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Electrochemical Proton Gradient across the Cell Membrane of *Halobacterium halobium*: Effect of *N,N'*-Dicyclohexylcarbodiimide, Relation to Intracellular Adenosine Triphosphate, Adenosine Diphosphate, and Phosphate Concentration, and Influence of the Potassium Gradient†

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ABSTRACT: The proton motive force across the cell membrane of halobacterial cells has been estimated and compared to intracellular values of ATP, ADP, and inorganic phosphate concentrations with respect to the chemiosmotic hypothesis. The accumulation of ^{14}C-labeled indicator substances, triphenylmethylphosphonium for the membrane potential and 5,5-dimethyloxazolidine-2,4-dione for the pH difference between the cell interior and the medium, has been measured in the cells. Values up to 270 mV for the proton motive force have been found in cells pretreated with *N,N'*-dicyclohexylcarbodiimide (DCCD, 10^{-4} M, 30 °C, 12 h). Upon illumination a high membrane potential is generated, which is then gradually replaced by a large pH difference. Cells treated with lower DCCD concentrations show only an enhancement of membrane potential upon illumination; the pH difference remains at a low level. Under anaerobic dark conditions, untreated cells maintain a proton motive force of 120–140 mV, which is equilibrated with the intracellular levels of ATP, ADP, and inorganic phosphate. The pH gradient is 1 unit at pH 6 but 0 at pH 8. The membrane potential is low (60–80 mV) at pH 6 and high (120–130 mV) at pH 8. We propose that the proton translocating ATPase compensates for the lowered pH difference at high external pH values by enhancing the membrane potential. The concentration difference of the potassium ions influences the proton motive force and the intracellular ATP levels, apparently via its action on the membrane potential. When the difference of the chemical potential of the potassium ion, expressed in millivolts, exceeds the preexisting membrane potential, the intracellular ATP level is enhanced. When the difference of the chemical potential of the potassium ion (millivolts) is smaller than the membrane potential, the ATP level is decreased.

During the last years halobacteria have been of much interest in biochemical and biophysical research. Under oxygen-limited growth conditions they synthesize a retinal–protein complex known as bacteriorhodopsin (Oesterhelt & Stoeckenius, 1971), which mediates light energy conversion (Oesterhelt & Stoeckenius, 1973).

In intact cells light has been found to drive ATP synthesis (Danon & Stoeckenius, 1974; Oesterhelt, 1974; Hartmann & Oesterhelt, 1977), to inhibit respiration (Oesterhelt & Krippahl, 1973), and to drive the uptake of amino acids (Hubbard et al., 1976) and potassium (Wagner et al., 1978). This light energy conversion of halobacteria is best explained by the chemiosmotic hypothesis (Mitchell, 1966, 1968): bacteriorhodopsin acts as an electrogenic proton pump by releasing protons at the outside of the cell membrane and taking up protons from the inside (Oesterhelt & Stoeckenius, 1973). This process creates an electric potential difference ($\Delta\psi$)[1] across the cell membrane, the so-called membrane potential,

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[1] Abbreviations and symbols used: DMO, 5,5-dimethyloxazolidine-2,4-dione; $TPMP^+$, triphenylmethylphosphonium ion; $TPMP^+_i$ and $TPMP^+_o$, intra- and extracellular $TPMP^+$; DCCD, *N,N'*-dicyclohexylcarbodiimide; $\Delta\psi$, electric potential difference across the cell membrane; pH_o, extracellular pH; pH_i, intracellular pH; ΔpH, pH_o minus pH_i; *n*, number of protons translocated per molecule of ATP synthesized or hydrolyzed; $\Delta\bar{\mu}_{H^+}/F$, electrochemical potential difference of the proton across the membrane or the proton motive force (expressed in millivolts); basal salt, 4.3 M NaCl, 27 mM KCl, and 81 mM $MgSO_4$; potassium basal salt, 2.7 M KCl, 1.6 M NaCl, and 81 mM $MgSO_4$.

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and a pH difference. Both are components of the electrochemical potential difference of the proton or the proton motive force according to the formula

$$\frac{\Delta\bar{\mu}_{H^+}}{F} = \Delta\psi - 2.3\frac{RT}{F}\Delta pH \quad (1)$$

where F = Faraday's constant, R = the gas constant, and T = the absolute temperature.

Recently, methods have been described which allow the estimation of the membrane potential and the pH difference across the cell membrane of halobacteria (Michel & Oesterhelt, 1976; Bakker et al., 1976). The accumulation of the permeant cation $TPMP^+$ in the cells has been used to determine the membrane potential according to the Nernst equation, whereas the pH difference has been evaluated from the distribution of the weak acid DMO.

In the first part of this communication we report the effects of treatment of the whole cells with the ATPase inhibitor DCCD; in the second part, we compare the levels of ATP, ADP, and phosphate with the electrochemical proton gradient. The influence of the potassium gradient on the ATP level is also considered.

Experimental Procedure

Bacteria. Culture conditions and the main features of the bacteria used in the experiments (*Halobacterium halobium* R_1M_1) have been described in detail previously (Hartmann & Oesterhelt, 1977).

pH Measurements. If glass electrodes are calibrated with a buffer of low ionic strength, measurement of pH at high ionic strength is in error by as much as 0.3 unit. Consequently, we calibrated our glass electrode (405-M-3, Ingold, Frankfurt) with a hydrogen electrode at high ionic strength. The glass electrode was small enough to measure the pH changes in parallel experiments directly in 4 mm diameter centrifuge tubes.

