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Effect of Extreme Salt Concentrations on the Physiology and Biochemistry of *Halobacteroides acetoethylicus*

S. RENGPIPAT,† S. E. LOWE, AND J. G. ZEIKUS*

Michigan Biotechnology Institute, Lansing, Michigan 48909, and the Departments of Biochemistry and Microbiology, Michigan State University, East Lansing, Michigan 48824

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Halobacteroides acetoethylicus **grew in media with 6 to 20% NaCl and displayed optimal growth at 10% NaCl. When grown in medium with an [NaCl] of 1.7 M, the internal cytoplasmic [Na^+] and [Cl^-] were 0.92 and 1.2 M, respectively, while K^+ and Mg^{2+} concentrations in cells were 0.24 and 0.02 M, respectively. Intracellular [Na^+] was fourfold higher than intracellular [K^+]. Since Na^+ and Cl^- ions were not excluded from the cell, the influence of high salt concentrations on key enzyme activities was investigated in crude cell extracts. Activities greater than 60% of the maximal activity of the following key catabolic enzymes occurred at the following [NaCl] ranges: glyceraldehyde-3-phosphate dehydrogenase, 1 to 2 M; alcohol dehydrogenase (NAD linked), 2 to 4 M; pyruvate dehydrogenase, 0.5 to 1 M; and hydrogenase (methyl viologen linked), 0.5 to 3 M. These studies support the hypothesis that obligately halophilic, anaerobic eubacteria adapt to extreme salt concentrations differently than do halophilic, aerobic eubacteria, because they do not produce osmoregulants or exclude Cl^-. This study also demonstrated that these halophilic, anaerobic eubacteria have a physiological similarity to archaebacterial halophiles, since Na^+ and Cl^- are present in high concentrations and are required for enzymatic activity.**

Halophiles include procaryotic bacteria and eucaryotic algae which have adapted to live in environments high in salt (i.e., [NaCl] greater than that in sea water). Most halophilic bacteria fall into one of two groups on the basis of the sodium chloride concentration range in which they will grow. Moderate halophiles grow in concentrations of sodium chloride ranging from 2 to about 20% (0.3 to 3.4 M). Extreme halophiles require at least 15% (2.6 M) sodium chloride for growth and grow even in saturated brines (about 30% [wt/vol] or 5 M) (14, 18). Some exceptions to these definitions exist (21, 22, 24). In general, obligate halophiles have evolved two different strategies for growth in an environment of concentrated salt. The internal salt concentration can be maintained at a level comparable to that of the environment, or the organism can actively exclude salt and produce a compatible osmoregulant such as betaine (8, 11).

Extremely halophilic archaebacteria usually possess an intracellular salt concentration approximately equivalent to the environmental concentration (5, 7, 16). The [Na^+] is usually slightly lower inside the cells, the [Cl^-] remains approximately equal to the external concentration, and [K^+] is much higher inside than outside the cell (5, 16, 17). Extremely halophilic eubacteria, such as *Ectothiorhodospira halochloris*, do not contain high intracellular concentrations of NaCl; instead, this species produces betaine as an osmoregulatory solute (8).

Enzymes from those halophilic archaebacteria which have so far been described have salt requirements greater than those of corresponding enzymes from either nonhalophilic bacteria or aerobic, eubacterial halophiles (10, 17, 18). In general, halophilic archaebacterial enzymes are denatured at low salt concentrations, especially in the complete absence of salt (15). By and large, enzymes from halophilic archaebacteria either maintain activity at high salt concentrations or are most active at their normal intracellular salt concentration (5). Nonetheless, reports exist of halophilic archaebacterial enzymes which are inactivated by salt (28). K^+ is at least as effective as Na^+ in stimulating halophilic archaebacterial enzyme activity, and together with Cl^-, these intracellular constituents play an important role in the cell by maintaining the enzymes in an active state (18).

Several halophilic, anaerobic eubacteria have been isolated and studied, although to a much lesser extent than their aerobic counterparts. *Haloanaerobium praevalens* was isolated from Great Salt Lake sediments (35), and in subsequent studies two more moderately halophilic anaerobes, *Sporohalobacter lortetii* (25) and *Halobacteroides halobius* (27), were described. Recent studies (26) on the intracellular sodium, potassium, and chloride concentrations in *Haloanaerobium praevalens* and *H. halobius* showed that the [Na^+] was lower inside the cells, [Cl^-] was equal to that in the environment, and K^+ was actively taken up by the cells.

