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Variation in Quantitative Requirements for Na^+ for Transport of Metabolizable Compounds by the Marine Bacteria *Alteromonas haloplanktis* 214 and *Vibrio fischeri*

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The rates of uptake by *Alteromonas haloplanktis* of 19 metabolizable compounds and by *V. fischeri* of 16 of 17 metabolizable compounds were negligible in the absence of added alkali-metal cations but rapid in the presence of Na^+. Only D-glucose uptake by *V. fischeri* occurred at a reasonable rate in the absence of alkali-metal cations, although the rate was further increased by added Na^+, K^+, or Li^+. Quantitative requirements for Na^+ for the uptake of 11 metabolites by *A. haloplanktis* and of 6 metabolites by *V. fischeri* and the characteristics of the Na^+ response at constant osmotic pressure varied with each metabolite and were different from the Na^+ effects on the energy sources used. Li^+ stimulated transport of some metabolites in the presence of suboptimal Na^+ concentrations and for a few replaced Na^+ for transport but functioned less effectively. K^+ had a small capacity to stimulate lysine transport. The rate of transport of most of the compounds increased to a maximum at 50 to 300 mM Na^+, depending on the metabolite, and then decreased as the Na^+ concentration was further increased. For a few metabolites, the rate of transport continued to increase in a biphasic manner as the Na^+ concentration was increased to 500 mM. Concentrations of choline chloride equimolar to inhibitory concentrations of NaCl were either not inhibitory or appreciably less inhibitory than those of NaCl. All metabolites examined accumulated inside the cells against a gradient of unchanged metabolite in the presence of Na^+, even though some were very rapidly metabolized. The transport of L-alanine, succinate, and D-galactose into *A. haloplanktis* and of L-alanine and succinate into *V. fischeri* was inhibited essentially completely by the uncoupler 3,5,3′,4′-tetrachlorosalicylanilide. Glucose uptake by *V. fischeri* was inhibited partially by 3,5,3′,4′-tetrachlorosalicylanilide and also by arsenate and iodoacetate.

All gram-negative marine bacteria that have been examined in detail have been found to require Na^+ for growth (2, 24, 25). In this respect they are different from most terrestrial bacteria but are similar to such selectively pressured species as moderate and extreme halophiles (22) and alkalophilic (19) and rumen (4) bacteria.

In marine bacteria the growth requirement for Na^+ reflects some specific and at least one nonspecific function of Na^+. *Alteromonas haloplanktis* has been shown to require Na^+ for the transport of the nonmetabolizable metabolite analogs α-aminoisobutyric acid (AIB) and D-fucose (6, 7) and for the uptake of a number of metabolites (7, 8, 11). *Vibrio fischeri* (7) and *Vibrio alginolyticus* (41) also require Na^+ for the transport of AIB. *V. alginolyticus* needs Na^+ for the transport of sucrose (15) and 19 amino acids (cited in reference 42). Similarly, moderate (20) and extreme (21) halophiles and alkalophilic bacteria (3) have been shown to have Na^+-dependent transport processes. *Escherichia coli*, a terrestrial species which normally does not require Na^+ for growth, has been shown to require Na^+ for the transport of melibiose (45), glutamate (10), and proline (5).

The marine bacteria *A. haloplanktis* (33) and *V. alginolyticus* (42) resemble a number of other types of cells, both procaryotic and eucaryotic (18), in that they possess Na^+/H^+ antiporters. Such antiporters serve as Na^+ pumps and regulate cytoplasmic pH (18).

V. alginolyticus, but not *E. coli*, requires Na^+ for the activation of NADH-quinone oxidoreductase in the respiratory chain (46, 47). Evidence has also been obtained that there is a requirement for Na^+ in the cytoplasm for the oxidation of NADH by *A. haloplanktis*, *V. fischeri*, *Vibrio natriegens*, and *Photobacterium phosphoreum* (16). Thus, there appears to be a requirement for Na^+ for the generation of energy by the oxidation of NAD-linked substrates in these marine bacteria which is separate from the requirement for Na^+ for membrane transport. In some moderate halophiles, the NADH-quinone oxidoreductase may also serve to generate a membrane potential at alkaline pH by the primary extrusion of Na^+ ions (42).

For *A. haloplanktis* NaCl can function nonspecifically to provide an osmotic pressure in the external medium which can act to prevent the loss of intracellular solutes (28).

One of the characteristics distinguishing marine bacteria from terrestrial species that do not require Na^+ for growth may well be the larger number of transport systems in marine bacteria requiring Na^+ for activation. Most of the characteristics of Na^+-dependent transport in *A. haloplanktis* have been determined by using the nonmetabolizable amino acid analog AIB (6, 7, 48). In this study we examined in more detail than that done previously the characteristics and specificity of the requirement of *A. haloplanktis* and *V. fischeri* for Na^+ for the transport of a number of metabolizable compounds. Careful attention was

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paid to distinguishing between a requirement for Na^+ for transport and requirements for Na^+ for energy generation, osmotic pressure, and subsequent metabolism of the transported compounds.

MATERIALS AND METHODS

Organism. *A. haloplanktis* 214, variant 3, ATCC 19855 (formerly referred to as marine pseudomonad B-16) was maintained at 4°C by monthly transfer on slants of medium containing 0.8% (wt/vol) nutrient broth (Difco Laboratories, Detroit, Mich.), 0.5% yeast extract (Difco), 300 mM NaCl, 26 mM $MgSO_4$, 10 mM KCl, and 1.5% Bacto-Agar (Difco).

V. fisheri MAC401, previously referred to as *Photobacterium fischeri*, was originally obtained from W. D. McElroy, Johns Hopkins University, Baltimore, Md., and has been used in our laboratory in previous studies (7, 37). Cultures were maintained at 20°C by bimonthly transfer on slants consisting of 0.8% nutrient broth (Difco), 0.1% yeast extract (Difco), 3% NaCl, 0.3% glycerol, and 1.5% Bacto-Agar (Difco) (37).