Determination of pH Differences and Membrane Potential. The pH difference across the cell membranes was calculated from the distribution of the weak acid [^{14}C]DMO between external medium and intracellular fluid (Addanki et al., 1968). The concentration of [^{14}C]DMO in the medium and in the cells was determined after silicone layer centrifugation as previously described (Michel & Oesterhelt, 1976).

With the same technique the accumulation of the lipophilic cation $TPMP^+$ in the cells was measured. The membrane potential was calculated from the Nernst equation

$$\Delta\psi = \frac{RT}{2.3F}\log\frac{[TPMP^+_i]}{[TPMP^+_o]} \quad (2)$$

Unless stated otherwise, cell suspensions with a turbidity of 5.5 at 578 nm (Eppendorf M 1 photometer) corresponding to 2.75 mg of protein per mL or 4.39 mg of intracellular water per mL of cell suspension have been used. For further experimental details, see Michel & Oesterhelt (1976).

One difficulty that arises when the membrane potential is calculated from the accumulation of [^{14}C]$TPMP^+$ is that a substantial amount of $TPMP^+$ cannot be released from the cells by uncouplers, which would normally abolish the outside-positive membrane potential and lead to a total loss of the indicator ion. This amount of $TPMP^+$ is bound to sedimentable material, as can be shown by high-speed centrifugation of detergent-lysed cells (Michel, 1977). High concentrations ($>10^{-5}$ M) of the uncoupler carbonyl cyanide *m*-chlorophenylhydrazone or 4,5,6,7-tetrachloro-2-(trifluoromethyl)benzimidazole as well as phloretin induce binding of $TPMP^+$ to sedimentable material (100000*g*, 1 h). For this reason we have corrected the accumulation of $TPMP^+$ for the amount of $TPMP^+$ that could not be released by 10^{-6} M carbonyl cyanide *m*-chlorophenylhydrazone. The membrane potential calculated without correction for uncoupler-insensitive binding gives an upper limit, $\Delta\psi_{max}$, which with correction yields a lower value, $\Delta\psi_{min}$. Thus, the true membrane potential lies between these two values. At high membrane potential (ca. 150 mV) the accumulation of $TPMP^+$ far exceeds the amount which is bound to the membranes, and $\Delta\psi_{max} - \Delta\psi_{min}$ is on the order of 3 mV. At low membrane potential ($\Delta\psi_{max}$ = 60 mV) the difference between the accumulated $TPMP^+$ and the amount of $TPMP^+$ which is bound to the membrane is far less, and $\Delta\psi_{max} - \Delta\psi_{min}$ is on the order of 50 mV.

Illumination. Illumination of cell suspensions was carried out as previously described (Michel & Oesterhelt, 1976). The irradiance was varied by neutral density glass filters (Schott) and was measured with a bolometer (Kipp and Zonen) connected to a Knick microvoltmeter.

Determination of ATP, ADP, and P_i. The luciferin–luciferase assay was used to determine ATP and ADP (Hartmann & Oesterhelt, 1977; Wagner et al., 1978). Phosphate was determined by the method of Bagnisky et al. (1967). All measurements were performed in duplicate.

Treatment of Cells with DCCD. "Intensively DCCD-treated" cells were obtained by incubating cell suspensions (2 mg of protein per mL) in the presence of 10^{-4} M DCCD at 30 °C overnight without stirring. "Moderately DCCD-treated" cells were incubated with 2.5×10^{-5} M DCCD at 0 °C for 30 h. Before use the cells were spun down and resuspended in a medium without DCCD.

Results

Effect of DCCD Treatment. In a previous paper (Michel & Oesterhelt, 1976), it was shown that treatment of halobacteria with the ATPase inhibitor DCCD leads to large differences in both membrane potential and pH difference, in darkness and when illuminated. A more careful inspection of the effect of DCCD showed that significant differences are obtained when DCCD is used in low concentrations and at low temperature (moderate DCCD treatment), just abolishing light-induced ATP synthesis, or in high concentrations and at higher temperature (intensive DCCD treatment), affecting more cellular functions than solely ATP synthesis. Both types of DCCD-treated cells seem to be suitable model systems, reducing the complexity of the intact cells.

Cells which had been intensively treated with DCCD undergo large increases in both membrane potential and pH gradient upon illumination, changes which are far larger than with untreated cells (see Table I). The dependence of the pH difference, expressed in millivolts obtained from the product of $\Delta pH \times 59$ mV, the membrane potential, and the proton motive forces of intensively DCCD-treated cells upon irradiance is shown in Figure 1. The pH difference in darkness is 0.1–0.2 unit and rises upon illumination by more than 2.5 units. Concomitantly the membrane potential rises from 40–80 mV in darkness to 110–130 mV at an irradiance of 5–11 mW/cm^2 and declines to 100–110 mV at higher irradiance. The decrease in membrane potential upon increase in irradiance seems to be partially compensated for by the increase of the pH difference. The dependence of the proton motive force on irradiance resembles a saturation curve, although saturation is still incomplete with the highest irradiance used. Under our conditions the maximal values for the proton motive force are around 270–280 mV.