Recently, *Halobacteroides acetoethylicus* was isolated from deep subsurface brine waters associated with injection water filters on offshore oil rigs in the Gulf of Mexico (S. Rengpipat, T. Langworthy, and J. G. Zeikus, Syst. Appl. Microbiol., in press). This eubacterial halophile grows at a salt range of 6 to 20% NaCl, displays a requirement of 10% NaCl for optimal growth, but does not require high external [K^+].

The purpose of the present report is to assess whether the physiological mechanism of this species for adaptation to high salt is a consequence of the production of compatible osmoregulants or of the evolution of enzymes that are adapted to function at high intracellular NaCl concentrations. The findings presented here support the general hypothesis that anaerobic, eubacterial halophiles that are chemoorganotrophs have evolved catabolic enzymes that function at high salt concentrations in lieu of compatible solutes, the synthesis of which requires energy.

* Corresponding author.
† Present address: Department of Food Science, University of Wisconsin-Madison, Madison, WI 53706.

MATERIALS AND METHODS

Chemicals. All chemicals used were of reagent grade and were obtained from either Mallinckrodt, Paris, Ky., or Sigma Chemical Co., St. Louis, Mo. Trypticase was obtained from BBL Microbiology Systems, Cockeysville, Md., and yeast extract was from Difco Laboratories, Detroit, Mich. Methyl viologen and other biochemicals were obtained from Sigma. All gases were supplied from Linde, Michigan Welding, East Lansing, Mich., and were scrubbed free of oxygen by passage over heated (370°C) copper filings. Tritium gas was obtained from Amersham Corp., Arlington Heights, Ill.

Bacterial strains and growth conditions. *H. acetoethylicus* EIGI (ATCC 43120) as described elsewhere (S. Rengpipat et al., in press) was used. *H. acetoethylicus* was grown on a glucose complex halophilic medium. Halo-Trypticase-yeast extract-glucose (TYG) medium was prepared by anaerobic methods with a gas phase of 95 N_2–5% CO_2 and contained the following (amounts are per liter of distilled water): NaCl, 10 g; KH_2PO_4, 0.3 g; $Na_2HPO_4 \cdot 7H_2O$, 2.1 g; NH_4Cl, 1 g; glucose, 5 g; $MgCl_2 \cdot 6H_2O$, 0.2 g; Tryptic, 10 g; yeast extract, 2.5 g; trace mineral solution (10 ml) and resazurin (0.002% [wt/vol]). Culture transfers on halophilic TYG medium were incubated at 34°C. Nonhalophilic anaerobes used as controls in enzyme studies included *Methanosarcina barkeri*, *Butyribacterium methylotrophicum*, *Clostridium thermohydrosulfuricum*, and *Sarcina ventriculi*.

All organisms were mass cultured in 20-liter glass carboys containing 16 liters of medium unless stated otherwise. *M. barkeri* (32) was grown on phosphate-buffered mineral medium containing 100 mM acetate as previously described (13); the headspace was N_2, and the incubation temperature was 37°C. *B. methylotrophicum* (13) was grown at 37°C on phosphate-buffered mineral medium containing 13 mM glucose. *C. thermohydrosulfuricum* 39E (23) was grown at 60°C in 1-liter, round-bottom flasks containing 500 ml of TYG medium containing 25 mM glucose and with a headspace of 95% N_2–5% CO_2. *S. ventriculi* (9) was grown on 12 liters of glucose complex medium (pH 5.0) in a fermentor (New Brunswick Scientific Co., Inc., Edison, N.J.). The medium pH was controlled by the addition of 4 M NaOH; the gas phase was N_2, and the temperature was 37°C.

Preparation of cell extracts. Cultures of *C. thermohydrosulfuricum*, *B. methylotrophicum*, and *H. acetoethylicus* were harvested during the exponential phase of growth with a Pellicon tangential-flow membrane system (Millipore Corp., Bedford, Mass.) fitted with a 100,000-molecular-weight cutoff membrane. The system was made anaerobic by flushing with N_2 gas and sulfide-reduced distilled water. The concentrated cells were pumped into a 250-ml polycarbonate centrifuge bottle which had been flushed with N_2. *S. ventriculi* and *M. barkeri* cells were harvested by allowing the cultures to sediment and then withdrawing the cells anaerobically into polycarbonate centrifuge bottles. The cells were centrifuged at 8,000 × *g* for 15 min and washed twice with distilled water containing 2 mM dithiotreitol. *H. acetoethylicus* cells were washed in 1.7 M NaCl (containing dithiothreitol) to avoid cell lysis.