Growth conditions. *A. haloplanktis* was grown in the medium used for culture maintenance except that the agar was omitted and the pH was adjusted to 7.2 with KOH. This medium was supplemented with 0.5% (wt/vol) citrate, succinate, L-malate, or D-galactose to obtain cells for use in transport studies of these metabolites (27). *V. fischeri* was grown in a medium containing 100 mM NaCl, 10 mM KCl, 2.5 mM $MgSO_4$, 5.5 mM NH_4Cl, 0.5 mM $CaCl_2$, 100 mM Tris buffer, 0.5% yeast extract (Difco), and 0.3% glycerol adjusted to pH 7.5 with HCl (37). Cultures were grown at 25°C in Erlenmeyer flasks with shaking on a rotary shaker (16).

Preparation of cells. Cells were harvested by centrifugation at 40,000 × *g* (4°C) for 10 min and washed 3 times with volumes of complete salts-Tris (CST) buffers equal to the volume of the original medium. These buffers contained 50 mM Tris; 50 mM $MgSO_4$; 9 mM KCl; 1 mM KH_2PO_4 (omitted from $^{32}P_i$ experiments); and combinations of choline chloride (ChCl), NaCl, KCl, or LiCl totaling 300 mM, as required for the different experimental conditions. The pH was adjusted to 7.2 with HCl. Concentrated stock cell suspensions were prepared from the washed cells and maintained on ice during the course of an experiment.

Measurement of transport. For all experiments except those for which the results are presented in Table 7, stock cell suspensions were diluted with the appropriate CST buffer and preincubated for 15 min at 25°C on a water bath shaker. Fractions of the suspensions were transferred to a reaction chamber consisting of the bottom half of a 25-ml graduated cylinder (TEKK brand; Fisher Scientific Co., Montreal, Quebec, Canada) from which the plastic base was removed. The cylinder was inserted through a rubber stopper into a jar through which water circulated at 25°C. Aeration was obtained by magnetic stirring. Chloramphenicol (100 μg/ml) was included in amino acid uptake experiments. Ethanol (25 mM) was provided as an exogenous electron donor for *A. haloplanktis* 1 min before the ^{14}C-labeled substrate was added. The final reaction volume was 3.5 ml and contained 100 μg (dry weight) of cells per ml. After the addition of the substrate, 0.5-ml samples were removed at 30-s intervals and filtered (pore size, 0.45 μm; HA; Millipore Corp., Bedford, Mass.). The cells retained on each filter were washed immediately at room temperature (20°C) with 5 ml of the same CST buffer used for washing and incubation. Uptakes were measured for 3 min after the addition of the substrates. Experiments were done in duplicate, and many were repeated several times. Experiments for which the results are shown in Table 7 were carried out as described previously (48).

The method used for assaying phosphate uptake was the same as that used for ^{14}C-labeled substrates with the following modifications. The membrane filters (Amicon Corp., Lexington, Mass.) were pretreated by boiling in 1 mM K_2HPO_4 (9). The reaction mixtures were contained in 50-ml disposable polystyrene beakers incubated at 25°C on a water bath shaker. All glassware was cleaned with a phosphate-free detergent and concentrated acid.

In all systems incubation mixture fractions containing no added cells were filtered, and the filters were subsequently washed to permit corrections to be made for the nonspecific binding of radioactivity to the filters.

Amino acids were added as L-U-^{14}C-labeled amino acids at 200 μM and a specific activity of 0.5 μCi/μmol, except for the experiments for which the results are presented in Table 7, for which the amino acid concentrations were 50 μM and the specific activity was 0.22 μCi/μmol. The other metabolites used were as follows: L-[U-^{14}C]malic acid, 100 μM, 2.5 μCi/μmol; D-[U-^{14}C]glucose and [1,5-^{14}C]citric acid, 50 μM, 2.5 μCi/μmol; [2,3-^{14}C]succinic acid (salt), 50 μM, 0.5 μCi/μmol; $K_2H^{32}PO_4$, 25 μM, 50 μCi/μmol.

Extraction of intracellular substrate pools. The transport of ^{14}C-labeled substrates was allowed to occur as described above, except that the final reaction volume was 1.1 ml and the specific activity of the metabolite added was increased to 10 μCi/μmol. Each reaction mixture at each Na^+ concentration was sampled twice (at 0.5 and 3 min). After the samples were filtered and rinsed with 5 ml of CST buffer, each filter for each time and Na^+ concentration was transferred as quickly as possible to a separate centrifuge tube containing 20 ml of water maintained at 90°C in a water bath. Each uptake experiment was repeated 6 times by using cells from the same stock suspension. The filters were added to the appropriate centrifuge tube to give a total of six filters with a known dry weight (300 μg) of adhering cells for each Na^+ concentration at each time. The filters were extracted at 90°C for 20 min, and the tubes were centrifuged for 15 min at 40,000 × *g* (4°C). The supernatants were collected, and the filters were extracted with another 20 ml of water. The supernatants from both extractions were pooled and lyophilized, and the residues were dissolved in a known volume (usually 1 ml) of water.

Intracellular P_i pools were extracted by soaking individual filters, containing 50 μg (dry weight) of cells, for 1 h in 2 ml of 5% (wt/vol) trichloroacetic acid at 0°C (9).

Analysis of cell extracts. Amino acids and tricarboxylic acid cycle intermediates were separated and isolated by thin-layer chromatography. A 5-μl volume of a concentrated radioactive extract was spotted on a thin-layer plate (0.1 mm cellulose; MN 300) together with 1 μl of a 50 mM solution of the metabolite to be isolated. The plate was developed in two dimensions, and the isolated spot was detected as described elsewhere (13, 31). The spot was scraped into a scintillation vial and the radioactivity was measured.