Corresponding results are obtained when membrane potential and pH difference are measured as a function of time

Table I: Dependence of Membrane Potential and pH Difference on DCCD Treatment with and without Illumination at pH 6.3 and 8[a]

pH	cells	illumination	$\Delta\psi_{max}$ (mV)	−59 mV × ΔpH (mV)
6.3	not treated	−	85	53
	with DCCD	+	100	65
	moderately treated	−	87	30
	with DCCD	+	162	*47*
	intensively treated	−	86	12[b]
	with DCCD	+	120	*153*
8.0	not treated	−	*131*	0[b]
	with DCCD	+	154	12[b]
	moderately treated	−	*93*	nd[c]
	with DCCD	+	163	nd
	intensively treated	−	*92*	0[b]
	with DCCD	+	109	106

[a] Values for membrane potential (without correction for uncoupler-insensitive accumulation of $TPMP^+$) and pH difference have been calculated from the accumulation of $TPMP^+$ or DMO after 1 h of incubation in the dark under exclusion of oxygen or an additional illumination for 10 min prior to centrifugation (irradiance 22 mW/cm²). Cells were suspended in strongly buffered basal salt (+75 mM Tris-maleate). [b] pH difference was too low to be measured accurately. [c] nd = not determined.

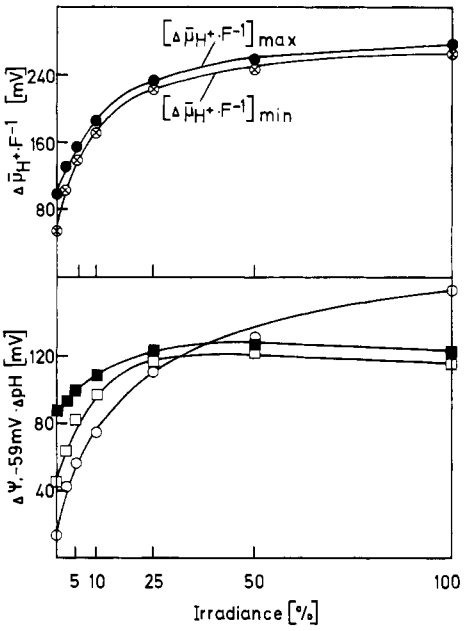

FIGURE 1: Dependence of membrane potential ($\Delta\psi_{max}$, ■; $\Delta\psi_{min}$, □), −59 mV × ΔpH (○, lower half of the figure), and proton motive force (upper half of the figure) on the irradiance measured after 10 min of illumination using intensively DCCD-treated cells. Strongly buffered (75 mM Tris-maleate, pH 6.3) cell suspensions in basal salt were incubated in the dark under anaerobic conditions for 1 h prior to illumination (100% irradiance = 22 mW/cm²). Then the cells were spun down, and the uptake of [^{14}C]DMO was determined for the measurement of ΔpH or that of [^{14}C]$TPMP^+$ for measurement of $\Delta\psi$.

at high irradiance (Figure 2). Immediately after onset of illumination the membrane potential reaches its maximum, but then it declines continuously. The decrease of the membrane potential is compensated for by an increase in the pH difference. The proton motive force remains constant at 260–270 mV during illumination. When the light is switched off, the membrane potential decreases at once to a value which is lower than that before the onset of illumination. The residual amount of the indicator ion $TPMP^+$ in the cells is then no larger than the amount which is normally bound to the cell membranes. Thus, it is probable that after switching off the

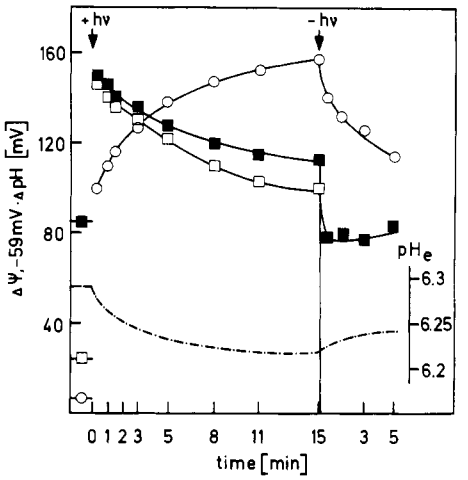

FIGURE 2: Time dependence of the membrane potential ($\Delta\psi_{max}$, ■; $\Delta\psi_{min}$, □) and −59 mV × ΔpH (○) at high irradiance (22 mW/cm²) with intensively DCCD-treated cells. Strongly buffered (75 mM Tris–maleate, pH 6.3) cell suspensions in basal salt were incubated anaerobically for 1 h prior to illumination and centrifugation. The time scale is not corrected for the time needed to spin down the cells (50% within 15 s). Therefore, in the figure curves for $\Delta\psi$ and −59 mV × ΔpH are interrupted where the light was switched on ($+h\nu$). When the light was switched off ($-h\nu$), $\Delta\psi_{min}$ became so small that an inside-positive value would be calculated after correction for uncoupler-insensitive binding of [^{14}C]$TPMP^+$. The light-induced change of the external pH is shown as an inset (−·−).

light, no membrane potential (inside negative) is maintained. No hint was found for the possibility that immediately after switching off the light the high pH difference establishes a diffusion potential of protons of OH^- ions (inside positive) since there was no accumulation of the permeant anion [^{14}C]SCN^-. [The SCN^- ion is accumulated in cells which have an inside-positive membrane potential [see e.g., Michels & Konings (1978) and Schuldiner et al. (1974)], but a high permeability of SCN^- ion in the halobacterial system has still to be proven.]

When moderately DCCD-treated cells are illuminated at an external pH of 6.3, the accumulation ratio of the indicator ion $TPMP^+$ rises from 30 (uncorrected for uncoupler-insensitive binding), corresponding to 87 mV, to 599, corresponding to 162 mV. In contrast to intensively DCCD-treated cells, the moderately DCCD-treated cells maintain a significant pH difference under anaerobic conditions in darkness. A 10-min illumination raises the pH difference from 0.5 to 0.8 unit, only one-third the value obtained by using intensively DCCD-treated cells. However, the membrane potential remains high and is not replaced by a pH gradient.

