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Anaerobic growth of halobacteria

(halophilism/photosynthesis/bacteriorhodopsin/substrate level phosphorylation)

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ABSTRACT **An energy-transducing pathway in halobacteria is described. Arginine mediates substrate level phosphorylation and allows the cells to grow anaerobically. Bacteriorhodopsin plus light can function as an alternative energy source under these conditions, provided the cells contain the pigment when transferred to the anaerobic environment. Therefore the selection of mutants functionally defective in ATP synthase or bacteriorhodopsin becomes possible.**

Halobacteria are chemoorganotrophic organisms that live mainly on amino acids in concentrated brines. In his review of their physiology, Dundas states that they "are aerobic and have respiratory, never fermentative, metabolisms" (1). In addition to oxidative phosphorylation, halobacteria have the capacity to photophosphorylate, which is mediated by bacteriorhodopsin (2, 3). This light–energy converting system is synthesized only under conditions of limited oxygen supply, when respiration is slowed down (4). However, the chromoprotein cannot be synthesized under strictly anaerobic conditions because retinal biosynthesis involves oxidative cleavage of β-carotene. Therefore, prolonged anaerobic growth of the halobacteria dependent on light energy is not expected to occur.

However, it now appears that anaerobic growth does occur with light as the energy source, provided either that the inoculating cells already contain large amounts of bacteriorhodopsin or that retinal is added to the growth medium.

Arginine degradation to ornithine has been amply demonstrated by Dundas, and the key enzyme ornithine carbamoyltransferase from halobacteria has been isolated and characterized (5–7). We show in this paper that this pathway can be used in a fermentative manner producing ATP from ADP and carbamoylphosphate and that this also allows strictly anaerobic growth of the bacteria.

MATERIALS AND METHODS

Halobacterium halobium R_1M_1 (8) containing no gas vacuoles or bacterioruberin was grown on complete medium (9) with or without 0.5% arginine at 40°C. For aerobic growth either 100-ml erlenmeyer flasks containing 35 ml of medium or 2-liter flasks containing 700 ml were shaken at 105 rpm on a rotatory shaker. These conditions lead to the production of large amounts of bacteriorhodopsin (1 nmol/mg of cell protein). Bacteriorhodopsin-free cells were produced under the same conditions but in the presence of 1 mM nicotine. For anaerobic growth a Gas Pak anaerobic system (Becton, Dickinson, Heidelberg-Wieblingen) was used that contained up to 1.5 liters of medium. The fermenting vessel had a septum for anaerobic sampling during growth. When cells were grown anaerobically under illumination, a 100-ml erlenmeyer flask containing 35 ml of medium was placed inside a Gas Pak vessel and the cell suspensions were stirred. Light from two 150-W projectors was filtered through orange glass filters (OG 515 Schott, Mainz, W. Germany) and directed onto the cell suspension.

Strictly anaerobic conditions were obtained by placing the fermenting vessel inside a vacuum desiccator filled with oxygen-free nitrogen. A change of pH during anaerobic growth from 7.0 to 7.5 was observed. The cellular levels of bacteriorhodopsin and ATP were assayed as described in ref. 10. Amino acid analyses were performed on a Kontron Liquimat III (Kontron, Eching/München).

Protein concentration was measured by the turbidity of the cell suspensions in an Eppendorf photometer model 1101 M at 578 nm. An OD of 4.0 corresponds to a cellular protein concentration of 2 mg/ml (11). The bacteriorhodopsin content of the cells was determined by difference spectroscopy of a cell suspension compared to the same suspension containing bleached bacteriorhodopsin (11).

A mutant unable to grow on arginine anaerobically was obtained by treatment of a cell suspension (OD_{578} 1.0) with 1-methyl-3-nitro-1-nitrosoguanidine (30 μg/ml suspension) for 15 min at 37°C. After the cells had been washed free of mutagen they were grown aerobically on arginine-containing medium for 25 hr and plated on agar. The colonies (about 100 per plate) were replicated after 5 days on three fresh plates, which then were incubated aerobically (with arginine and without arginine) and anaerobically (with arginine) for selection of the mutant.

For dicyclohexylcarbodiimide (DCCD) treatment, the cells were incubated at 30°C overnight in the presence of 0.1 mM DCCD.

