Influence of Na⁺ on Synthesis of Macromolecules by a Marine Bacterium

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Resting cells of Vibrio natriegens acquired the ability to take up ¹⁴C-labeled mannitol in media containing Na⁺ and K⁺. But, the cells took up a significant quantity of the label as well in the presence of 0.3 M K⁺ and no Na⁺. The label was distributed throughout the cells in both systems. Cells incubated in mannitol minimal culture medium proliferated and synthesized approximately ten times as much protein in the presence of Na⁺ and K⁺ as those incubated in the presence of mannitol and 0.3 M K⁺. The bacteria did not proliferate in the absence of Na⁺. Cells incubated in medium containing mannitol and Na⁺ and K⁺ synthesized approximately twice the quantity of deoxyribonucleic acid and ribonucleic acid as those incubated in medium containing mannitol and 0.3 M K⁺ but no Na⁺. A significant amount of mannitol-binding protein was synthesized in the membranes of V. natriegens incubated in the presence of mannitol and Na⁺ and K⁺, but only a small quantity was produced in medium containing mannitol and 0.3 M K⁺ but no Na⁺. A binding fraction comprising at least two proteins (both with molecular weight near 34,000) was isolated by gel electrophoresis from other components of a K₂CO₃-extract of membrane protein from mannitol-grown cells. This binding fraction mediated phosphorylation of mannitol at the expense of either adenosine triphosphate or phosphoenolpyruvate. It was then found that mannitol-grown, but not broth-grown, cells contained nicotinamide adenine dinucleotide-linked mannitol-1-phosphate dehydrogenase. Neither contained mannitol dehydrogenase.

A minimal quantity of Na⁺ not replaceable by other ions or osmotic agents is required for the growth of a number of marine bacteria (5, 12, 13, 17, 20, 25). This ion affects the functioning of the substrate penetration mechanisms of both growing and resting marine bacteria (4, 16, 17, 30). Investigations in this laboratory showed that elevated K⁺ concentrations could, in part, replace Na⁺ as the agent influencing penetration of cells by the nonionizing organic substrates, L-arabinose and mannitol, but not by organic acids (21, 22). Although ¹⁴C-labeled mannitol that was taken into the cells in the presence of a high K⁺ concentration and a small amount of Na⁺ was dissipated and the label was evenly distributed throughout the cellular constituents, the marine bacteria did not grow. A minimal quantity of 0.06 M Na⁺ was required for proliferation of the bacteria, regardless of the presence of other ions (21).

It was further observed that Na⁺ influenced both synthesis and operation of penetration mechanisms for uptake of mannitol by a marine bacterium (22). Moreover, synthesis of a mannitol-binding membrane protein fraction was found to be dependent upon the simultaneous presence of Na⁺ and mannitol in the culture or suspending medium in which cells were incubated (24), regardless of whether K⁺ was added. These latter studies have now been extended, and the current paper demonstrates a requirement for Na⁺ in the marine bacterium not only for synthesis of binding protein specifically but also for the net synthesis of cellular protein generally. The means by which mannitol catabolism is initiated by this bacterium have also been determined.

MATERIALS AND METHODS

Bacteria. For some years we have designated the marine bacterium used in this study Pseudomonas natriegens (18). But, we have classified (2, 10) the bacterium Vibrio natriegens for the following reasons. (i) Certain of the criteria originally invoked to justify this placement are now known to be less critical than once thought (28), (ii) the bacterium can grow anaerobically, and (iii) the guanine-cytosine ration of isolated deoxyribonucleic acid (DNA) is 45.3% (J.

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DeLey, personal communication). Evaluation of the taxonomic characters originally noted (16, 18) revealed them to be stable and appropriate to this new designation. Only the ability to utilize glycerol as sole carbon source had been lost since the original description in 1961.

Stock cultures of *V. natriegens* were maintained at room temperature on slants of seawater nutrient agar (SWNA). For experiments requiring noninduced cells and extracts from them, the organism was grown in seawater nutrient broth (SWNB). For other experiments, the bacteria were grown on a minimal seawater medium containing 0.25% mannitol or glucose as sole carbon and energy source (16).