The membrane potentials of moderately DCCD-treated cells, intensively DCCD-treated cells, and untreated cells are compared in Table I. The high membrane potential of the untreated cells at pH 8 in darkness is abolished by both moderate and intensive DCCD treatment. This can be explained by considering the inactivation of the proton-translocating ATPase (see Discussion).

Electrochemical Proton Gradient and ATP, ADP, and P_i Levels under Anaerobic Dark Conditions. The membrane potential, pH difference, and intracellular ATP concentration of untreated cells in darkness under anaerobic conditions are shown as a function of pH in Figure 3. The intracellular levels of phosphate and ADP are shown in Table II. When the cells are incubated at pH 6, both components of the proton motive force are of similar size: the pH difference is 1 pH unit, corresponding to 59 mV, and the membrane potential is 60–80 mV. When the pH of the medium is increased, the pH difference decreases simultaneously with an increase in the

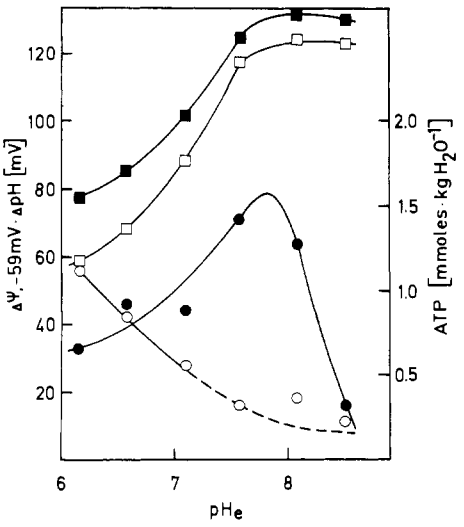

FIGURE 3: Membrane potential ($\Delta\psi_{max}$, ■; $\Delta\psi_{min}$, □), −59 mV × ΔpH (○), and intracellular ATP level (●) as a function of external pH. Strongly buffered cell suspensions (75 mM Tris–maleate, pH 6.3) in basal salt were incubated anaerobically for 1 h in the dark before $\Delta\psi$, ΔpH, or ATP level were determined. (--○--) Remember that −ΔpH values below 0.4 cannot be accurately measured.

Table II: Values of [ATP], [ADP], [P_i], and 59 mV × log [[ATP]/([ADP][P_i])] Taken from the Experiment in Figure 3

pH_o	[ATP] (nmol/kg of H_2O)	[ADP] (nmol/kg of H_2O)	[P_i] (nmol/kg of H_2O)	59 mV × log [[ATP]/([ADP][P_i])] (mV)
6.2	0.65	1.64	51.6	52.1
6.6	0.90	1.58	50.7	63.3
7.1	0.89	1.53	53.2	61.2
7.6	1.43	1.66	52.0	71.8
8.1	1.29	1.69	53.5	69.6
8.5	0.32	0.73	52.7	54.0

membrane potential [similar results in the measurement of $\Delta\psi$ and ΔpH have already been published; see Michel & Oesterhelt (1976) and Bakker et al. (1976). We present the results here mainly for a comprehensive discussion of the relation of the electrochemical proton gradient to intracellular levels of ATP, ADP, and P_i].

Surprisingly, the intracellular ATP level increases at higher pH, reaches a maximum value at around pH 8, and then declines sharply at still higher pH. This increase of the ATP levels also occurs upon shift of the pH to higher values with cells incubated anaerobically in the dark. As shown in Figure 4, the new ATP level is reached slowly, taking approximately 90 min. When the cell suspension is illuminated, a maximal ATP level is attained by photophosphorylation very quickly; upon cessation of illumination the ATP level returns rapidly to its value prior to illumination. Lowering the external pH from pH 7.9 to 6.9 leads to a slow decrease in ATP level. Thus, the changes of ATP level in the dark caused by pH shifts between pH 6 and 8 are very slow and reversible.

The increase in ATP level due to alkalinization of the medium has two other notable features. First, before the increase in ATP occurs, it is preceded by a small decrease. Second, the increase in ATP is delayed by between 1 and 60 min. This would indicate that some event other than the pH increase itself causes the change in ATP level. We must stress that neither respiration nor light could cause the increase of ATP level. The possibility of ATP formation due to fermentation is ruled out, since the cells were suspended in basal salt and, additionally, no fermentation is known in *H. halobium*. Nitrate reduction can also be excluded, since nitrate

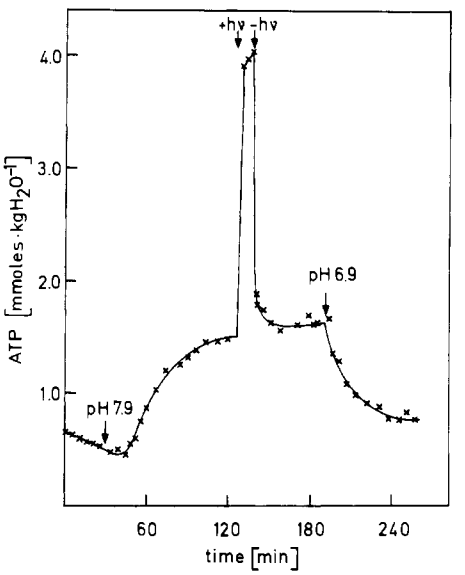

FIGURE 4: Time dependence of the changes of intracellular ATP levels, expressed as millimoles per kilogram of intracellular water, upon pH shifts. 10 mL of an anaerobic cell suspension with a turbidity of 6.9 at 578 nm was adjusted to pH 6.5 with 3.8 mM Tris–maleate buffer at 25 °C. After 90 min the pH was brought to 7.9 by addition of 1 mL of anaerobic 375 mM Tris–maleate buffer in basal salt. 100 min later the cells were illuminated for 10 min at an irradiance of 57 mW/cm². 50 min after illumination the pH was reduced to 6.9 by addition of 70 μL of 0.5 N HCl. Every 5 min 0.1-mL aliquots were taken for ATP levels determination.