ATP was obtained from Boehringer Mannheim, DCCD from Schuchard (Darmstadt, W. Germany), carbonylcyanide *m*-chlorophenylhydrazone (CCCP) from Sigma, and methylnitronitrosoguanidine from Ega-Chemie (Steinheim, W. Germany), and L-arginine was a gift of Degussa (Konstanz, W. Germany). Other chemicals used were from Merck (Darmstadt) and were analytical grade. *H. salinarium* and *H. cutirubrum* were obtained from the American Type Culture Collection.

RESULTS

Anaerobic growth in light

Photophosphorylation in halobacteria is already maximal in the presence of less than 10% of the bacteriorhodopsin content found in cells grown under limited oxygen supply. One would therefore expect anaerobic growth under illumination, provided that the cells in the inoculum contain enough bacteriorhodopsin to be distributed among the daughter cells. Light-dependent growth should continue until bacteriorhodopsin is diluted to

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Abbreviations: DCCD, dicyclohexylcarbodiimide; CCCP, carbonylcyanide *m*-chlorophenylhydrazone.

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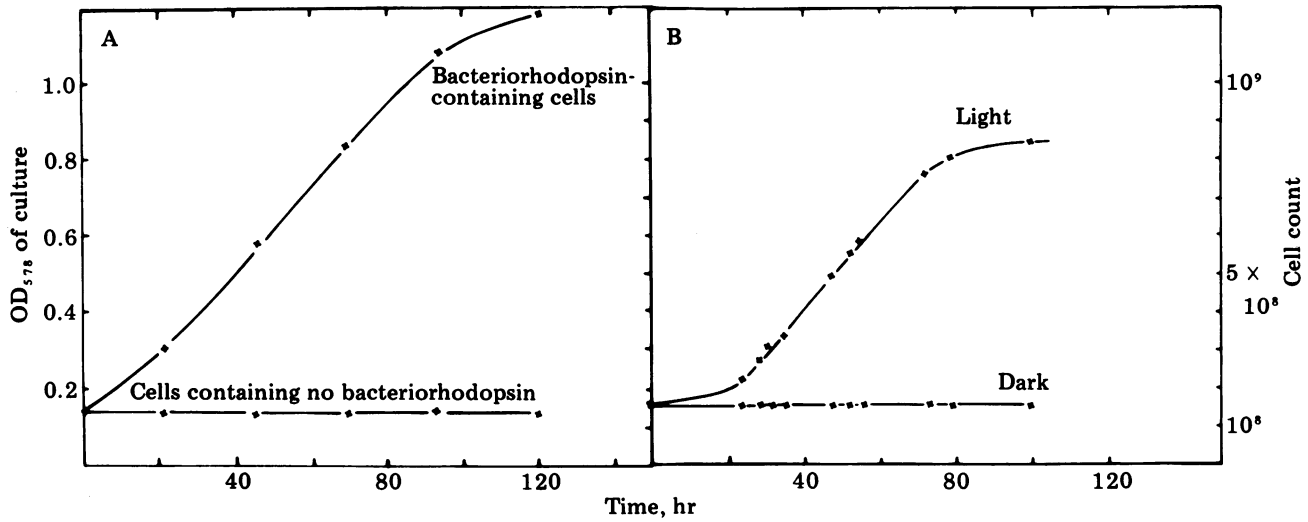

FIG. 1. Anaerobic growth of *H. halobium* mediated by bacteriorhodopsin. (*A*) Comparison of cells with and without bacteriorhodpsin. (*B*) Comparison of cells containing bacteriorhodopsin when illuminated and in darkness. Inocula were prepared under standard conditions with and without 1 mM nicotine. The cell suspension was then diluted 1:10 with fresh medium and 0.1 mM nicotine was added to the bacteriorhodopsin-containing cells in *A*. Growth was measured by taking samples anaerobically from the suspension (35 ml) in erlenmeyer flasks (100 ml) placed inside an anaerobic chamber. Light from two 150-W lamps filtered through glass filter OG 515 was projected from a distance of 4 cm onto the stirred samples.

an extent that no longer allows a sufficient rate of photophosphorylation.

