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Relationship Between Ion Requirements for Respiration and Membrane Transport in a Marine Bacterium

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Intact cells of the marine bacterium *Alteromonas haloplanktis* 214 oxidized NADH, added to the suspending medium, by a process which was stimulated by Na^+ or Li^+ but not K^+. Toluene-treated cells oxidized NADH at three times the rate of untreated cells by a mechanism activated by Na^+ but not by Li^+ or K^+. In the latter reaction, K^+ spared the requirement for Na^+. Intact cells of *A. haloplanktis* oxidized ethanol by a mechanism stimulated by either Na^+ or Li^+. The uptake of α-aminoisobutyric acid by intact cells of *A. haloplanktis* in the presence of either NADH or ethanol as an oxidizable substrate required Na^+, and neither Li^+ nor K^+ could replace it. The results indicate that exogenous and endogenous NADH and ethanol are oxidized by *A. haloplanktis* by processes distinguishable from one another by their requirements for alkali metal ions and from the ion requirements for membrane transport. Intact cells of *Vibrio natriegens* and *Photobacterium phosphoreum* oxidized NADH, added externally, by an Na^+-activated process, and intact cells of *Vibrio fischeri* oxidized NADH, added externally, by a K^+-activated process. Toluene treatment caused the cells of all three organisms to oxidize NADH at much faster rates than untreated cells by mechanisms which were activated by Na^+ and spared by K^+.

Alteromonas haloplanktis 214 (previously referred to as marine pseudomonad B-16) is a strictly aerobic marine bacterium which has been shown to require Na^+ for the accumulation of the non-metabolizable amino acid analog α-aminoisobutyric acid (AIB) (4) and other metabolites (5–7). Since transport against a gradient requires the expenditure of energy, it was necessary in initial studies to distinguish between a requirement for Na^+ for respiration and for transport. This was done by showing that 50 mM Na^+ is required by the cells for maximum respiratory activity in the presence of galactose as an oxidizable substrate, whereas with galactose present, 200 mM Na^+ is needed for the maximum rate of uptake of AIB (4). Further work in this laboratory has shown that in the absence of respiratory activity and in the presence of an artificially imposed membrane potential, AIB accumulation by *A. haloplanktis* is absolutely Na^+ dependent (16).

Unemoto et al. (24) have observed that the NADH oxidase in membrane fractions from the marine bacterium *Vibrio alginolyticus* and the moderate halophile *Vibrio costicola* require 0.3 and 0.5 M Na^+, respectively, for maximum activity, whereas that from *Escherichia coli* has no specific requirement for Na^+. Evidence has also been obtained that NADH oxidase in membrane fractions from *A. haloplanktis* 214 is activated by Na^+ (T. Unemoto, personal communication).

Intact cells of *V. alginolyticus* show no NADH oxidase activity (24). Exogenous NADH, however, can stimulate the accumulation of AIB and K^+ by intact cells of *A. haloplanktis* 214 and of L-alanine into membrane vesicles of this organism by a mechanism sensitive to the presence of respiratory inhibitors (20, 22). Since Unemoto et al. have observed that an obligatory step in the electron transport chain involved in the oxidation of NAD-linked substrates, the NADH-quinone oxidoreductase step requires Na^+ for activation in a marine bacterium (23), the relation between the Na^+ requirement for NADH oxidation by and the transport of AIB into cells of *A. haloplanktis* has been investigated.

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MATERIALS AND METHODS

Cultures used. The organisms used were: *A. haloplanktis* 214, variant 3 (ATCC 19855); *Vibrio natriegens* 107; *Photobacterium phosphoreum* 404; and *Vibrio fischeri* MAC401. The sources of these cultures and the methods used to maintain them have been previously described (5, 11).

Preparation of bacterial suspensions. The growth medium used contained 0.8% nutrient broth (Difco Laboratories, Detroit, Mich.), 0.5% yeast extract (Difco), 300 mM NaCl, 50 mM $MgSO_4$, and 10 mM KCl and was adjusted to pH 7.2 with KOH. A 10-ml volume of the medium in a 50-ml Erlenmeyer flask was inoculated from a slant culture of the organism and incubated for 24 h at 25°C on a rotary shaker. One milliliter of this culture was used to inoculate 100 ml of fresh medium, which was incubated with shaking for 16 h. The cells were harvested by centrifugation (16,300 × *g* for 10 min at 4°C) and washed twice by suspension in and centrifugation from volumes of an appropriate wash solution equal to the volume of the growth medium. The composition of the wash solution used varied depending on the experiment. The cells were finally suspended in the wash solution used at a density of either 10 or 7 mg (dry weight) of cells per ml for addition to the incubation mixtures used in the respiration and transport experiments, respectively. The suspensions were freshly prepared for each experiment and were then stored on ice.