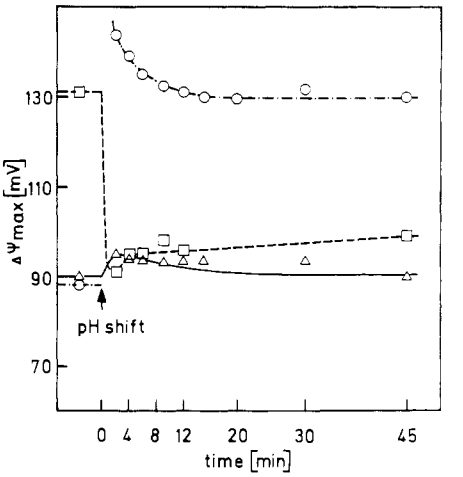

FIGURE 5: Time dependence of the changes of membrane potential ($\Delta\psi_{max}$) upon pH shifts. 160-μL cell suspensions (with a turbidity of 6.9 at 578 nm), containing 20 mM Tris–maleate and 4 μM [^{14}C]TPMP$^+$, were incubated in centrifuge tubes at room temperature under nitrogen in the dark. 40 μL of anaerobic 375 mM Tris–maleate buffer in basal salt was added after 1 h and mixed. At various times after addition of the buffer, the accumulation of TPMP$^+$ in the cells was determined. External pH was shifted from 7.9 to 6.55 (--□--) and from 6.4 to 8.0 (·–○–·) by using cells not treated with DCCD and from 6.3 to 8.1 (△) when intensively DCCD-treated cells were used.

was not present in our experiments, nor is nitrate reductase induced under our growth conditions. We have thus considered the possibility that the chemical potential difference of the K^+ ion is the energy source for the increase of the ATP level (see next section).

Shifts in the pH of the medium also cause changes of the membrane potential. An upward pH shift causes the potential to increase rapidly to a maximum and then to relax to a constant value (Figure 5). Decreasing the pH causes the potential to shift downward. These changes in the membrane potential upon increasing or decreasing the extracellular pH

Table III: Membrane Potential, pH Difference, and Intracellular ATP, ADP, and P_i Concentrations of *H. halobium* at pH 8 in Basal Salt and Potassium Basal Salt[a]

	$\Delta\psi_{max}$ (mV)	$\Delta\psi_{min}$ (mV)	ΔpH	[ATP] (nmol/kg of H_2O)	[ADP] (nmol/kg of H_2O)	[P_i] (nmol/kg of H_2O)	59 mV × log [[ATP]/([ADP][P_i])] (mV)
basal salt	133	125	0	1.78	1.50	66	74.1
K^+ basal salt	119	112	0	0.29	0.90	69	39.5

[a] $TPMP^+$ and DMO accumulation was measured in the presence of 75 mM Tris-maleate after 1 h of incubation under anaerobic dark conditions.

occur so quickly that they cannot be resolved kinetically by the method used. This clearly demonstrates that the ATP increase and the increase of membrane potential upon alkalinization are not correlated.

A point worthy of note is that either moderate or intensive treatment of the cells with the ATPase inhibitor DCCD blocks the changes in the membrane potential (Figure 5 and Table I).

Influence of the Extracellular Potassium Concentration on the ATP Level and the Proton Motive Force. It is well-known that halobacteria accumulate large amounts of potassium (Christian & Waltho, 1962; Ginzburg et al., 1970; Lanyi & Silverman, 1972; Wagner et al., 1978). The resulting potential difference of the potassium ion could possibly serve as an energy source for ATP synthesis. Under our conditions the intracellular K^+ concentration (3 mol/kg of cell water; Wagner et al., 1978) is approximately 100 times the extracellular concentration (30 nmol/kg of extracellular water). Since the membrane potential is strongly pH dependent (Figure 3), some important conclusions can be drawn about the possible relation between membrane potential, chemical potential difference of potassium ion, and ATP level.

The electrochemical potential difference of the potassium ions, assuming an activity coefficient of 1, is

$$\frac{\Delta\bar{\mu}_{K^+}}{F} = \Delta\psi - 59\ \text{mV} \times \log \frac{[K^+_i]}{[K^+_o]} \qquad (3)$$

where $[K^+_i]$ and $[K^+_o]$ are the intra- and extracellular potassium concentrations. At the equilibrium of the potassium ion ($\Delta\bar{\mu}_{K^+} = 0$), the membrane potential is equal to 59 mV × log ($[K^+_i]/[K^+_o]$). When the membrane potential is smaller than this term, the potassium ions would attempt to reach equilibrium by flowing out of the cell and would enhance the membrane potential. Such an enhancement of a preexisting membrane potential would drive protons into the cell via the proton-translocating ATPase and cause ATP synthesis. When the membrane potential is larger than 59 mV × log $[K^+_i]/[K^+_o]$, potassium ions would flow into the cell and lower the membrane potential. If the intracellular levels of ATP, ADP, and P_i were in equilibrium with the proton motive force, such a lowering of the membrane potential would force the ATPase to hydrolyze ATP in an attempt to restore equilibrium.