Fig. 1 shows that cells containing large amounts of bacteriorhodopsin (PM cells) grow anaerobically in light, whereas cells containing no bacteriorhodopsin (nicotine cells) do not. The inocula were prepared by growth under conditions of optimal bacteriorhodopsin synthesis, both with and without 1 mM nicotine. To start the experiment, both cell suspensions were diluted 1:10 with fresh medium and nicotine was added to the PM cells so that the final growth medium contained 0.1 mM nicotine in both cases. This concentration does not interfere with growth or retinal synthesis. Fig. 1*A* shows that bacteriorhodopsin allows the PM cell suspension to reach the standard cell density observed under aerobic conditions. The nicotine-treated cells containing no bacteriorhodopsin do not grow at all. If the PM cells are kept in the dark no growth occurs (Fig. 1*B*).

The cells are able to synthesize bacteriorhodopsin in the presence of nicotine if retinal is added to the growth medium (Table 1). Therefore, as expected, halobacterial cells will grow anaerobically under illumination in the presence of nicotine when 3 μM retinal is present in the medium (Fig. 2). This is of importance for the isolation of mutants with functionally defective bacteriorhodopsin. It should be possible to isolate such mutants on agar plates by simple replication techniques.

Anaerobic growth with arginine

Because halobacteria are able to grow anaerobically with ATP produced from light energy by bacteriorhodopsin, one might question the statement that they do not possess a fermentative metabolism. Arginine degradation in halobacteria was reported to occur via the arginine deiminase pathway (1, 6). The resulting citrulline could be converted into ornithine and carbamoylphosphate, which is a valuable ATP source. In fact, Dundas reported that arginine will sustain halobacterial motility under anaerobic conditions (1). Under aerobic conditions in peptone media, halobacterial cells grow slightly better in the absence than in the presence of 0.5% arginine (Fig. 3*B*). Anaerobic conditions were provided by a closed system in which oxygen was removed from the fermenting vessel by reaction with hydrogen on a platinum catalyst. The vessel, made of translucent plastic, was modified in order to allow growth in 1500-ml anaerobic suspensions and withdrawal of samples via a septum. Fig. 3*A* shows anaerobic growth of *H. halobium* in darkness in the presence of arginine. Omission of arginine from the medium totally prevented growth. On agar plates colonies of halobacteria grew anaerobically in the presence of arginine as well as they did aerobically (arginine absent). After mutagen

Table 1. Growth of *H. halobium* in the presence of nicotine (1 mM) and various amounts of retinal

Sample	Retinal, μM	Cell density, OD_{578}	Bacteriorhodopsin, nmol/mg of cell protein
1	0	1.44	0.04
2	1	1.38	0.39
3	3	1.58	0.44
4	5	1.47	0.66

Growth was for 120 hr.

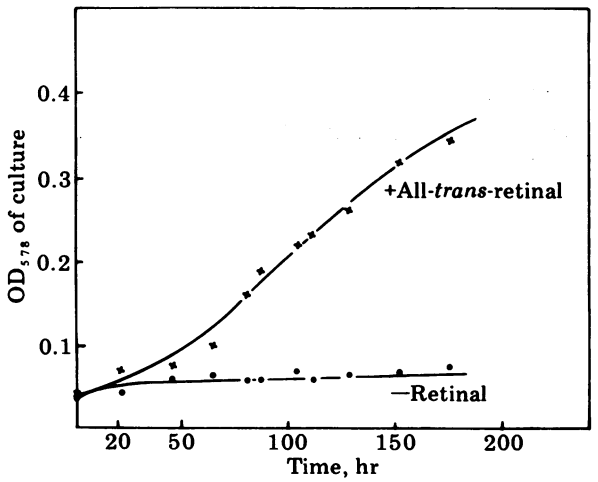

FIG. 2. Retinal-induced anaerobic growth of *H. halobium*. Medium containing 1 mM nicotine was inoculated with bacteriorhodopsin-free cells as described for Fig. 1. All-*trans*-retinal (3 μM final concentration) was added to one of the suspensions, and the suspensions were then incubated anaerobically at 40°C.