**Influence of cations on substrate uptake.** Cells cultured aerobically in SWNB for 18 hr at 30°C were harvested by centrifugation, washed three times with 0.052 M MgCl₂, and suspended [16.5 mg (dry weight)/ml] in 0.04 M potassium phosphate buffer, pH 7.0. These suspensions were supplemented with 20 μmole of NaCl and KCl to provide the desired molarities. After incubation for 4 hr at 30°C, the cells were washed five times with 0.052 M MgCl₂, suspended in distilled water, and fractionated by the method of Roberts et al. (26). Radioactivity of each of the fractions was determined with a Packard Tri-Carb Spectrometer by using a scintillation fluid containing 0.4% 2,5-bis-[2-(5-tert-butyl-benzoxazo)yl]-thiophen in 70% toluene-30% Triton X-100. Where activity was low, counting was prolonged to insure validity. Each value was corrected for background activity (average 25 counts/min).

**Influence of cations on growth and macromolecular synthesis.** Cells grown on glucose-minimal medium were harvested, washed twice with 0.052 M MgCl₂ and suspended [1.65 mg (dry weight)/ml] in culture media, pH 7.0, containing 0.08 M (NH₄)₂HPO₄, 0.02 M (NH₄)₂SO₄, 0.01 M MgCl₂, 0.02 M mannitol, and various quantities of NaCl and KCl. Chloramphenicol (100 μg/ml) was added to a duplicate of the system containing 0.25 M Na⁺ and 0.1 M K⁺ as a control. These suspensions were incubated aerobically at 30°C with constant agitation. To assay for growth, samples of 1 ml were appropriately diluted in seawater and plated out on SWNA at 30-min intervals from 0 to 6 hr. Colonies were counted after incubation of the plates for 48 hr at 30°C. Total protein content of cells in 10-ml samples of the cultures taken at hourly intervals was determined as described by Smith and Pardee (29). DNA and ribonucleic acid (RNA) contents of the cells in identical 10-ml samples were determined as well. For these assays, hot trichloroacetic acid extracts were prepared and subjected to the diphenylamine and orcinol tests described by Dische (3).

**Extraction and fractionation of membrane protein.** Spheroplasts of cells grown on SWNB or mannitol-minimal medium were prepared and ruptured; the membranes were harvested, treated with deoxyribonuclease, and washed (23, 24). Protein contents were determined by the method of Lowry et al. (11) with crystalline ovalbumin as standard. To extract binding protein, the membranes were homogenized at room temperature for 30 min in suspensions containing 0.5 g (wet weight) of cells per 2.5 ml of 0.05 M K₂CO₃ (15) and centrifuged for 60 min in the cold at 105,000 × g. The clarified supernatant was neutralized with HCl and assayed for mannitol-binding capacity or for the enzymatic ability to phosphorylate mannitol. Equilibrium dialysis was employed as previously described (24) to test for binding.

Samples of extract in 0.05 M tris(hydroxymethyl)-aminomethane (Tris)-hydrochloride, pH 7.0, were placed on Sephadex G-150 in columns (2.5 by 45 cm) packed to a height of 40 cm. Before use, the gel was deaerated in the columns and swelled in 0.05 M Tris-hydrochloride buffer containing 100 nmoles of ethylenediaminetetraacetic acid (pH 7.2) per ml. Extract proteins were eluted with 0.05 M Tris-hydrochloride, pH 7.0, at room temperature at a rate of 15 ml/hour, 2.5-ml samples being collected. Migration rates of purified samples (5 mg each) of ovalbumin, molecular weight 45,000; pepsin, 34,000; trypsin inhibitor, 21,600; lysozyme, 14,600; and blue dextran dye, which is excluded by the gel, were assayed for calibration of the column. Elution of the proteins was assayed by measuring light absorption of the samples at 280 nm. Samples of both the crude, K₂CO₃-extract and the mannitol-binding fraction that was eluted from Sephadex were subjected to gel electrophoresis as described by Brewer and Ashworth (1). Protein bands were located by staining with Buffalo Black in 7% acetic acid. To test for mannitol-binding and phosphorylating capacity of the protein represented by the stained bands, several unstained gels were sliced into sections 15 mm long. Homologous segments were pooled and extracted by homogenization with small lots of 0.04 M sodium phosphate buffer, pH 7.0. Supernatants obtained from these homogenates after 1 hr of settling in the cold were then used as sources of enzyme for the assays.