Cell disruption. Cell extracts for enzymatic studies were prepared by passage through a French pressure cell (American Instrument Co., Inc., Silver Spring, Md.) at 20,000 lb/in^2. The French pressure cell was made anaerobic with N_2 for 20 min prior to the addition of cells. The disrupted cells were collected in an anaerobic tube and centrifuged at 20,000 × *g*. The supernatant was stored under N_2 in rubber-bunged vials at −70°C until used. Protein was determined by the Lowry method (19), with bovine serum albumin as the standard.

Determination of internal salt concentrations and osmoregulants. *H. acetoethylicus* was grown to mid-exponential phase in TYG medium containing different concentrations of salts. The cells were harvested at 3,000 × *g* for 30 min, and the supernatant was decanted. The pellet was suspended in medium lacking salt, yeast extract, and tryptic and was centrifuged at 10,000 × *g* for 4 min on a centrifuge (Eppendorf Geratebau, Netheler + Hinz GmbH, Hamburg, Federal Republic of Germany). The supernatant was discarded, and the pellet was resuspended in either 10% perchloric acid (for the determination of $[Na^+]$, $[K^+]$, and $[Mg^{2+}]$) or distilled water (for the determination of $[Cl^-]$). The cells were broken in Eppendorf tubes by ultrasonic treatment for 30 min. These tubes were placed at −20°C for 2 h and then allowed to thaw to room temperature before being heated at 95°C for 30 min in an Eppendorf heating block. The contents were mixed and centrifuged at 10,000 × *g* for 4 min, and the Na^+, K^+, Mg^{2+}, and Cl^- contents of the supernatant were determined. Samples for analysis were diluted either with 10% perchloric acid for $[Na^+]$, $[K^+]$, and $[Mg^{2+}]$ determinations or with distilled water for determination of $[Cl^-]$. $[Na^+]$, $[K^+]$, and $[Mg^{2+}]$ were determined by using inductively coupled argon plasma emission spectroscopy methods (30). $[Cl^-]$ was determined by the method described by Oren (26).

The internal volume of the cell was determined by the procedure described by Goodwin and Zeikus (9). *H. acetoethylicus* cells were concentrated by centrifugation to give a final optical density at 660 nm of greater than 1.5. The concentrated cells were incubated with ^3H_2O and [^{14}C]sorbitol at 34°C for 15 to 20 min. Samples (1 ml) were withdrawn and rapidly separated by centrifugation through 0.2 ml of silicone oil in 1.5-ml centrifuge tubes at 3,000 × *g* for 5 min. Radioactivity in the cell pellet and supernatant fractions was determined after incubation in Instagel scintillation cocktail at 5°C for 24 h. Radioactivity was determined on a Tricarb 4530 liquid scintillation counter programmed with a ^{3}H-^{14}C dual-label quench curve. The internal cell pellet volumes were measured as outlined by Kashket et al., (12), and intracellular Na^+, K^+, Mg^{2+}, and Cl^- concentrations were calculated as described by Oren (26).

The presence of osmoregulants within cells of *H. acetoethylicus* was examined with methods used to demonstrate the presence of these solutes in halophilic eubacteria. Glycerol content determination was by the method of Wieland (33), and betaine and choline contents were determined by adsorption of their periodide derivatives (31). None of these osmoregulants were found in significant concentrations (<2 mM was detected) within *H. acetoethylicus*.

Enzyme assays. All enzyme assays were performed by strict anaerobic techniques described previously (34). Glass cuvettes (1.7-ml final volume) sealed with grey stoppers were made anaerobic by being repeatedly evacuated and refilled with the desired gas phase. All buffers and substrates were prepared in glass vials sealed with rubber stoppers and rendered anaerobic by the above-described procedure. All additions to the cuvettes were made with a microliter syringe. Specific activities were calculated from the linear part of the reaction, and values for activity were determined from a minimum of two measurements. Enzyme activities in *H. acetoethylicus* were determined from two separate extracts. All assays were performed at the optimal growth temperature for each organism in a Cary 219 spectrophotometer. The wavelengths and millimolar extinction coefficients for NAD,

NADH, NADP, and NADPH were 340 nm and 6.22 cm^{-1} mM^{-1}, respectively, and for methyl viologen they were 578 nm and 9.78 cm^{-1} mM^{-1}, respectively.