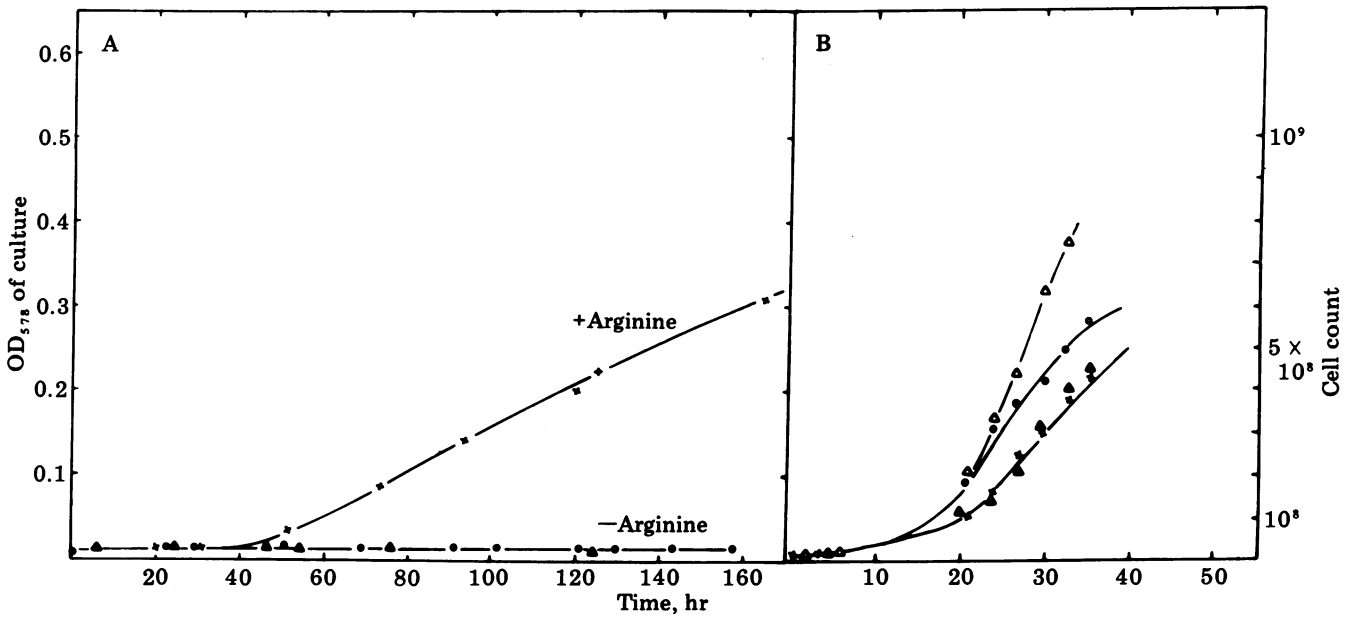

FIG. 3. Anaerobic and aerobic growth of *H. halobium*. (*A*) Anaerobic growth in the presence of 0.5% arginine (X). Inoculum 1:100. O, Without arginine; ▲, mutant unable to metabolize arginine. (*B*) Comparison of initial aerobic growth of the wild type and the arginine mutant with and without arginine. ▲, Mutant + arginine; △, mutant − arginine; X, wild type + arginine; O, wild type − arginine.

treatment with 1-methyl-3-nitro-1-nitrosoguanidine, colonies that did not grow anaerobically in the presence of arginine were selected by standard replication techniques.

Table 2 shows that a variety of halobacteria can grow anaerobically in darkness in the presence of arginine.

The fact that arginine sustains anaerobic growth immediately suggests the fermentative pathway involving reactions of the urea cycle that is mentioned above. The reaction sequence arginine → citrulline → ornithine provides carbamoylphosphate for ATP synthesis. It should be mentioned that this pathway has already been demonstrated in some bacteria (12). Fig. 4 shows that cell suspensions incubated under nitrogen took up arginine from the medium and secreted an equivalent amount of ornithine. When the suspensions were illuminated, neither arginine consumption nor ornithine secretion was observed. This can be explained by the combined action of bacteriorhodopsin and ATP synthase, which keeps the ATP level of the cells at its maximum and therefore makes ADP unavailable for phosphorylation by carbamoylphosphate. Consequently, arginine degradation is prevented. This is another example of bioenergetic competition in halobacteria, comparable to light inhibition of respiration (13).