The difference of the chemical potential of the K^+ ions can be lowered experimentally by replacement of NaCl in the external medium by equimolar amounts of KCl. This will decrease the pH value at which the membrane potential is equal to $[K^+_i]/[K^+_o]$. This is a corollary of the strong pH dependence of the membrane potential (Figure 3). With increasing K^+ concentration in the medium, the intracellular ATP concentrations should be lowered preferentially at high pH. For instance, at pH 8 the membrane potential is approximately 125 mV (Figure 3 and Table III), then K^+ would be in equilibrium when the internal K^+ concentration is 3.5 M and the extracellular 27 mM, as in basal salt. Upon an increase of the extracellular K^+ concentration, the K^+ ions would no longer be in equilibrium and would be driven into the cell and lower the membrane potential. The vectorial ATPase would hydrolyze ATP to restore the equilibrium of the ATPase reaction and thus lower the internal ATP level. In contrast, at pH 6 the membrane potential is around 60 mV ($\Delta\psi_{min}$). With the same intracellular K^+ concentration of 3.5 M, the external equilibrium concentration of the K^+ ions is 0.33 M. Thus, an enhancement of the external K^+ concentration up to 0.33 M should not decrease the preexisting membrane potential. A decrease of the ATP concentration should occur only beyond this K^+ concentration. The anticipated result is found experimentally, as shown in Figure 6. Enhancement of the extracellular K^+ concentration lowers the intracellular ATP level, particularly at high pH. Table III shows, additionally, that in a salt solution in which 2.7 M NaCl of the basal salt solution was replaced by KCl, the effect on the membrane potential is not drastic and can well be explained by the changes of ATP, ADP, and P_i levels (see Discussion).

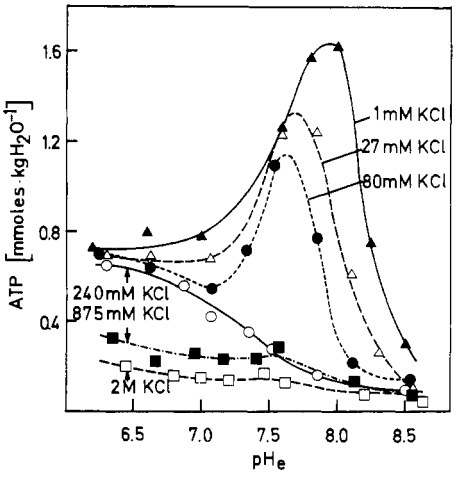

FIGURE 6: Dependence of the intracellular ATP level on the extracellular pH at different external potassium concentrations. Cells from a late logarithmic culture were centrifuged down and resuspended in modified basal salt solutions, in which some of the NaCl had been replaced by equimolar amounts of KCl. 0.1 volume of 375 mM Tris-maleate buffer in potassium-free basal salt was added. After a 1-h anaerobic incubation in the dark, aliquots were taken for ATP level determination. The potassium concentrations given in the figure refer to the values present in the modified basal salt before resuspension of the cells, neglecting K^+ outflow from the cells.

Discussion

An average cell of *H. halobium* is 5 μm long with a diameter of 0.6 μm. The surface area is thus about 10^{-7} cm^2, and the internal volume is 1.4×10^{-14} cm^3. On the assumption that the insulating cell membrane has a thickness of 5 nm and the dielectric constant is 3 [see, e.g., Huang & Levitt (1977)], the electric capacitance is approximately 5.3×10^{-14} F. Because the capacitance of the cell is so small, a 1 mV change in membrane potential requires transport of only 330 charges.

On the other hand, titration experiments with lysed and unlysed cells show that at an intracellular pH of 7, where the buffer capacity is at its minimum value, the transport of 8×10^6 protons/cell would shift the intracellular pH by only 0.1 unit (experiment not shown).

Since a halobacterial cell contains 10^5 molecules of bacteriorhodopsin [calculated from Hartmann & Oesterhelt (1977)], enough protons can be pumped within milliseconds to reach the maximal membrane potential which can be built up by bacteriorhodopsin. With negligible passive counter current across the membrane, this would correspond to the electromotive force of bacteriorhodopsin. For instance, to reach a membrane potential of 260 mV, less than 10^5 protons/cell have to be translocated. Due to the high intracellular buffer capacity, these protons alter the internal pH by only 0.001 unit. The membrane potential generated acts as a strong driving force for negatively charged ions (e.g., Cl^-) to flow out of the cell and for positively charged ions (K^+ and Na^+) to flow into the cell, but for every single passive ion flow one more proton can be pumped out of the cell. Repetition of this process would lead to a measurable pH gradient. This pH gradient would lower the maximal membrane potential which can be obtained by bacteriorhodopsin, by the amount 59 mV $\times$ ΔpH (assuming that the maximum value of $\Delta\bar{\mu}_{H^+}$ is constant).

The maximal electrochemical proton gradient found with DCCD-treated cells is about 270 mV. This is a minimal value for the electromotive force of bacteriorhodopsin at zero pH gradient, since passive ion fluxes lower the membrane potential and fluxes of H^+ and OH^- also lower the pH gradient. In spite of these limitations, our value is in good agreement with the 300-mV value found for the electromotive force of bacteriorhodopsin, when measured after incorporation into planar films (Drachev et al., 1976).

As a conclusion, the results with the intensively DCCD-treated cells could have been expected from a closed system which possesses an electrogenic proton pump, low electric capacitance, high buffer capacity, and passive permeabilities for other ions. Furthermore, the convincing and clear results with the intensively DCCD-treated cells substantiate the methods of measurement of membrane potential and pH gradient.