Triethanolamine hydrochloride buffer was used for all the enzyme assays and was prepared with various concentrations of NaCl, KCl, and $MgCl_2$. The final reaction volume for each assay was 1 ml.

Glyceraldehyde-3-phosphate dehydrogenase (EC 1.2.1.12) was assayed by monitoring the reduction of NAD. The reaction mixture consisted of 0.1 M triethanolamine hydrochloride buffer (pH 8.1), 5 mM K_2HPO_4, 20 mM sodium arsenate, 2 mM glutathionine (reduced form), 1 mM NAD, 2 mmol of glyceraldehyde-3-phosphate, and cell extract (gas phase, N_2). The reaction was initiated by the addition of glyceraldehyde-3-phosphate, and NAD reduction was monitored at 340 nm.

Pyruvate dehydrogenase activity (coenzyme-A acetylating) (EC 1.2.7.1) was determined by measuring the reduction of methyl viologen with pyruvate. The assay contained 0.1 M triethanolamine hydrochloride buffer (pH 8.1), 10 mM sodium pyruvate, 0.1 mM coenzyme A, 5 mM methyl viologen, cell extract, and sufficient sodium dithionite to obtain a pale blue color. The gas phase was N_2. The reaction was initiated by the addition of pyruvate, and methyl viologen reduction was monitored at 578 nm.

Alcohol dehydrogenase (EC 1.1.1.12) activity was assayed by monitoring the oxidation of NADH or NAD(P)H. The reaction mixture consisted of 0.1 M triethanolamine hydrochloride buffer (pH 7.8), 2 mM dithiothreitol, 0.3 mM NADH or NAD(P)H, 5 mM acetaldehyde (for *C. thermohydrosulfuricum*) or 20 mM acetaldehyde (for *H. acetoethylicus*), and cell extract (gas phase, N_2). The reaction was initiated by the addition of acetaldehyde, and the oxidation of NADH or NAD(P)H was monitored at 340 nm.

Hydrogenase activity (EC 1.12.1.1) was determined by monitoring the reduction of methyl viologen. The assay system contained 0.1 M triethanolamine hydrochloride buffer (pH 7.8), 2 mM methyl viologen, cell extract, sufficient sodium dithionite to obtain a pale blue color, and a substrate gas phase of hydrogen. The reaction was initiated by the addition of cell extract, and methyl viologen reduction was monitored at 578 nm.

The in vivo hydrogenase activity of growing cells was measured by the tritium exchange assay described by Schink et al. (29). Cells were first grown to the mid-exponential phase in a glass round-bottom flask which contained TYG medium and a nitrogen headspace. The cells were centrifuged anaerobically at 3,000 × *g* for 20 min and then suspended in TYG medium without salt to give a cell concentration of 10 mg/ml. From the cell suspension, 0.5 ml was transferred to sterile 58-ml serum bottles, which were filled with N_2 and contained 9.5 ml of TYG medium with various NaCl concentrations. A tritium-hydrogen gas mixture (1 ml; specific activity, 21.7 μCi/ml) was then added by gas-tight syringe to each bottle, and the cultures were shaken at 34°C. Liquid samples (0.1 ml) were removed by syringe at 30-min intervals, and tritiated-water formation was quantified by liquid scintillation techniques.

RESULTS

The intracellular concentrations of sodium, potassium, magnesium, and chloride in *H. acetoethylicus* were determined (Table 1). The organism was grown in media containing different concentrations of NaCl, and the concentrations of salts outside the cell were compared with the intracellular salt concentrations. Compatible solutes such as glycerol, choline, and betaine were not detected in cells by standard methods. When *H. acetoethylicus* was grown in increasing concentrations of NaCl, the intracellular concentrations of Na^+ and Cl^- increased, and at the highest concentration of NaCl in the medium, the intracellular levels of K^+, Cl^-, and Mg^{2+} increased. The Na^+ concentration of the cell was approximately 60% of the external concentration, with little change in the ratio of external to internal concentration with increasing levels of Na^+. Potassium and magnesium were incorporated by the cell against a concentration gradient, with [K^+] increasing approximately 8- to 23-fold over the external concentration, and magnesium levels were approximately three to seven times the external concentrations. The Cl^- content of the cell was almost the same as the external concentration of Cl^-, regardless of the initial Cl^- content of the medium.