Phosphorylation of ADP, mediated by arginine catabolism, was demonstrated by measurements of ATP level in cell suspensions under various conditions. Oxidative phosphorylation was observed in darkness upon aeration, photophosphorylation under nitrogen upon illumination, and substrate level phosphorylation upon addition of arginine as shown in Fig. 5. As expected, the mutant unable to grow anaerobically on arginine showed no increase in the ATP level upon addition of arginine. Furthermore, the quantitative difference in arginine-mediated phosphorylation found between aerobically and anaerobically grown cells suggests that the enzymes responsible for arginine degradation are induced under anaerobic growth conditions.

Direct proof for substrate level phosphorylation during arginine degradation was obtained by the experiment shown in Fig. 6. DCCD prevents ATP synthesis by inactivation of the reversible proton translocating ATPase (ATP synthase). It was shown earlier that this agent inhibits oxidative phosphorylation as well as photophosphorylation in *H. halobium* (3). In contrast,

Table 2. Anaerobic growth of different halobacterial strains in the dark

Strain	OD_{578} Initial	OD_{578} After 7 days
H. halobium R_1M_1	0.02	0.38
H. halobium R_1	0.02	0.37
H. halobium wild type	0.02	0.36
H. salinarium	0.02	0.38
H. cutirubrum	0.02	0.22

Flasks with 25 ml of complete medium (9) containing 0.5% arginine were inoculated with different strains from colonies on agar plates and the flasks were sealed under nitrogen. The cultures were shaken at 39°C for 7 days and then their turbidities were determined at 578 nm. A control (*H. halobium* R_1M_1) without arginine remained at the initial OD_{578} of 0.02.

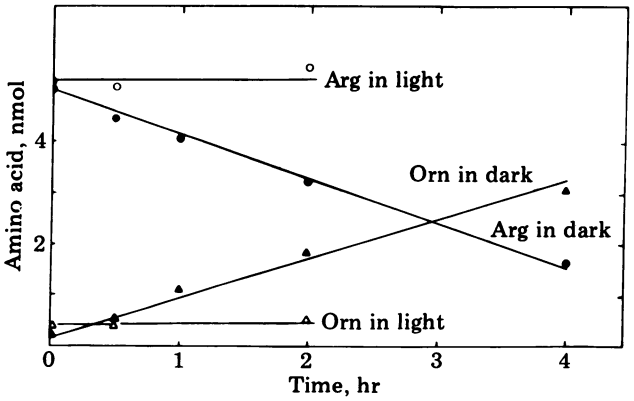

FIG. 4. Conversion of arginine to ornithine by *H. halobium* cells under anaerobic conditions. Cells of *H. halobium* containing bacteriorhodopsin were suspended in basal salts in the presence of 50 mM Tris/maleate, pH 7, and kept under nitrogen. Arginine (0.2%) was added and the cell suspension (2 mg of protein per ml) was incubated at 39°C in the dark. At the indicated times 10-ml samples were taken, the cells were removed by centrifugation, and aliquots of the supernatant were analyzed for arginine (Arg, ●) and ornithine (Orn, ▲). In a parallel experiment the cell suspension was illuminated (50 mW/cm^2, filter OG 515) during incubation (Arg, O; Orn, △).