An interesting question is why moderately DCCD-treated cells build up only a small pH gradient when illuminated. It would certainly be lethal for a cell to experience such a large pH gradient as is found for the experiment of Figure 2, where the intracellular pH rose from 6.3 to 9.0 within 15 min of illumination with a physiological light intensity. Such a high internal pH would slow down metabolism; therefore, the existence of an electrogenic proton pump forces the cell to develop effective systems for the regulation of the internal pH.

Such a role could be played by the Na^+/H^+ antiport, whose existence has been postulated in halobacteria (Lanyi & MacDonald, 1976), *Streptococcus faecalis* (Harold & Papineau, 1972b), and *Escherichia coli* (West & Mitchell, 1974). A reasonable explanation for the differences between moderately and intensively DCCD-treated cells is that moderate treatment with DCCD blocks mainly the ATPase, whereas intensive DCCD treatment also blocks the pH regulation systems. In agreement with this explanation, moderately DCCD-treated cells still take up protons during the first minute of illumination ["alkaline overshoot"; see, e.g., Hartmann & Oesterhelt (1977)], which has been attributed to the function of the H^+/Na^+ antiport, and the pH gradient developed upon illumination remains small. Intensively DCCD-treated cells do not show an alkaline overshoot (Figure 2, inset) but do develop large pH gradients when illuminated.

Relationship between Proton Motive Force and ATP, ADP, and P_i Levels. The chemiosmotic hypothesis (Mitchell, 1968) postulates a reversible proton-translocating ATPase to be present in the membranes of mitochondria, bacteria, and thylakoids. It catalyzes the reaction

$$ATP + nH^+_i + H_2O \rightleftharpoons ADP + nH^+_o + P_i \qquad (4)$$

The proton motive force and the energy stored in the terminal anhydride bond of ATP are in equilibrium when the following equation is met:

$$\frac{\Delta\bar{\mu}_{H^+}}{F} = \frac{\Delta G^{\circ\prime}}{nF} + \frac{RT}{nF} \log \frac{[ATP]}{[ADP][P_i]} \qquad (5)$$

$\Delta G^{\circ\prime}$ is the standard free energy of ATP hydrolysis per mol at pH 7. $\Delta G^{\circ\prime}$ depends on the ionic environment and has not been determined under conditions which correspond to the cell interior of halobacteria, but it can be anticipated that there is no decrease in $\Delta G^{\circ\prime}$ at high ionic strength (Vogt & Rechnitz, 1974). At pH 7–8 and high ionic strength, a free energy of 7–11 kcal, corresponding to 304–434 mV for n = 1, seems to be a reasonable value.

Table II shows the values of 59 mV $\times$ log [[ATP]/([ADP][P_i])] from the experiment presented in Figure 3; they vary between 52 and 73 mV. For the sum of the right-hand terms of eq 5, values between $350/n$ and $510/n$ mV are found, whereas the proton motive force is always between 120 and 140 mV. This means that proton motive ATP, ADP, and P_i levels would be in equilibrium if n = 3, or less plausible 4. This is close to the value of 2.9 found by Bogomolni et al. (1976) when they compared the transient inflow of protons upon illumination and light-induced ATP synthesis. This approach, however, became questionable since it was demonstrated that the transient proton inflow can occur without ATP synthesis (Hartmann & Oesterhelt, 1977).

The following hypothetical experiment shows that such a relatively constant proton motive force is expected from the chemiosmotic hypothesis. Consider a halobacterial cell in a medium of pH 7, assuming a pH difference of 0.5 and a membrane potential of 90 mV. The electrochemical proton gradient is then 119.5 mV; the ATPase-catalyzed electrogenic reaction is assumed to be in equilibrium. If then the extracellular pH is shifted to 8, the pH difference is lowered by 1 unit. The resulting electrochemical proton gradient is now 60.5 mV. The ATPase has to hydrolyze ATP with a concomitant transport of protons out of the cell, which leads to an enhancement of the membrane potential, in order to regain equilibrium among the intracellular levels of ATP, ADP, and P_i and proton motive force. Knowing the electric capacitance of one cell (see above), one can calculate that transport of approximately 20 000 protons is sufficient to enhance the membrane potential by 59 mV. With n = 3, hydrolysis of 6667 ATP molecules would be necessary. Since the internal volume of one cell is approximately 1.4×10^{-12} cm^3, the 6667 ATP molecules correspond to a concentration of 8×10^{-6} M. The necessary decrease of the internal ATP concentration would not be measurable, since the ATP concentration is around 10^{-3} M. These considerations are still correct if the electrical capacitance has been underestimated by a factor of 10.

Thus, the results presented in Figure 5 are exactly what would have been predicted by the chemiosmotic hypothesis that there is an immediate increase of the membrane potential upon an upward pH shift, within a time scale on which no changes of ATP level are observed. The assumption of a rapid equilibration of ATP, ADP, and P_i levels with the electro-

chemical proton gradient is realistic, because, due to the low electric capacitance of the cell, only small amounts of ATP have to be hydrolyzed or synthesized. Furthermore, the interpretation that the ATPase is responsible for the increase of the membrane potential upon alkalinization is substantiated by the fact that the cells treated with the ATPase inhibitor DCCD do not show this increase (Table I and Figure 5).

During the preparation of this manuscript, R. Hartmann and D. Oesterhelt (unpublished results) did an experiment which demonstrated that the intracellular ATP level and the electrochemical proton gradient in *H. halobium* are in or close to equilibrium. Addition of arginine to halobacteria leads to an increase of the intracellular ATP level due to the reversal of some reactions of the urea cycle. The increase also occurs in cells after treatment with DCCD. With untreated cells the newly established high ATP level is sensitive to uncouplers; in contrast, uncouplers have no influence on the new ATP level in cells treated with DCCD.