Toluene-treated cells were prepared as follows. Toluene (1%, vol/vol) was added to a suspension containing 10 mg (dry weight) of washed cells per ml. The suspension was then mixed for 1 min with a vortex mixer and incubated on ice for 30 min.

Respiratory studies. Respiration was measured at 25°C with an oxygen electrode (Rank Bros., Bottisham, Cambridge, England) coupled to a chart recorder (model 8383-00; Cole-Parmer Instrument Co., Chicago, Ill.). Washed cells with or without toluene treatment were suspended in 2.95 ml

of incubation medium contained in the reaction chamber of the oxygen electrode and was maintained at 25°C with circulating water. After the rate of endogenous oxygen uptake had become constant, the rate of endogenous respiration was determined. Substrate was then added in a 50-μl volume, and the rate of respiration in the presence of substrate was recorded. Details of suspension media and concentrations of additives accompany the relevant figures.

Transport studies. Washed cells were suspended in 3.5-ml volumes of incubation medium contained in a reaction chamber maintained at 25°C with circulating water. The contents of the reaction chamber were aerated by being stirred with a Teflon-coated magnetic stirring bar. The incubation mixture was equilibrated for 15 min. At −1.0 min, the oxidizable substrate was introduced. At zero time, [^{14}C]AIB was added. At 30-s intervals for 3 min, 0.5-ml volumes of the suspension were removed and filtered with membrane filters (type HA, 0.45-μm pore size; Millipore Corp., Bedford, Mass.). The filters were washed immediately with 5-ml volumes of wash solution and were dried under an infrared lamp.

Radioactivity measurements. Ten-milliliter volumes of Aquasol (New England Nuclear Corp., Boston, Mass.) were added to the filters contained in scintillation vials, and radioactivity was determined with a Beckman LS 7500 liquid scintillation counter (Beckman Instruments, Inc., Irvine, Calif.).

Cell density determination. The cell densities of suitably diluted suspensions were determined turbidimetrically at 660 nm (microsample spectrophotometer, model 300-N; Gilford Instrument Laboratories, Inc., Oberlin, Ohio). An E_{660} of 1.0 was equivalent to a density of 0.385 mg of dry cells per ml.

Chemicals. NADH (cyclohexylamine salt), NADH (Tris salt), and NAD (grade III, free acid) were obtained from Sigma Chemical Co., St. Louis, Mo. [1-^{14}C]AIB was a product of New England Nuclear Corp. All other reagents used were of analytical grade.

RESULTS

Oxidation of NADH. Intact cells of *A. haloplanktis* oxidized NADH added to the suspending medium (Fig. 1A). The rate of oxidation increased to a maximum as the Na^+ concentration in the suspending medium was increased to 200 mM and then decreased again at 300 mM. Li^+ was nearly as effective as Na^+ in stimulating respiratory activity, whereas K^+ was essentially inactive.

In intact cells, electron donors such as NADH are produced in the cytoplasm, and oxidation occurs by dehydrogenases that are accessible from the cytoplasmic side of the membrane. It was therefore of interest to determine the relation between exogenously and endogenously oxidized NADH in *A. haloplanktis*. For this purpose, the cells were treated with toluene to render them penetrable by small molecules such as NADH (8). After treatment, the same number of cells oxidized NADH at approximately 2.5 times the rate of untreated cells (Fig. 1B). The oxidation required Na^+ for maximum activity. Neither Li^+ nor K^+ could replace Na^+ for this reaction. That the stimulation of respiration by NADH was due to the oxidation of NADH and not to the breakdown products of NADH was indicated by the failure of NAD to stimulate respiration by either intact or toluene-treated cells (data not shown).

A. haloplanktis contains K^+ at a high concentration and Na^+ at a low concentration. When K^+ is lost from the cells, it is replaced by Na^+ when Na^+ is present in the suspending medium (21). To determine the effect of K^+ on the response of the intracellular NADH oxidase to Na^+, Na^+ was added at increasing concentrations to suspensions of toluene-treated cells in which the total concentration of added salt was maintained at a constant 400 mM by the addition of K^+ as KCl. When the response to Na^+ in the presence of K^+ is compared with that in its absence (Fig. 2), it is evident that at a low Na^+ concentration, K^+ has an sparing action on the Na^+ requirement.