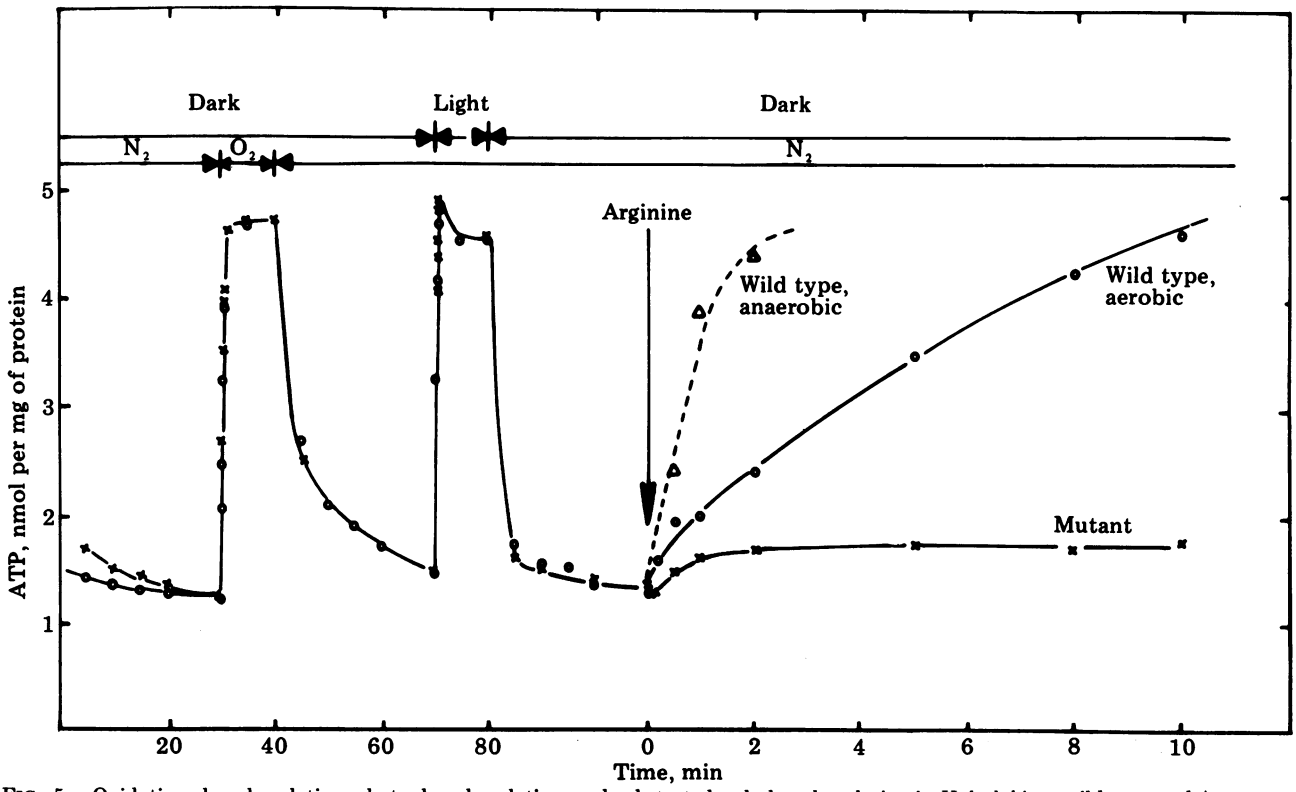

FIG. 5. Oxidative phosphorylation, photophosphorylation, and substrate level phosphorylation in *H. halobium* wild type and Arg$^-$ mutant. For each experiment a 10-ml suspension (2 mg of protein per ml) in basal salts, buffered with 50 mM Tris/maleate, pH 7, was used. Experimental details are described in ref. 10. Light intensity was 50 mW/cm^2; 0.2% L-arginine was added for induction of ATP synthesis. "Aerobic" and "anaerobic" refer to conditions of growth of the cells.

ATP synthesis induced by arginine was unaffected by DCCD, indicating that it is substrate level phosphorylation. Furthermore, and in contrast to untreated control cells, the ATP level did not decrease when the uncoupler CCCP was added to cells whose ATP synthase had been blocked with DCCD. This can be explained by the equilibration of the ATP/ADP/P_i system