Amino acids were detected by spraying the plates with ninhydrin, a reagent that causes the loss of some ^{14}C label from these metabolites. Recovery experiments were run to determine the extent of this loss and the loss when a spot was transferred to a vial. Corrections for these losses were made in calculating the intracellular metabolite concentrations.

D-Galactose was isolated from extracts by descending

paper chromatography (50). Samples of 10 μl of 10 mM D-galactose and 5 μl of radioactive extract were spotted, and chromatograms were developed by using isopropanol-water (4:1) or *n*-butanol-pyridine-water (2:2:1) as the solvent. The isolated galactose spot was detected by spraying with aniline phthalate reagent. The galactose spot was cut out and placed in a scintillation vial, and the radioactivity was measured.

The P_i in extracts was precipitated in the form of its phosphomolybdate salt (39). The precipitates were filtered on 0.45-μm-pore-size membrane filters and rinsed with 5 ml of water, and their radioactivity was measured.

Radioactivity measurements. Filters with ^{14}C-labeled cells were placed under a heat lamp immediately after filtration of the cells to stop metabolic activity. Scintillation fluid (Aquasol; New England Nuclear Corp., Boston, Mass.) was added, and the radioactivity was determined by using a liquid scintillation spectrometer (Isocap/300; Nuclear-Chicago Corp., Des Plains, Ill.). For the measurement of $^{32}P_i$, 10 ml of water was added to each vial, and Cerenkov light emission was measured by using a liquid scintillation counter (LS 7500; Beckman Instruments, Inc., Fullerton, Calif.).

Intracellular metabolite concentrations. From the amount of radioactivity extracted as unchanged metabolite from a known weight of cells and the specific activity of the metabolite added to the cell suspension, the amount of unchanged radioactive metabolite present in the intracellular pool was determined. Values of 1.6 μl/mg (dry weight) of cells determined previously (40) for *A. haloplanktis* and 1.26 μl/mg (dry weight) of cells determined in this study for *V. fischeri* were used as intracellular fluid volumes to calculate the intracellular concentrations of unchanged metabolites.

Cell dry weight determination. The cell dry weights 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 0.385 and 0.549 mg (dry weight) of cells per ml of *A. haloplanktis* and *V. fischeri*, respectively. To obtain these values, fractions of cell suspensions were centrifuged to obtain packed cell preparations that were then lyophilized and dried to constant weight. The [^{14}C]inulin space in packed cell preparations was also determined and used to correct for the weight of salts in the extracellular fluid in the packed cells by previously described procedures (40).

Intracellular fluid volume of *V. fischeri*. The procedures used to determine the intracellular fluid volume of *V. fischeri* were essentially those described previously (40), except that the intracellular volume of *V. fischeri* was determined as the difference between the [^{14}C]lactose and ^3H_2O spaces in a packed cell preparation.

Flame spectrophotometric analysis of Na^+. Na^+ contamination of the solutions used in this study was determined by flame emission spectrophotometry with an atomic absorption spectrophotometer (SP90A; Pye Unicam Ltd., Cambridge, England).

Chemicals. The L-[^{14}C]malic acid used was obtained from Amersham/Searle Corp. (Arlington Heights, Ill.). All other radioactive materials were products of New England Nuclear.

RESULTS

Requirements of *A. haloplanktis* for Na^+ for membrane transport. The effect of the Na^+ concentration on the uptake by *A. haloplanktis* of each of a variety of metabolites is shown in Fig. 1. The initial rate of uptake of each substrate at each Na^+ concentration was determined by measuring the amount of radioactivity accumulated by the cells after 1 min of incubation in the presence of the radioactive substrate. At NaCl concentrations below 300 mM the total salt added to the incubation mixture was maintained constant at 300 mM by adding ChCl. Ethanol was included in the suspending medium as an energy source. Under these conditions none of the 11 metabolites actively accumulated in the absence of added Na^+, although significant quantities of glutamate, arginine, and citrate became associated with the cells (data not shown). This association was attributed to binding rather than transport because the process was complete by the time the first samples were taken at 30 s. A correction was made for the amounts bound when the uptake rates of these metabolites were calculated. Because the uptake rates of the different metabolites tested varied considerably, the scales in each of the panels of Fig. 1 are different.

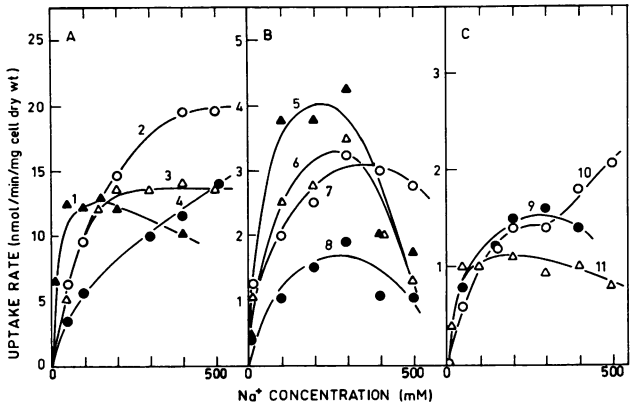

FIG. 1. Effect of Na^+ concentration on the rate of uptake of various metabolites by *A. haloplanktis* 214. The metabolites tested were as follows: curve 1, D-galactose; curve 2, L-alanine; curve 3, succinate; curve 4, L-leucine; curve 5, L-glutamate; curve 6, L-aspartate; curve 7, L-lysine; curve 8, L-arginine; curve 9, L-malate; curve 10, citrate; curve 11, phosphate. Below 300 mM NaCl the total concentration of salts added to the incubation medium was maintained constant by adding ChCl. Note that the scale of the ordinate changes from panels A to B to C.

The results indicate that the quantitative requirement for Na^+ for the maximum rate of transport varied markedly, depending on the metabolite tested, and ranged from 50 mM for galactose to in excess of 500 mM for L-leucine. As the Na^+ concentration was increased above that concentration which gave a maximum rate, the rate of transport of most metabolites was progressively reduced. This was particularly apparent for aspartate and glutamate transport.