Unemoto et al. observed that Mg^{2+}, like K^+, spared the requirement for Na^+ for NADH oxidation by membrane preparations of *V. alginolyticus* (24). We have been unable to demonstrate a sparing action of Mg^{2+} on the Na^+ requirement for NADH oxidation by toluene-treated cells of *A. haloplanktis*. The presence of Mg^{2+} at 50 mM was actually found to depress the rate of oxidation of NADH (data not shown). This action may be related to the observation that after toluene-treatment, some clumping of cells occurred when Mg^{2+} was present.

Oxidation of ethanol. In other transport experiments with cells and membrane vesicles of *A. haloplanktis*, ethanol has been used as the energy source (20, 22). The effects of ions on ethanol-stimulated respiration of *A. haloplanktis* were therefore examined. The results (Fig. 3) show that in the presence of ethanol, very small amounts of Na^+ (<10 mM; Fig. 3, inset) strongly stimulated respiration. Increasing the Na^+ concentration above 10 mM produces only a small additional increase in the rate of respiration. The response to Na^+ could be partially replaced by Li^+ at a low concentration, but at 300 mM, Li^+ is appreciably more effective than Na^+.

Uptake of AIB. When the effects of Na^+, K^+, and Li^+ on the uptake of AIB by *A. haloplanktis* were examined with NADH as the oxidizable substrate, only Na^+ was effective in promoting transport (Fig. 4). The same experiment was run with ethanol as the oxidizable substrate. Again, Na^+ was required specifically for the uptake process, and the quantitative requirements for Na^+ were the same as when NADH was present (data not shown).

Response of other species. Unemoto et al. have suggested that the requirement for Na^+ for the activation of NADH oxidase may be a characteristic feature of marine and moderately halophilic bacteria (24). We therefore examined the capacity of three additional species of marine bacteria, *V. natriegens*, *P. phosphoreum*, and *V. fischeri*, to oxidize NADH in the presence and absence of Na^+. Intact cells of all three organisms oxidized NADH added externally, although *P. phosphoreum* oxidized the compound at about one-half the rate of the other two organisms or of *A. haloplanktis*. The oxidation of exogenous NADH by all of the organisms except *V. fischeri* was stimulated by added Na^+. The oxidation by *V. fischeri*, on the other hand, was stimulated by added K^+. Toluene-treated cells of the three organisms oxidized NADH at a greatly increased rate compared with intact cells, and the rate increased with added Na^+ in the manner reported for *A. haloplanktis* (Fig. 1). Also, as for *A. haloplanktis* (Fig. 2), K^+ spared but did not replace the requirement for Na^+ for all three organisms. The NADH-oxidizing system in *V. fischeri* was found to be unstable in the presence of toluene even at 4°C. If not examined immediately after toluene treatment, NADH-oxidizing activity became greatly reduced or was lost completely. This was also true to a lesser extent for the other organisms and can account for the variability in the NADH-oxidizing activity of different preparations of toluene-treated cells of *A. haloplanktis* (Fig. 1 and 2).