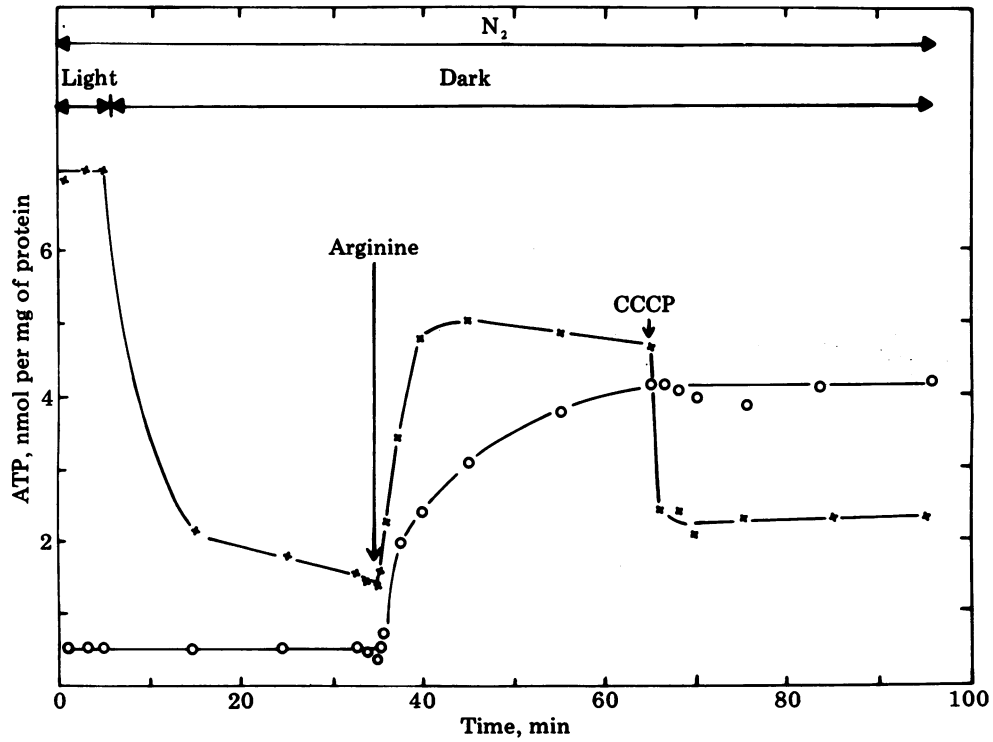

Fig. 6. Evidence for substrate level phosphorylation induced by arginine. Experimental details as for Fig. 2. X, Control cells; O, cells treated with 0.1 mM DCCD. Cell suspensions were illuminated for 5 min at 50 mW/cm^2, then kept in the dark for 90 min. Arginine and CCCP were added to final concentrations of 0.2% and 10 μM, respectively. Strictly anaerobic conditions were maintained during the experiment. Sampling and ATP determination were as in ref. 7.

with the electrochemical proton gradient across the cell membrane through the mediation of ATP synthase. Thus, when the addition of an uncoupling agent lowers the electrochemical proton gradient, ATP synthase will hydrolyze ATP and transfer protons from the cell, equilibrating both systems. DCCD-inactivated synthase is not able to carry out this role and therefore the ATP level remains unaffected.

DISCUSSION

Halobacteria seem to be well equipped with metabolic machinery for the production of ATP: oxidative phosphorylation, photophosphorylation, and substrate level phosphorylation. Thus the bacteria have the capacity to grow under aerobic, semiaerobic, and anaerobic conditions. Although retinal synthesis would be blocked under anaerobic conditions and the bacteriorhodopsin progressively diluted out by cell multiplication, it can be shown that even serial dilution by a factor of 100 during growth produces cells that still show measurable photophosphorylation. This is in accordance with data indicating that the bacteriorhodopsin molecules normally present in a cell need to work at only 1% of their maximal pump capacity (11) to energize the ATP synthase system. In addition, the oxygen requirement for the retinal synthesis that is necessary for appreciable bacteriorhodopsin formation is so small that in the natural habitat of halobacteria synthesis would usually be possible. At least in the fermenting vessel used in these experiments enough oxygen diffused through the plastic walls to allow measurable synthesis of retinal—i.e., bacteriorhodopsin. Only when the vessel was placed under oxygen-free nitrogen were the last traces of oxygen removed and no measurable retinal synthesis observed.

Under total lack of oxygen, arginine can replace light as an energy source for ATP synthesis. This fermentative pathway was already implicated in experiments on motility of halobacteria, in which arginine was shown to sustain motility under anaerobic conditions (1). The discovery of substrate level phosphorylation in *Halobacterium* is of key importance because it completes the set of complementary growth conditions sufficient to allow the selection of conditional lethal mutations in the energy-transducing pathways. Growing in the presence of arginine, cells are sustained by substrate level phosphorylation and have no need for either ATP synthase or bacteriorhodopsin. In contrast, in the absence of arginine cells are totally dependent on a functional ATP synthase and protonmotive force. In addition, anaerobic growth in light renders the cells absolutely dependent on bacteriorhodopsin. Thus, the isolation of both ATP synthase and bacteriorhodopsin mutants, hampered in the past by the lack of a proper selection technique, is now possible.

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