TABLE 1. Intracellular and supernatant concentrations of sodium, potassium, magnesium, and chloride in *H. acetoethylicus* cultures grown with different NaCl concentrations in the culture medium[a]

Ion and source	Ion concn (M) in medium with NaCl concn (M) of:	
	1.70	2.32
Na^+		
Supernatant	1.16	2.52
Intracellular	0.92	1.50
K^+		
Supernatant	0.032	0.034
Intracellular	0.240	0.780
Mg^{2+}		
Supernatant	0.006	0.006
Intracellular	0.020	0.040
Cl^-		
Supernatant	1.4	2.7
Intracellular	1.2	2.5

[a] Cells were grown to mid-exponential phase in TYG medium with the [NaCl] indicated. Each culture was processed into a supernatant and an intracellular fraction prior to analysis of ions.

Since the above data indicated that the internal salt concentration of *H. acetoethylicus* was high, the effect of salt on the activity of its key catabolic enzymes was investigated. The enzymes studied were oxidoreductases, including glyceraldehyde-3-phosphate dehydrogenase, pyruvate dehydrogenase, hydrogenase, and alcohol dehydrogenase. Controls consisting of cell extracts prepared from nonhalophilic anaerobes that contained these enzymatic activities were used.

The activity of *H. acetoethylicus* glyceraldehyde-3-phosphate dehydrogenase, the key dehydrogenase of the Embden-Meyerhof-Parnas glycolysis pathway, was determined in buffers with various NaCl or KCl concentrations and compared with the activity of glyceraldehyde-3-phosphate dehydrogenase from a nonhalophile, *B. methylotrophicum* (Fig. 1). Glyceraldehyde-3-phosphate dehydrogenase from *H. acetoethylicus* showed considerably higher activity in the presence of NaCl than in its absence (5.81 U at 1.7 M NaCl compared with 0.27 U when no salt was present). The enzyme retained high activity at 2.3 M NaCl, but the activity decreased at 3.4 M NaCl. A similar trend of glyceraldehyde-3-phosphate dehydrogenase activity was observed with increasing KCl concentration, although the activity was lower

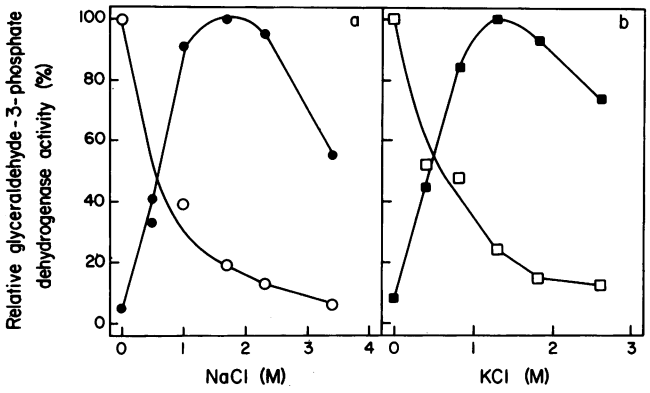

FIG. 1. Comparison of the effects of different salt concentrations on the glyceraldehyde-3-phosphate dehydrogenase activities of cell extract prepared from either *H. acetoethylicus* (closed symbols) or *B. methylotrophicum* (open symbols). Highest activities (100%) in panels a and b correspond to 5.81 and 4.53 U/min per mg of protein, respectively, for *H. acetoethylicus* and 1.44 and 1.32 U/min per mg of protein, respectively, for *B. methylotrophicum*. Conditions: volume of cell extract of *H. acetoethylicus* used, 2 μl (0.089 mg of protein); volume of cell extract of *B. methylotrophicum* used, 5 μl (0.125 mg of protein); total volume, 1 ml; gas phase, nitrogen; temperature, 34°C for *H. acetoethylicus* and 37°C for *B. methylotrophicum*.

than compared to the activity in the presence of NaCl. This enzyme showed maximum activity at 1.3 M KCl, with a 10-fold increase over activities measured in the absence of KCl. Glyceraldehyde-3-phosphate dehydrogenase activity in cell extract of *B. methylotrophicum* was severely inhibited in the presence of 1 M NaCl or KCl.

Pyruvate dehydrogenase is a key dehydrogenase in the *H. acetoethylicus* pathway for synthesis of acetate, H_2, CO_2, and ethanol from fermentation substrates. The activities of this enzyme from *H. acetoethylicus* and *S. ventriculi* in different concentrations of NaCl, KCl, or $MgCl_2$ were compared (Fig. 2). Pyruvate dehydrogenase activity of *H. acetoethylicus* was stimulated by the presence of moderate concentrations of NaCl, although the activity decreased above 1.7 M NaCl compared with enzyme activity in the absence of salt. Increasing concentrations of KCl resulted in higher levels of pyruvate dehydrogenase activity than were found in the absence of salt (Fig. 2b). Pyruvate dehydrogenase activity remained high in the presence of a low concentration of $MgCl_2$ (0.15 M) but was severely inhibited at concentrations above 0.3 M. Controls with *S. ventriculi*, a nonhalophilic anaerobe, showed significant inhibition of pyruvate dehydrogenase by increasing salt concentration.