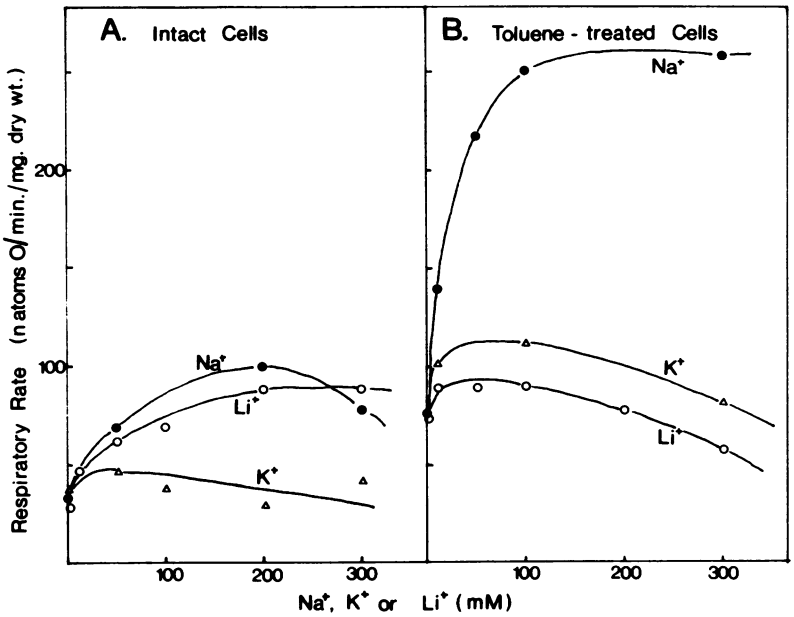

FIG. 1. Effects of Na^+, K^+, and Li^+ on the rate of oxidation of NADH by intact cells (A) and by toluene-treated cells (B) of *A. haloplanktis*. Cells were washed in a solution (adjusted to pH 7.2 with HCl) containing 300 mM choline chloride, 50 mM $MgSO_4$, 50 mM Tris buffer, 9 mM KCl, and 1 mM KH_2PO_4. The incubation medium (pH 7.2) contained 50 mM $MgSO_4$, 50 mM Tris buffer, 9 mM KCl, 1 mM KH_2PO_4, and the indicated concentrations of Na^+, K^+, and Li^+ added as their chloride salts together with sufficient choline chloride to bring the total concentration of the added salts to 300 mM in each incubation mixture. NADH was added as the cyclohexylamine salt at 2.5 mM. The suspension contained the equivalent of 1 mg of dry bacterial cells in a final volume of 3 ml. The values recorded have been corrected for endogenous respiratory activity (negligible in the case of toluene-treated cells).

DISCUSSION

The finding that an obligatory step (NADH-quinone oxidoreductase) in the electron transport chain for NAD-linked substrates in a marine bacterium requires 0.3 to 0.5 M Na^+ (23) raised the question of how it is possible to distinguish between a Na^+ requirement for respiration and a Na^+ requirement for transport in *A. haloplanktis*. The observations made in this study show that it is possible because the NAD-linked oxidation system that drives the transport is acting internally and responding to internal rather than external concentrations of Na^+ and K^+. The results show that in *A. haloplanktis*, NADH can be oxidized by both intact cells and toluene-treated cells by processes that can be distinguished by the difference in their response to Na^+ and Li^+. When exogenous NADH is oxidized by intact cells, the process is activated by Na^+ and Li^+ but not by K^+. After toluene treatment, a procedure designed to make the cytoplasmic membrane permeable to NADH (8), NADH is oxidized at a much faster rate by a mechanism activated by Na^+, but not by Li^+, and spared by K^+. The best explanation for these observations is that there are two sites of oxidation of NADH in *A. haloplanktis*, one on the outside and one on the inside surface of the membrane of the cells. A second possible explanation is that there is an NADH translocation system in the cytoplasmic membrane of the cells which is activated by either Na^+ or Li^+ and which can transport NADH to the site of oxidation on the inside surface of the membrane. Although with the data presented, one cannot eliminate the latter possibility, if a translocation mechanism were involved, one might expect the rates of oxidation by intact cells and toluene-treated cells to be more nearly equal. Whatever the explanation, the system permitting oxidation of NADH by intact cells can account for the capacity of the intact cells to use exogenous NADH as an

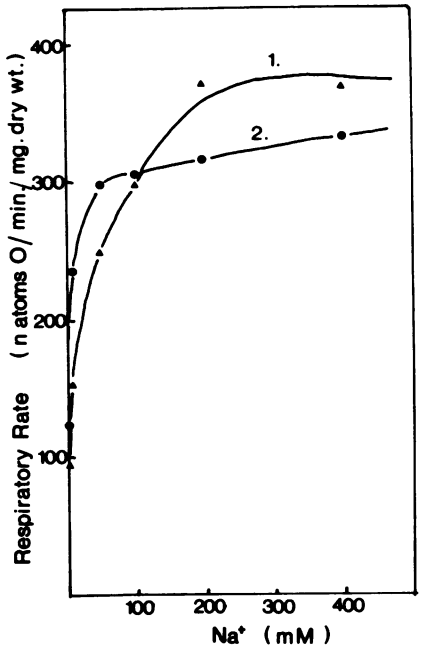

FIG. 2. Effect of K^+ on the capacity of Na^+ to stimulate the rate of oxidation of NADH by toluene-treated cells of *A. haloplanktis*. Curve 1, Response to Na^+ alone; curve 2, response to Na^+ when the total concentration of Na^+ plus K^+ was maintained at a constant 400 mM. Cells were washed in a solution (adjusted to pH 7.2 with HCl) containing 300 mM choline chloride, 50 mM Tris buffer, and 0.5 mM H_3PO_4. The incubation medium (pH 7.2) contained 50 mM Tris buffer and 0.5 mM H_3PO_4, and the indicated concentrations of Na^+ were added as NaCl. For the results in curve 2, KCl was added to bring the total concentration of NaCl plus KCl to 400 mM in each incubation mixture. Other conditions were the same as those described in the legend to Fig. 1.

