mM potassium in the medium, stimulated ATP synthesis in the dark even at 10^{-8}M. Addition of HCl at the same time as valinomycin greatly enhanced the stimulation (Fig. 4), while without valinomycin the stimulation by HCl alone was markedly lower (Fig. 5). High external potassium concentrations reduced considerably the degree of stimulation due to the combined effects of valinomycin and HCl (Fig. 5). CCCP (10^{-6} M) added at the same time as valinomycin and HCl in the presence of 10 mM K^+ prevented ATP synthesis (Fig. 6). In the pH range 5–3 the inhibition by CCCP, in the absence of valinomycin, ranged from 100 % to 71 %, while in the presence of 10 mM valinomycin only some 33 % inhibition was obtained at pH 3.

The physical separation between the light-driven proton pump (bacteriorhodopsin complex) and the ATP-synthesizing system in the cell membrane of *H. halobium* makes it highly probable that the electrochemical potential gradient of the protons is indeed used as an intermediate store of energy as has been suggested [1]. In any given situation in which energy conversion takes place, the membrane must tend towards a stationary state such that the two functions are coupled together by a continuously circulating proton current. The total electrochemical potential difference generated by the pump between protons on either side of the membrane is the immediate driving force for ATP synthesis, and since the membrane of the intact *Halobacterium* is evidently extremely ion-tight, the electrical component of this potential difference appears to be predominant. However, the experiments shown in Figs 4–6 confirm that ATP synthesis on this organism can be induced by either a pH difference or an electrical potential difference of suitable magnitude, or a combination of both.

The results presented above demonstrate two important characteristics of anaerobic *H. halobium*. Firstly, there is a strong correlation between the initial light-induced proton uptake and the ATP synthesis. Secondly, the proton-motive force presumably created by acidification of the medium and addition of valinomycin in the

presence of low external potassium is able to drive ATP synthesis in the dark. Very recently, intracellular ATP was shown to increase in *E. coli* and *S. lactis* due to the imposition of a transmembrane K^+ gradient [17], and in *E. coli* due to the imposition of a proton-motive force [18]. In our system, as in chromatophores, the valinomycin-induced membrane potential increases the proton electrochemical potential gradient driving the ATP synthesizing apparatus [14], but as in mitochondria the direction of the fluxes is inside to out for K^+ and outside to in for protons passing through the phosphorylation pathway [9, 10].

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