For *V. fischeri*, five of the six metabolites tested were not taken up by the cells at a measurable rate unless Na^+ was added to the medium (Fig. 2A and B). As with *A. haloplanktis* the concentration of Na^+ required for the maximum rate of transport varied, depending on the metabolite transported, and ranged from 50 mM for L-alanine to over 500 mM for L-glutamate and P_i. For three of the metabolites, L-alanine, succinate, and L-arginine, an increase in the Na^+ concentration beyond the optimum caused the rate of uptake to be reduced. The sixth metabolite, D-glucose, was transported by *V. fischeri* at an appreciable rate without added Na^+, although the addition of 100 to 200 mM Na^+ further increased the rate of uptake of D-glucose by the cells (Fig. 2C, curve 6). Because in separate experiments ethanol was not found to be metabolized by *V. fischeri* (data not shown),

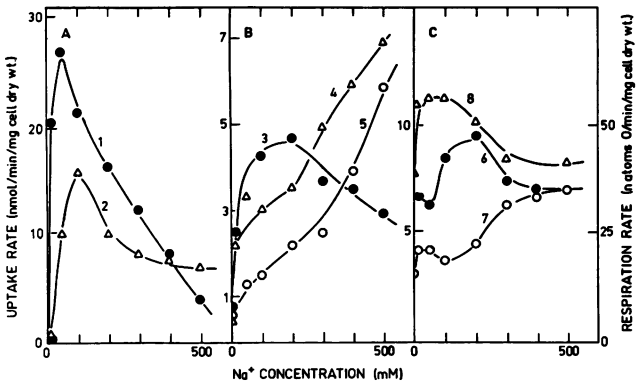

FIG. 2. Effect of Na^+ concentration on the rate of uptake of various metabolites by *V. fischeri* and on endogenous respiration and respiration in the presence of D-glucose. Metabolites tested were as follows: Curve 1, L-alanine; curve 2, succinate; curve 3, L-arginine; curve 4, L-glutamate; curve 5, phosphate; curve 6, D-glucose. Curves 7 and 8 show the effects of Na^+ concentration on endogenous respiration and on respiration in the presence of 10 mM D-glucose (corrected for endogenous), respectively. Note that the scale of the ordinate changes from panels A to B to C.

the experiments for which the results are shown in Fig. 2 were conducted without an added energy source; endogenous respiration was relied on to provide the energy for transport. Endogenous respiration of *V. fischeri* occurred in the absence of Na^+ but was stimulated by added Na^+ to various extents, depending on the concentration of Na^+ added (Fig. 2C, curve 7). The effects of the Na^+ concentration on endogenous respiration, however, did not parallel its effects on the transport of any of the metabolites tested. Similarly, D-glucose respiration occurred without added Na^+ but was stimulated by appropriate concentrations of added Na^+ (Fig. 2C, curve 8).

Specificity of the Na^+ requirement for transport. The specificity of the Na^+ requirement for transport in *A. haloplanktis* was examined by comparing the ability of cells of the organism to take up the metabolizable substrates when the incubation medium prepared without added NaCl was supplemented in each of five ways (Table 1). Supplement 1 consisted of 300 mM NaCl. This provided Na^+ at the concentration required for the maximum rate and extent of growth of *A. haloplanktis*. The rate of uptake of each metabolite under this set of conditions was assigned a value of 100. Supplement 2 was 300 mM ChCl. This served as the Na^+-free control and provided sufficient ChCl to maintain the total salt concentration (and medium osmotic pressure) at a constant level. Supplement 3, a combination of 10 mM NaCl and 290 mM ChCl, provided sufficient Na^+ to stimulate ethanol oxidation to the extent that it occurs with 300 mM NaCl (16). Enough ChCl was added so that the external osmotic pressure was equal to that provided by 300 mM NaCl. A rate of uptake of a metabolite in the presence of 300 mM NaCl that was greater than that with 10 mM NaCl–290 mM ChCl should thus be due to the positive effect of Na^+ on the uptake process. Supplement 4 was 10 mM NaCl plus 290 mM KCl, and supplement 5 was 10 mM NaCl plus 290 mM LiCl. These supplements were designed to determine whether K^+ or Li^+ could replace the Na^+ requirement for transport. A sixth supplement, 300 mM LiCl, was tested with those metabolites that exhibited a stimulation of transport by LiCl in the presence of 10 mM NaCl. In all experiments uptake was measured for 3 min after the addition of the radioactive metabolites because uptake was found either to be linear or to drop off only slightly when samples were removed at 30-s intervals over this period of time. The results in Table 1 indicate that none of the metabolites were taken up by *A. haloplanktis* at significant rates in the absence of NaCl, except for D-galactose and phosphate in the presence of 300 mM LiCl. In the presence of 10 mM NaCl and 290 mM ChCl, rates of transport of most metabolites increased over those in the presence of 300 mM ChCl. This was particularly evident for D-galactose and phosphate, as would be expected from the relatively low quantitative requirements of *A. haloplanktis* for Na^+ for the maximum rate of transport of these metabolites (Fig. 1). All of the metabolites were transported at a faster rate in the presence of 300 mM NaCl than in the presence of 10 mM NaCl–290 mM ChCl. This increased rate at the higher Na^+ concentration can be attributed to the effect of Na^+ on transport into *A. haloplanktis*, which is distinct from any that it might have on respiration or osmotic pressure. The rates of uptake of all of the metabolites in the presence of 10 mM NaCl–290 mM KCl never exceeded those in 10 mM NaCl–290 mM ChCl, except in the presence of L-lysine, in which a small stimulation was obtained. These results indicate that K^+ is unable to replace Na^+ in any of the transport processes that were examined, although it had some sparing action on the Na^+ requirement for L-lysine uptake. In the presence of 10 mM NaCl, LiCl had various effects on uptake by *A. haloplanktis*, depending on the metabolite. For this organism Li^+ had no effect on the uptake rates of L-alanine and L-leucine, whereas the ion inhibited the uptakes of L-glutamate and L-aspartate. The rate of transport of all of the other metabolites was greater in the presence of a combination of 10 mM NaCl–290 mM LiCl than with 10 mM NaCl–290 mM ChCl. Phosphate uptake was even faster with the LiCl-NaCl combination than with 300 mM NaCl alone. When LiCl was tested in the absence of NaCl, however, it was only half as effective as NaCl alone in promoting both phosphate and D-galactose transport.