Alcohol dehydrogenase activity from *H. acetoethylicus* was significantly increased by extreme concentrations of NaCl or KCl (Fig. 3). Enzyme activity increased with increasing concentrations of either salt, although much higher activity was observed with NaCl (0.133 U) than with KCl (0.042 U). Unlike the other enzymes, alcohol dehydrogenase showed an increase in activity in the presence of 0.15 M $MgCl_2$, and 30% of the maximum activity remained at high concentrations of $MgCl_2$ (0.5 to 0.7 M). Alcohol dehydrogenase activity in the *C. thermohydrosulfuricum* control decreased with increasing concentrations of Na^+, K^+, or Mg^{2+}.

Figure 4 compares hydrogenase activity of *H. acetoethylicus* with that of *M. barkeri* in relation to an increasing NaCl or KCl concentration. In the presence of NaCl, the hydrogenase of *H. acetoethylicus* (but not of *M. barkeri*) showed maximum activity at 0.5 M NaCl, and hydrogenase activity remained high in the presence of 2.3 M NaCl. Hydrogenase activity of *H. acetoethylicus* also increased with increasing concentrations of KCl, with the highest activity at 1.8 M KCl. The specific activities of hydrogenase in the presence of NaCl and KCl were comparable.

Since *H. acetoethylicus* produces H_2 and is not known to consume H_2, the influence of salt concentration on in vivo hydrogenase activity was further studied. Figure 5 presents data on in vivo hydrogenase activity from mid-exponential-phase cells of *H. acetoethylicus* suspended in different concentrations of NaCl. The effect of [NaCl] on in vivo hydrogenase activity was similar to its effect on hydrogenase activity in cell extracts. The highest activity of in vivo hydrogenase occurred near 1.0 M NaCl, with enzyme activity significantly inhibited at 0.023 or 2.3 M NaCl.

DISCUSSION

By and large, the data from this study support the general hypothesis that obligately halophilic eubacterial anaerobes

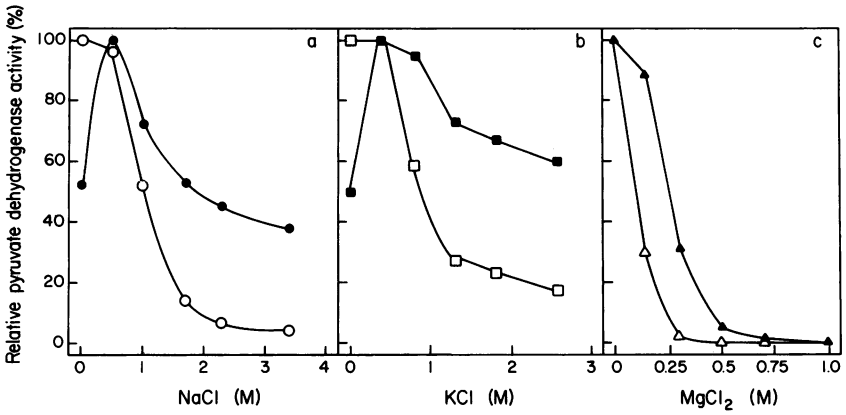

FIG. 2. Comparison of the effects of different salt concentrations on the pyruvate dehydrogenase activities of cell extract prepared from either *H. acetoethylicus* (closed symbols) or *S. ventriculi* (open symbols). Highest activities (100%) in panels a, b, and c correspond to 0.72, 0.61, and 0.35 U/min per mg of protein, respectively, for *H. acetoethylicus* and 1.69, 1.21, and 1.22 U/min per mg of protein, respectively, for *S. ventriculi*. Conditions: volume of cell extract of *H. acetoethylicus* used, 5 μl (0.24 mg of protein); volume of cell extract of *S. ventriculi* used, 3 μl (0.06 mg of protein); total volume, 1 ml; gas phase, nitrogen; temperature, 34°C for *H. acetoethylicus* and 37°C for *S. ventriculi*.