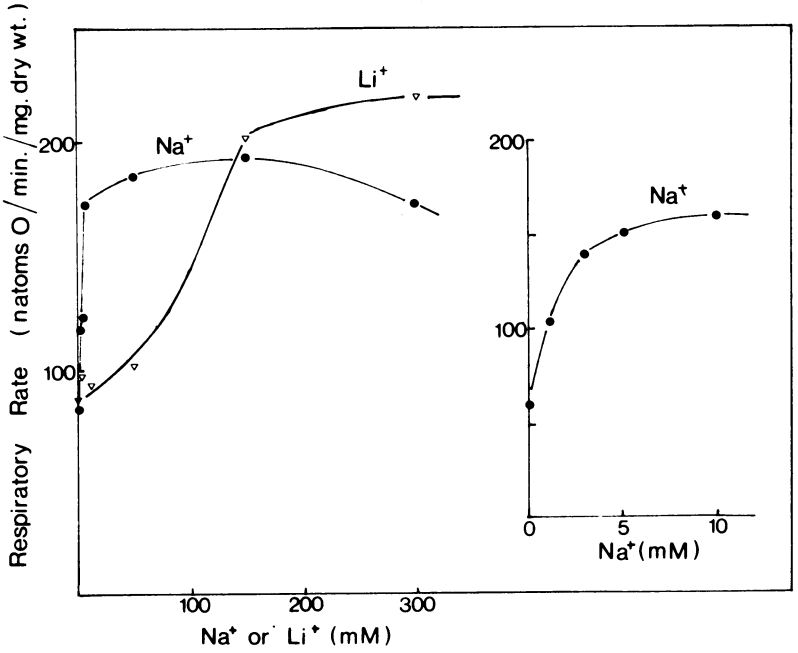

FIG. 3. Effects of Na^+ and Li^+ on the rate of oxidation of ethanol by intact cells of *A. haloplanktis*. Cells were washed in a solution containing 300 mM choline chloride, 50 mM $MgSO_4$, 50 mM Tris buffer, and 0.5 mM H_3PO_4 (pH 7.5). The incubation medium (pH 7.5) contained 50 mM Tris buffer, 50 mM $MgSO_4$, 10 mM KCl, 0.5 mM H_3PO_4, and the indicated concentrations of Na^+ or Li^+ added as their chloride salts together with sufficient choline chloride to bring the total concentration of the added salts to 300 mM in each incubation mixture. Ethanol was added to 10 mM. Other conditions were the same as those described in the legend to Fig. 1.

energy source to drive the active transport processes (20, 22), whereas the internal NADH-oxidizing system would permit the oxidation of, and hence the energy to be derived from, NAD-linked substrates present in the cytoplasm. The ability of both Na^+ and Li^+ to stimulate NADH oxidation by intact cells (whereas only Na^+ is active with toluene-treated cells) suggests that the Na^+-dependent step(s) in NADH oxidation by intact cells involves an enzyme(s) or transport protein(s) which can be activated by Na^+ or Li^+. In toluene-treated cells, however, the enzyme(s) required for oxidation is activated by Na^+ and cannot use Li^+ in its place.

NADH oxidation by toluene-treated cells of *A. haloplanktis* is activated by Na^+, and the requirement for Na^+ can be reduced but not eliminated (i.e., spared) by K^+. The rationale for sparing actions of this type in multienzyme systems has been considered previously (13, 14). A sparing action of K^+ on the Na^+ requirement, however, has been observed in a single enzyme system (NADH:quinone oxidoreductase) of *V. alginolyticus* (23). The cooperative activity of Na^+ and K^+ in this step suggests that the enzyme may catalyze partial reactions differing in their capacity to be activated by Na^+ and K^+. Partial reactions differing in their response to metal ions have been observed in yeast aldolase catalysis (10).