The possibility was considered that Li^+ stimulation of transport might be due to contaminating Na^+ in the Li^+ salt that was used. Flame photometric analysis showed, however, that with 300 mM LiCl the salt would have added only

TABLE 1. Effect of salts or salt combinations on the relative uptake rates of various metabolites into *A. haloplanktis* 214

Metabolite	Relative uptake rate with the following added salts or salt combinations (mM)[a]:					
	300 NaCl	300 ChCl	10 NaCl–290 ChCl	10 NaCl–290 KCl	10 NaCl–290 LiCl	300 LiCl
D-Galactose	100	0	55	55	88	55
L-Alanine	100	2	34	33	30	—[b]
Succinate	100	0	0	0	13	0
L-Leucine	100	1	1	1	1	—
L-Glutamate	100	4	24	24	15	—
L-Aspartate	100	3	14	3	3	—
L-Lysine	100	1	46	59	65	—
L-Arginine	100	5	28	23	33	—
L-Malate	100	4	18	7	31	—
Citrate	100	7	7	7	15	—
Phosphate	100	0	62	58	112	56

[a] Salts were added to an incubation medium containing no added Na^+. The concentration of Na^+ present as a contaminant in the incubation medium supplemented with either 300 mM ChCl or 300 mM LiCl was 10 to 12 μM. For each metabolite the uptake rate with 300 mM NaCl was taken as 100.

[b] —, Not tested.

10 to 12 μM Na^+ to the incubation medium, which is a concentration that is insufficient to account for the effects that were obtained.

Similar experiments to determine the specificity of the Na^+ response were carried out with *V. fischeri* (Table 2). The salt combinations selected for this determination were modified to allow for differences in the Na^+ requirements of *V. fischeri* for transport. The results indicate that except for D-glucose, K^+ had no capacity to replace Na^+ in the transport of any of the metabolites tested into *V. fischeri*, while Li^+ stimulated the transport of L-glutamate but only in the presence of Na^+. The results in Table 2 also suggest that the uptake by *V. fischeri* of phosphate and, to a lesser extent, of L-arginine and L-glutamate occurred at an appreciable rate in the absence of added Na^+. For phosphate and L-glutamate this was an illusion created by the selection of the rate obtained with 100 mM Na^+–200 mM ChCl as the rate that gave a relative uptake rate of 100 (Fig. 2B). When rates of uptake with 500 mM NaCl were taken as 100, relative rates in the absence of Na^+ were lower. The L-arginine response, on the other hand, may merely reflect the low requirement for Na^+ for the uptake of this amino acid (Fig. 2B). D-Glucose uptake, however, occurred at an appreciable rate in the absence of Na^+, but its uptake rate was increased by the presence of Na^+. The results in Table 2 indicate that this stimulation was not specific for Na^+ but was achieved equally well by adding K^+ or Li^+.

Effect of ChCl on membrane transport. In the previous experiments ChCl was used to maintain the osmotic pressure of the suspending medium constant as the NaCl concentration was varied below 300 mM. It was important to know if the failure of *A. haloplanktis* and *V. fischeri* to take up metabolites in the absence of Na^+ was due to a lack of Na^+ or to the presence of an inhibitory concentration of choline. The results in Table 3 indicate that when ChCl was added to maintain the salt concentration constant at the level obtained by adding 300 mM NaCl, ChCl had a slight stimulatory effect on the transport of L-alanine and D-galactose into *A. haloplanktis* when the incubation mixture contained 10 mM NaCl but had no effect when it contained 50 mM NaCl. Of particular interest from the standpoint of the mechanism of Na^+-dependent transport is the observation that in the presence of 300 mM NaCl, the further addition of 300 mM ChCl had either a slight stimulatory effect or an appreciably smaller inhibitory effect than the addition of another dose of 300 mM NaCl. For *V. fischeri* (Table 4) ChCl had either no effect or small effects on transport rates that varied with the metabolite and with the ChCl concentration. The addition of 400 mM ChCl to a medium containing 100 mM NaCl had no significant inhibitory effects on transport and actually was slightly stimulatory. On the other hand, the further addition of 400 mM NaCl to a medium already containing 100 mM NaCl reduced the uptake rate of all three substrates.

Evidence for active transport. To establish whether active transport occurred, the ability of cells to accumulate metabolites against a gradient in an unchanged state was determined. Intracellular concentrations of each metabolite were compared with the initial extracellular concentrations at two Na^+ levels in the suspending medium: 10 and 300 mM for *A. haloplanktis* and 10 and either 100 or 200 mM Na^+ for *V. fischeri*. Again, the total concentration of added salts was maintained constant at 300 mM by adding ChCl. The results for *A. haloplanktis* are reported in Table 5 as internal to external ratios. At 10 mM Na^+, *A. haloplanktis* was able to concentrate 7 of 11 metabolites against a gradient within 0.5 min. After 3 min all of the ratios had further increased. At 10 mM Na^+, citrate and succinate were not transported. At 300 mM Na^+, the ratios showed that the internal concentrations of all of the metabolites were higher than at 10 mM Na^+ after 0.5 min of incubation, and the outwardly directed gradients increased as the time of incubation increased to 3 min. Because of the low concentrations of free succinate detected in *A. haloplanktis* cells, ratios for this compound were determined after 3 and 6 min. The response pattern with succinate was similar to that with the other metabolites and showed that at 300 mM Na^+ this compound also reached a