The explanation is as follows. The uncouplers lower the electrochemical proton gradient and thus disturb the equilibrium between ATP, and ADP, P_i, and the electrochemical proton gradient.

In cells not treated with DCCD, the vectorial ATPase hydrolyzes ATP so as to restore equilibrium, which results in a futile cycle. This series of events is not possible with cells which have been treated with DCCD, since the ATPase is blocked.

Similar values for the membrane potential and its increase upon alkalinization have been found with *S. faecalis* (Harold & Papineau, 1972a,b) and *E. coli* (Griniuviene et al., 1975), but no interpretation was advanced. When the accumulation of Rb^+ ions in the presence of valinomycin was measured (Padan et al., 1976), a high membrane potential at alkaline pH was not found. This latter experiment was done in the presence of 1 mM KCl, which would have led to a massive valinomycin-mediated K^+ influx, decreasing the membrane potential. The ATPase would then hydrolyze ATP to restore equilibrium. Since the amounts of K^+ flowing into the cell would be larger than the internal ATP level, all the intracellular ATP would be hydrolyzed without restoration of the original membrane potential. This corresponds to the strong decrease of the membrane potential observed in halobacterial cells when $TPMP^+$ concentrations up to 1 mM were used (Michel & Oesterhelt, 1976).

It is much more difficult to explain the slow rise of the intracellular ATP concentration upon alkalinization of the medium (Figure 4). This rise is not related to the quick rise of the ATP level upon acidification to pH 3–4, which occurs in halobacteria (Hartmann & Oesterhelt, 1977) and other systems (Jagendorf & Uribe, 1966). The presence of a proton-translocating ATPase and an enhancement of the membrane permeability at pH 3–4 both have to be postulated to explain the results of this type of experiment. Without enhanced membrane permeability, positive charging of the cell, corresponding to a lowering of the membrane potential and negligible ATP synthesis, would occur, as first stated by Mitchell (1966). Additionally, at pH 4 an enhanced membrane permeability explains the delay of the onset of photophosphorylation (Hartmann & Oesterhelt, 1977). The energy source for the ATP synthesis in the Jagendorf-type experiment is the enhanced pH difference, whereas in the experiment of Figure 4 the pH difference was diminished. Furthermore, the pH-dependent influence of the extracellular K^+ concentration on the intracellular ATP level makes it plausible that the K^+ gradient is the energy source for the slow rise of the ATP level caused by alkalinization. On the assumption that the efflux of K^+ ions is pH independent (or increased at high pH by a regulation process) and that the ATP consumption at an internal pH of 8 is lower than that at pH 7 (which is the intracellular pH at an external pH of 7), then the ATP maximum at pH 8 would be explained.

Such an interpretation is not contradicted by the equilibrium of ATP, ADP, and P_i levels and proton motive force. Of course, both the electrochemical proton gradient and ATP level would rise.

The influence of a high external K^+ concentration on membrane potential and ATP, ADP, and P_i levels at pH 8 is shown in Table III. At this pH the influence on the membrane potential is most drastic. Nevertheless, the difference ($\Delta\psi$ in basal salt minus $\Delta\psi$ in potassium basal salt) of the membrane potential is only 12 mV, much less than expected from the high external K^+ concentration for a high K^+ permeability. The term 59 mV × log [[ATP]/([ADP][P_i])] in basal salt is 34.6 mV higher than that in the potassium basal salt. With $n = 3$ this difference corresponds to the 12-mV difference of the membrane potential in basal salt and potassium basal salt. This proves that the K^+ diffusion across the cell membrane is slower than the ATPase-catalyzed equilibration of the proton motive force with ATP, ADP, and P_i and that it must be the ATPase which is mainly responsible for the excess membrane potential. Furthermore, the influence of a slow K^+ diffusion on the membrane potential, and thus on the ATPase-mediated equilibrium of proton motive force and ATP level, allows us to explain the pH-dependent influence of the extracellular K^+ concentration on the intracellular ATP concentration as explained under Results.

Conclusion

Due to the large difference of electric capacity and buffer capacity for protons, the electrogenic, proton-translocating ATPase can equilibrate proton motive force and phosphate potential very rapidly. This leads to an immediate enhancement of the membrane potential by the ATPase upon reduction of the pH difference. The proton motive force remains nearly constant.

These considerations also hold for other bacteria.

The potassium gradient normally is not in equilibrium, but influences the level (height) of the equilibrium between phosphate potential and proton motive force. Under normal conditions (pH 7, basal salt) a slow K^+ efflux enhances the membrane potential. Since the membrane potential as part of the proton motive force is rapidly equilibrated with the phosphate potential, the intracellular ATP level is also enhanced.

If the pH is raised to 8 (in basal salt), the ATPase extrudes protons immediately, leading to a higher membrane potential. Independently, the K^+ efflux might be enhanced, via a regulation process, which has to be postulated, or the intracellular ATP consumption might be reduced. The K^+ efflux therefore leads to a slow increase of the ATP level. If the pH is raised further, the ATPase enhances the membrane potential further. The membrane potential becomes higher than the maximal possible K^+ diffusion potential. Consequently, K^+ is driven electrophoretically into the cells, thereby lowering $\Delta\psi$. The ATPase then consumes ATP to restore the original membrane potential, thereby reducing the intracellular ATP level. With higher extracellular K^+ concentrations this already occurs at lower pH values.

Therefore, both extracellular pH and K^+ concentration modulate the intracellular ATP concentration.

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