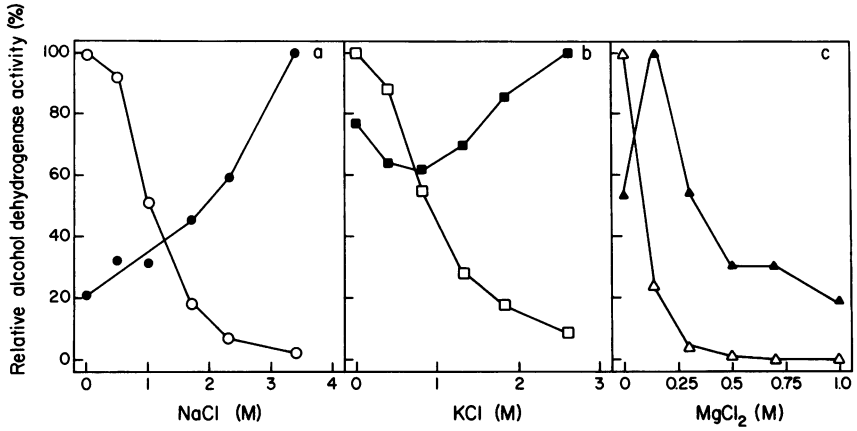

FIG. 3. Comparison of the effects of different salt concentrations on the alcohol dehydrogenase activities of cell extracts prepared from either *H. acetoethylicus* (closed symbols) or *C. thermohydrosulfuricum* (open symbols). Highest (100%) activities in panels a, b, and c correspond to 0.133, 0.042, and 0.045 U/min per mg of protein, respectively, for *H. acetoethylicus* and to 4.45 U/min per mg of protein for *C. thermohydrosulfuricum*. Conditions: volume of cell extract of *H. acetoethylicus* used, 15 μl (0.72 mg of protein); volume of cell extract of *C. thermohydrosulfuricum* used, 5 μl (0.18 mg of protein); total volume, 1 ml; gas phase, nitrogen; temperature, 34°C for *H. acetoethylicus* and 60°C for *C. thermohydrosulfuricum*.

have physiologically adapted to high-salt environments by evolving enzymes which function at high intracellular salt concentrations resulting from the external concentration and not by producing compatible solutes (e.g., betaine or glycerol), which would further tax their already constrained energy conservation mechanisms. Although the halophilic anaerobes have been placed in the kingdom of the eubacteria, they share some physiological and biochemical similarities with the halophilic archaebacteria.

The intracellular salt concentrations in *H. acetoethylicus* were similar to those reported for the other anaerobic halophiles, *Haloanaerobium praevalens* and *H. halobius* (26). In these haloanaerobes, the Cl^- levels inside the cells were comparable with those in the media, demonstrating a marked difference from haloaerobic eubacteria, from which Cl^- is excluded (7, 20). The intracellular salt contents of the halophilic archaebacteria and haloanaerobic eubacteria are dependent on the external salt concentration. The total internal concentration of monovalent ions either equals or exceeds the external concentration (6, 26). In haloaerobic eubacteria, an increase in salt above the optimum for growth will not result in a corresponding increase in the internal salt content (20, 21).

Comparison of the effect of salt on the in vitro activities of a number of enzymes from *H. acetoethylicus* revealed some striking similarities with enzymes from haloaerobic archaebacteria. Baxter and Gibbons (4) investigated the effect of salt on a number of enzymes from *Halobacterium salinaria* (*Pseudomonas salinarium*). Further comparison of the effect of salt on enzymes from extreme and moderate halophiles has been made (17).

The catabolic enzymes studied in *H. acetoethylicus* varied considerably in sensitivity to specific concentrations of salt.