TABLE 2. Effect of salts or salt combinations on the relative uptake rates of various metabolites into *V. fischeri*

Metabolite	Relative uptake rate with the following added salts or salt combinations (mM)[a]:					
	100 NaCl–200 ChCl	300 ChCl	10 NaCl–290 ChCl	10 NaCl–90 KCl–200 ChCl	10 NaCl–90 LiCl–200 ChCl	100 LiCl–200 ChCl
L-Alanine	100	0	69	69	59	—[b]
Succinate	100	3	3	3	19	7
L-Arginine	100	19	63	63	68	19
L-Glutamate	100	10 (7)	46	22	80	7
Phosphate	100	65 (11)	65	65	70	52

[a] Conditions were as described in footnote *a* of Table 1. For each metabolite except D-glucose, the uptake rate with 100 mM NaCl–200 mM ChCl was taken as 100. The values in parentheses represent uptake rates of L-glutamate and phosphate relative to uptake rates of these compounds at 500 mM NaCl (from Fig. 2). For D-glucose the salt or salt combinations added and the corresponding relative rates of uptake were as follows: 200 NaCl–100 ChCl, 100; 300 ChCl, 52; 200 KCl–100 ChCl, 95; 200 LiCl–100 ChCl, 94.

[b] —, Not tested.

TABLE 3. Effect of ChCl on the relative uptake rates of metabolites by *A. haloplanktis* at different NaCl concentrations

Concn (mM) of added salt[a]		Relative uptake rate of the following transported metabolites[b]:		
NaCl	ChCl	L-Alanine	D-Galactose	Succinate
10		12 ± 1	22 ± 7	0
10	290	16 ± 2	34 ± 9	0
50		40 ± 0	59 ± 3	32 ± 2
50	250	39 ± 1	59 ± 8	34 ± 1
300		100	100	100
300	300	91 ± 6	92 ± 14	115 ± 17
600		55 ± 15	68 ± 5	77 ± 6

[a] Salts were added to an incubation medium containing no added NaCl.

[b] The uptake rate with 300 mM NaCl was taken as 100. Results indicate the average and average deviation of at least two experiments.

TABLE 4. Effect of ChCl on the relative uptake rates of metabolites by *V. fischeri* at different concentrations of NaCl

Concn (mM) of added salt[a]		Relative uptake rate of the following transported metabolites[b]:		
NaCl	ChCl	L-Alanine	D-Glucose	Succinate
10	–	32 ± 3	55 ± 4	9 ± 2
10	290	39 ± 5	51 ± 5	9 ± 2
100	–	90 ± 3	96 ± 4	87 ± 5
100	200	100	100	100
100	400	110 ± 14	103 ± 3	107 ± 7
500	–	70 ± 1	80 ± 2	88 ± 4

[a] See footnote *a* to Table 3.

[b] The uptake rate with 100 mM NaCl–200 mM ChCl was taken as 100. Results indicate the average and average deviation of two experiments.

TABLE 5. Effect of Na^+ concentration on the capacity of *A. haloplanktis* to concentrate unchanged metabolites inside the cells against a gradient

Metabolite	I/E ratios at the following extracellular Na^+ concn (mM) and uptake times (min)[a]:				% Recovery[b]
	10		300		
	0.5	3.0	0.5	3.0	
D-Galactose	3.4	4.6	4.2	10.3	2.2
L-Alanine	4.2	9.6	28	101	64
L-Leucine	2.1	3.4	24	104	
L-Glutamate	4.6	8.9	15	74	68
L-Aspartate	0.7	1.4	1.6	23.3	
L-Lysine	4.2	11.3	4.7	16.5	59
L-Arginine	1.5	2.2	6	13.9	75
L-Malate	0.4	1.3	1.6	2.6	12
Citrate	0	0	1.6	16.4	26
Phosphate	5	18	5	33.6	36

[a] The I/E ratio is the ratio of intracellular concentration of unchanged metabolite (I) to initial extracellular concentration (E). For succinate the I/E ratios after 3 and 6 min at 10 mM Na^+ were both 0, and at 300 mM Na^+ they were 6.9 and 18.5, respectively. Recovery of unchanged succinate was 1.4% after 3 min. Na^+ was added to the incubation medium as NaCl. The total concentration of added salt was maintained constant at 300 mM by adding ChCl.

[b] Recovery of radioactivity extracted from cells as unchanged metabolite expressed as a percentage of total radioactivity present in cells after incubation for 3 min in the presence of 300 mM NaCl.

higher concentration inside than outside the cells at both incubation times tested.

The results with *V. fischeri* (Table 6) presented a similar pattern. All of the compounds tested were concentrated inside the cells against a gradient; and except for L-alanine and L-arginine, which had low Na^+ optima for the maximum rate of transport (Fig. 2), the gradient increased as the Na^+ concentration increased. Free D-glucose could not be detected in *V. fischeri* after 0.5 min with 10 mM Na^+, although the cells were found to have accumulated considerable amounts of radioactivity under these conditions.

The percentage of radioactivity in the cells that was recovered as unchanged metabolite after 3 min of incubation with 300 mM NaCl is shown in Tables 5 and 6. For some compounds, such as D-galactose, L-malate, and succinate for *A. haloplanktis* and succinate and D-glucose for *V. fischeri*, the percent recovery was poor, thus indicating a rapid conversion of these compounds to intermediates and end products of metabolism. Other compounds such as L-arginine and L-glutamate for *V. fischeri* were recovered almost quantitatively as unchanged metabolite. In spite of such differences, all metabolites that were examined accumulated inside the cells against a gradient of unchanged metabolite in the presence of Na^+, even though some were very rapidly metabolized.