Exogenous NADH is oxidized by suspensions of intact plant (3), yeast (9), and rat liver mitochondria (2), as well as by *E. coli* (12) and *Bacillus subtilis* (1). In each case, evidence has been obtained indicating the presence of two sites for NADH oxidation, one accessible to the inside and the other accessible to the outside of the organelle or cell. The four marine bacteria examined in this study all oxidize exogenous NADH, and it appears likely that two sites for NADH oxidation occur in the cells of these organisms as well.

The size and solubility of the ethanol molecule suggest that it can enter cells readily by diffusion through aqueous channels or pores in the cytoplasmic membrane (18). An NAD-linked alcohol dehydrogenase has been shown to be present in the cytoplasm of *A. haloplanktis* (20). The intracellular oxidation of ethanol would thus be expected to require Na^+ for the oxidation of the intracellular NADH generated by the alcohol dehydrogenase, and this requirement for Na^+ could not be replaced by Li^+. Since ethanol oxidation by intact cells of *A. haloplanktis* is activated by

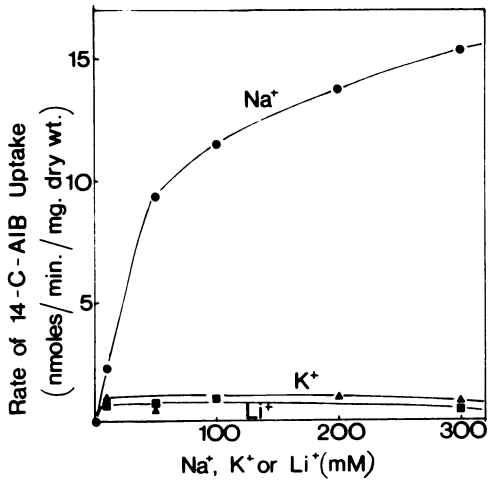

FIG. 4. Effects of Na^+, K^+, and Li^+ on the rate of AIB uptake by intact cells of *A. haloplanktis* in the presence of NADH as an energy source. Wash solution was the same as that described in the legend to Fig. 1. Incubation medium was the same as that described in the legend to Fig. 1, except that cells equivalent to 700 μg of dry weight were suspended in a final volume of 3.5 ml, and [^{14}C]AIB (0.5 μCi/μmol; 200 μM) was added.

either Na^+ or Li^+ (Fig. 3) and since membrane vesicles have been shown to oxidize ethanol and to lack NAD-linked alcohol dehydrogenase activity (20), it seems likely that exogenous ethanol is being oxidized by a membrane-bound alcohol oxidase activated by either Na^+ or Li^+ and that it is situated at the outer surface of the cytoplasmic membrane of these cells. A similar membrane location has been suggested for the methanol dehydrogenase of *Pseudomonas* sp. strain AM1 (17). A less likely possibility is that there is a Na^+- or Li^+-activated translocation mechanism for ethanol which can increase the rate of transfer of ethanol to its site of oxidation inside the cell.

With energy for active transport provided by NADH or ethanol, both of which are oxidized by intact cells using systems activated by either Na^+ or Li^+, the uptake of AIB by the cells was activated by Na^+ but not by Li^+, thus confirming that there is a process requiring Na^+ for membrane transport which is distinct from the requirement for Na^+ for energy generation.

It is also of interest that there appears to be a small but absolute intracellular requirement for Na^+ for respiration in cells of *A. haloplanktis*. When *A. haloplanktis* was analyzed directly by using a thick cell suspension technique, values for intracellular Na^+ and K^+ of 90 and 440 mM, respectively, were obtained (18). That these values might not reflect the true concentration, particularly of Na^+, in actively metabolizing cells was indicated by the observation that no flux of $^{22}Na^+$ into the cells with AIB could be demonstrated (19). The suspicion was that in packed-cell preparations, when anaerobic conditions could be expected, some exchange of K^+ for Na^+ might occur, whereas, under aerobic conditions, actively metabolizing cells might extrude Na^+ as fast as it entered, and the absolute intracellular Na^+ concentration under these conditions would be negligible. Since the oxidation of NAD-linked substrates by *A. haloplanktis* appears to require the presence of some Na^+ in the cells, even in the presence of K^+, clearly, a small amount of Na^+ must be present in the cells at equilibrium if these compounds are to be metabolized. Since Na^+ can stimulate respiration, which leads to the extrusion of protons, and since the Na^+/proton antiporter known to be present in this organism (15) can use the extruded protons to pump Na^+ from the cells, it would appear that the intracellular Na^+ level could be one of the factors exercising metabolic control in the cells.

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