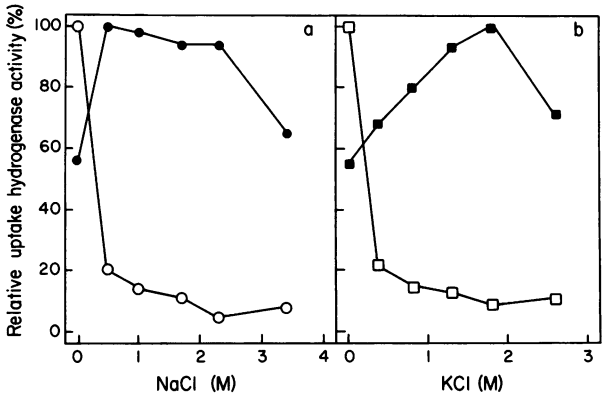

FIG. 4. Comparison of the effects of different salt concentrations on the hydrogenase activities of cell extracts prepared from either *H. acetoethylicus* (closed symbols) or *M. barkeri* (open symbols). Highest (100%) activities in panels a and b correspond to 0.069 and 0.081 μmol of H_2 consumed per min per mg of protein, respectively, for *H. acetoethylicus* and 0.383 μmol of H_2 consumed per min per mg of protein for *M. barkeri*. Conditions: volume of cell extract of *H. acetoethylicus* used, 5 μl (0.31 mg of protein); volume of cell extract of *M. barkeri* used, 5 μl (0.15 mg of protein); total volume, 1 ml; gas phase, hydrogen; temperature, 34°C for *H. acetoethylicus* and 37°C for *M. barkeri*.

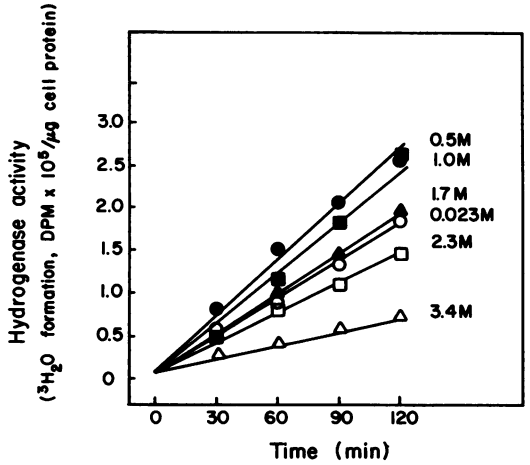

FIG. 5. Relation of NaCl concentration (on the right) to in vivo hydrogenase activity in whole cells of *H. acetoethylicus*. Cells were grown to the mid-exponential phase at 34°C in 58-ml serum bottles. Before the assay, the headspace was evacuated and flushed with N_2 gas, and 1 ml of $^3H_2$ was added. The assays were performed at 34°C.

All but one, pyruvate dehydrogenase, showed maximum activity at concentrations of Na^+ similar to or above those normally found in the cell. Pyruvate dehydrogenase was unlike the other enzymes in that maximum activity was shown at K^+ concentrations equivalent to those in vivo. The enzymes varied in activity in the absence of salt, with glyceraldehyde-3-phosphate dehydrogenase showing the greatest stimulation by the presence of NaCl and KCl. Hydrogenase remained active across a broad range of NaCl and KCl concentrations. Alcohol dehydrogenase activity continued to increase with increasing salt concentrations, and, notably, Na^+ stimulated total activity more than K^+ did.

The tolerance by enzymes of *H. acetoethylicus* to concentrations of salts above those normally found within the cells reveals a similarity to enzymes from haloaerobic archaebacteria. Of the enzymes studied from *P. salinarium* (1–4) and *Halobacterium cutirubrum* (5), the majority displayed maximum enzyme activities at sodium chloride concentrations above 1.5 M. Indeed, enzymes from *Halobacterium cutirubrum* showed a maximum activity at salt concentrations between 2 and 4 M. Only one enzyme, fatty acid synthetase from *Halobacterium cutirubrum*, was inhibited by salt, and it was concluded that the activity of this enzyme in vivo was normally repressed, which is consistent with the trace levels of fatty acids present in the organism (28). Studies on the effect of salt on enzymes from haloaerobic eubacteria (3, 4) have shown that most of the enzymes were active at salt concentrations below those found inside the cell. For example, the activities of succinate dehydrogenase and malate dehydrogenase from *Micrococcus halodenitrificans* were inhibited by the presence of salt (4).

Further study is required to determine whether the physiological requirement of 10% (1.7 M) NaCl for maximal growth of *H. acetoethylicus* can be related to specific catabolic enzyme requirements and whether growth inhibition in >10% NaCl is related to inhibition of specific enzymes (e.g., pyruvate dehydrogenase or other enzymes such as acetate kinase). The effect of higher sodium versus potassium concentration on physiological functions bears further examination and may lead to understanding at the molecular level of high $[Na^+]$ dependence and the activation of certain oxidoreductases (e.g., alcohol dehydrogenase). In most other kinds of organisms, including *H. halobius*, a haloanaerobe isolated from the high-$[K^+]$ Dead Sea environment (27), K^+ is usually higher than Na^+ in the cytoplasm. *H. acetoethylicus* was isolated from an environment high in Na^+ but very low in K^+.

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