TABLE 6. Effect of Na^+ concentration on the capacity of *V. fischeri* to concentrate unchanged metabolites inside the cells against a gradient

Metabolite	I/E ratios at the following extracellular Na^+ concn (mM) and uptake times (min)[a]:				% Recovery[b]
	10		100		
	0.5	3.0	0.5	3.0	
L-Alanine	35.2	108	27.3	105	45
Succinate	3.3	0.9	4.2	143	11
L-Arginine	7.9	22.9	6.8	25.5	90
L-Glutamate	10.1	28.6	25.3	65.6	90
Phosphate	9.7	79.2	29.9	137	36

[a] See footnote *a* to Table 5. For D-glucose the I/E ratios after 0.5 and 3 min at 0 mM Na^+ were 0 and 6.5, respectively, and at 200 mM Na^+ were 2.5 and 21.5, respectively. Recovery of unchanged D-glucose was 7.7% based on uptake after 3 min in the presence of 200 mM NaCl–100 mM ChCl.

[b] Recovery of radioactivity extracted from cells as unchanged metabolite expressed as a percentage of total radioactivity in cells after incubation for 3 min in the presence of 100 mM NaCl–200 mM ChCl, except for D-glucose.

TABLE 7. Comparison of the effect of NaCl and ChCl on the uptake of various amino acids by *A. haloplanktis* 214 and *V. fischeri* MAC401

Metabolite	Uptake (nmol/mg [dry wt] of cells per 10 min) by the following organisms in the presence of the indicated salts[a]:			
	A. haloplanktis		*V. fischeri*	
	NaCl	ChCl	NaCl	ChCl
L-Isoleucine	44.5	2.0	16.5	3.8
L-Serine	41.8	2.3	90.8	6.3
Glycine	40.6	2.7	94.9	5.4
L-Valine	36.8	0.1	17.0	3.7
L-Tyrosine	32.5	0.5	40.7	6.6
L-Threonine	19.1	1.9	33.9	6.0
L-Phenylalanine	14.5	3.4	13.1	3.7
L-Histidine	7.6	2.0	36.4	3.6
L-Proline	2.8	1.9	45.4	9.3
L-Leucine			120.0	3.0
L-Aspartate			21.4	7.0
L-Lysine			8.3	4.1

[a] Measured by previously described procedures (48).

Effect of Na^+ on the uptake of other compounds. In addition to the compounds examined in Tables 1 and 2, results of earlier experiments not previously published indicate that Na^+ has a marked capacity to stimulate the uptake of other amino acids by cells of both *A. haloplanktis* and *V. fischeri* (Table 7).

Effect of inhibitors. The effect of inhibitors on the uptake rates of selected metabolites was examined (Tables 8 and 9). For both organisms, the uncoupler 3,5,3′,4′-tetrachlorosalicylanilide (TCS) inhibited the transport of all metabolites essentially completely, except for the uptake of D-glucose by *V. fischeri*, in which only partial inhibition was obtained. The respiratory inhibitor KCN, the next most effective inhibitor, was only partially effective in preventing succinate uptake into both organisms and D-glucose transport into *V. fischeri*. Arsenate, a phosphate analog, and iodoacetate, a sulfhydryl reagent, inhibited the transport of most metabolites to various degrees, depending on the metabolite and the organism.

TABLE 8. Effect of inhibitors on initial rates of uptake of metabolites by *A. haloplanktis*

Inhibitor (concn)	% Inhibition of the following transported metabolites[a]:		
	L-Alanine	Succinate	D-Galactose
Arsenate (10 mM)[b]	55 ± 9	12 ± 12	16 ± 6
TCS (10 μM)	97 ± 1	92 ± 3	97 ± 1
KCN (10 mM)	96 ± 1	83 ± 7	94 ± 2
Iodoacetate (20 mM)	36 ± 0	16.7 ± 0	4 ± 4

[a] Inhibition in an incubation medium containing 300 mM NaCl.

[b] Added as Na_2HAsO_4.

DISCUSSION

Results of early experiments with *A. haloplanktis* showed that the requirements for Na^+ for maximum rates of oxidation of a number of metabolites varied with the compound oxidized (27). The requirements for Na^+ for the oxidation of D-alanine and D-galactose were different and similar to those for the transport of the corresponding nonmetabolizable analogs AIB and D-fucose (7). These findings led to the conclusion that the requirements for Na^+ for the oxidation of the metabolites reflected their requirements for Na^+ for transport. The extension of the results of these studies to include other metabolites has been limited by the availability of transportable analogs. The use of membrane vesicles was considered, because these permit a distinction between the requirements of a compound for transport and for subsequent metabolism (14). This also has limitations because the requirements for transport into membrane vesicles and intact cells may be quantitatively different.

The transport into *A. haloplanktis* 214 and *V. fischeri* of all the metabolites tested, except D-glucose into *V. fischeri*, required the presence of an alkali-metal cation in the suspending medium. Of the alkali-metal cations examined, Na^+ promoted the transport of all of the metabolites examined and promoted them at the highest rates. Li^+ permitted the transport of some metabolites, although at lesser rates. K^+ was ineffective, except in two circumstances; it stimulated L-lysine uptake by *A. haloplanktis* and replaced Na^+ in further stimulating D-glucose uptake by *V. fischeri*. The rate of transport of each of the metabolites into each of the organisms was affected differently by the Na^+ concentration in the medium. For most metabolites, the addition of increasing concentrations of Na^+ to a Na^+-free medium increased the rate of transport to a maximum, above which further increases in Na^+ concentration caused the rate of transport to be progressively reduced. The concentration of Na^+ giving rise to a maximum rate of uptake varied markedly, depending on the metabolite being transported. Because kinetic data suggest (48) that Na^+ affects the conformation of a transport protein, causing it to assume a spatial configuration with affinity for the substrate, the concentration of Na^+ producing the conformation with the highest affinity could be expected to vary both with the transport protein and the metabolite transported. Concentrations of Na^+ above the optimum might distort the conformation, thus reducing the affinity of the transport protein for the metabolite and hence the rate of transport. The tendency of increasing concentrations of Na^+ above the optimum to inhibit transport more than the addition of equimolar concentrations of choline (Tables 3 and 4) supports this possibility. We assumed previously that the inhibitory effects of higher NaCl concentrations were due to increased osmotic pressure, but they now appear to be due to the more specific effect of Na^+. This effect of Na^+ may at least in part explain the frequent observation (e.g., see reference 29) that many marine bacteria grow faster in half-strength rather than full-strength seawater, with the latter containing 460 mM Na^+.

The uptake of citrate into *A. haloplanktis* and L-glutamate and phosphate into *V. fischeri* responded to Na^+ concentrations differently from the other metabolites, with rates continuing to increase with Na^+ concentration in a biphasic manner. One possible explanation for this is that at lower concentrations Na^+ affects transport specifically, while at higher concentrations transport of these particular metabolites may also be responding to increased osmotic pressure.

TABLE 9. Effect of inhibitors on initial rates of uptake of metabolites by *V. fischeri*

Inhibitor (concn)	% Inhibition of the following transported metabolites[a]:		
	L-Alanine	Succinate	D-glucose
Arsenate (10 mM)[b]	50 ± 0	7 ± 1	60 ± 2
TCS (10 μM)	94 ± 1	98 ± 0	79 ± 0
KCN (10 mM)	90 ± 0	38 ± 4	71 ± 1
Iodoacetate (20 mM)	83 ± 3	93 ± 1	61 ± 1

[a] Inhibition in an incubation medium containing 100 mM NaCl–200 mM ChCl.
[b] Added as Na_2HAsO_4.

Li^+ has been found to spare the Na^+ requirement for the growth of *Vibrio parahaemolyticus* (30) and to function in place of Na^+ in some transport systems (36, 38, 43). Lithium ion-sugar cotransport via the melibiose transport system has been demonstrated in *E. coli* (44), and knowledge of the molecular biology of this transport process is well advanced (32, 49). In this study Li^+ was found to stimulate transport more frequently in the presence of suboptimal concentrations of Na^+ than it was to replace Na^+ completely. When it did do the latter, Li^+ was appreciably less effective than Na^+. It is difficult to interpret these data in whole cells because of the possibility of multiple transport systems for the uptake of any one metabolite (8, 35), each of which may have different capacities to respond to Na^+ and Li^+.

Only one metabolite, D-glucose, that was taken up by *V. fischeri* was transported at reasonable rates in the absence of an alkali-metal cation, although the addition of Na^+, K^+, or Li^+ but not choline strongly stimulated transport. Baumann and Baumann (1) have shown that the marine bacterium *Pseudomonas doudoroffii* assimilated fructose by a phosphoenolpyruvate-phosphotransferase system. Hodson and Azam (12) found a similar system for the uptake of glucose by the marine bacterium *Serratia marinorubra*. The latter system did not require nor was it stimulated by alkali-metal cations. It is possible that the phosphoenolpyruvate-phosphotransferase system is responsible for the non-Na^+-dependent transport of D-glucose into *V. fischeri*. The partial inhibition of this transport by arsenate and iodoacetate supports this hypothesis. The further stimulation of D-glucose uptake by alkali-metal cations suggests that there may also be an alkali-metal D-glucose cotransport system in this organism. This possibility is supported by the accumulation of unchanged D-glucose in the cell against a gradient and by the partial inhibition of D-glucose uptake by TCS and KCN. It is difficult, however, to understand how K^+ could replace Na^+ in the transport system because the intracellular concentration of K^+ in *V. fischeri* has been found to be 400 mM (unpublished data) when the external concentration is 10 mM.

The complete or nearly complete inhibition of transport of all compounds examined, except for D-glucose by the uncoupler TCS, indicates that the active transport of these compounds requires a membrane potential and is probably energized by an electrochemical gradient of Na^+ (26). *A. haloplanktis* has been shown to accumulate AIB by a Na^+ solute symport mechanism that is energized by an electrochemical potential of Na^+ ions (34) while a Na^+/H^+ antiporter maintains a downhill gradient of Na^+ into the cells (33).

When ChCl was added as an osmotic support, it showed little tendency to increase the rate of transport at suboptimal concentrations of Na^+. Results of previous studies (48) have

suggested a more marked effect of osmotic pressure under these conditions. The apparent discrepancy can be attributed to differences in the methods used to measure rates of transport. In the earlier study (48) uptakes of the nonmetabolizable compound AIB were measured, and the uptake time used to measure rates was 10 min. In this study uptake rates of metabolizable compounds were measured, and the time used to determine the rates was 1 min. The greatest sparing effects of osmotic pressure on the Na^+ requirement for transport were observed in *A. haloplanktis* after long periods of incubation (90 min) (28). It appears that osmotic pressure at suboptimal concentrations of Na^+ exerts its effects primarily on the capacity of cells to take up and maintain high concentrations of unchanged compound and has little effect on initial rates of uptake of compounds which, because of metabolism, have a limited opportunity to accumulate in the cells.

The results presented here indicate that the rates of uptake into *A. haloplanktis* of 19 metabolizable compounds tested and into *V. fischeri* of 16 of 17 compounds tested were markedly stimulated by the addition of Na^+ to the medium. In addition, the uptake of K^+ by *A. haloplanktis* is Na^+ dependent (11). The transport of 20 amino acids into cell envelope preparations of the extremely halophilic *Halobacterium halobium* (23) and of 13 amino acids into membrane vesicles of the alkalophilic *Bacillus* sp. strain 8-1 (17) has been found to be absolutely dependent on the presence of Na^+ in the medium. Thus, organisms growing in the presence of Na^+ appear to have acquired Na^+-dependent transport processes for most metabolites.

Our results indicate that the quantitative requirements for Na^+ for transport and the characteristics of the response to Na^+ were unique for each metabolite in each organism. Thus, the capacity of a bacterial strain or species to compete with other strains or species for available nutrients in the environment varies with the salinity of the environment and with the complement and particular characteristics of the transport systems of the competing organisms.

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