**Dehydrogenase assays.** Cell-free extracts of (i) bacteria grown on SWNB, (ii) cells incubated with mannitol and 0.3 M K⁺, and (iii) cells grown on Na⁺-containing mannitol-minimal medium were prepared by passing cells that were washed three times with 0.052 M MgCl₂ twice through a French pressure cell at 1,400 psi in the cold. The extracts were centrifuged in the cold successively at 12,000, 34,000, and 200,000 × g for 1, 1, and 2 hr, respectively. The clarified extracts were assayed for mannitol-1-phosphate dehydrogenase [M-1-PDH; d-mannitol-1-phosphate: nicotinamide adenine dinucleotide (NAD) oxidoreductase, EC 1.1.1.17] activity (7) and for mannitol dehydrogenase (MDH; mannitol: NAD oxidoreductase, EC 1.1.1.67) activity as well (14). Both the capacity for reduction of fructose by reduced NAD (NADH) and the ability to couple oxidation of mannitol with NAD (or NAD phosphate) reduction were assayed.

**Chemicals.** d-Mannitol-1,6-¹⁴C (specific activity, 0.2 mCi/9.1 mg) was obtained from New England Nuclear Corp. Inorganic compounds were reagent grade (Baker). Mannitol-1-phosphate and Tris (Trizma Base and Trizma HCl) were obtained from Sigma Chemical Co.
RESULTS

Uptake and distribution of $^{14}$C-labeled mannitol. Resting cells of *V. natriegens* grown on SWNB were induced during a 4-hr incubation period to take up radioactive mannitol and to distribute the labeled carbon most effectively when incubated in suspending media containing both Na$^+$ and K$^+$ (Table 1). Cells incubated in media containing labeled mannitol and 0.3 M K$^+$ but no added Na$^+$ acquired the ability to incorporate 27% of the label that was taken up by the cells in the presence of Na$^+$ and K$^+$. Fractionation of the cells revealed that the radioactivity was distributed throughout the macromolecular structures. Cells incubated in the presence of Na$^+$ but no K$^+$ have previously been found (22) to take up no more labeled mannitol than control cells lacking Na$^+$. The small quantity of mannitol that entered the cells under those conditions was sufficient to induce synthesis of the penetration and oxidation mechanisms since such cells took up and oxidized mannitol linearly when they were incubated with labeled mannitol, Na$^+$, K$^+$ and chloramphenicol. Thus, in the current study, the amount of labeled mannitol that entered the cells in the system containing 0.3 M K$^+$ (but no Na$^+$) should have been more than sufficient to induce enzymes for growth, if entry were the only barrier to utilization of mannitol as a growth substrate.

**Growth and macromolecular synthesis in media containing Na$^+$ and K$^+$.** The bacteria grew rapidly during the 6-hr incubation period in mannitol-minimal medium containing 0.25 M Na$^+$ and 0.01 M K$^+$ (Fig. 1) and produced a significant net increase in protein (Fig. 1). Simultaneously, there was an appreciable increase in net synthesis of both DNA and RNA by cells incubated in the presence of 0.25 M Na$^+$ and 0.01 M K$^+$ (Fig. 2).

**Lack of growth and minimal macromolecular synthesis in media containing high K$^+$ and no Na$^+$.** Even though the membrane barrier was penetrated (Table 1), the cells did not proliferate during a 6-hr period in mannitol-minimal medium with 0.3 M K$^+$ provided as a possible substitute for Na$^+$ (Fig. 1). Minimal net protein synthesis occurred in the medium containing mannitol and 0.3 M K$^+$ (Fig. 3), reflecting in some degree the considerable amount of substrate that gained entry (Table 1). This synthesis, which amounted to 11% of that observed in cells incubated with mannitol and 0.25 M Na$^+$ and 0.01 M K$^+$ (Fig. 1), may have been influenced by endogenous supplies of Na$^+$ that enabled the cells to function to this limited extent. As expected, there was no growth, little entry, and no detectable synthesis of protein in the control cells incubated with mannitol and 0.01 M K$^+$ but without Na$^+$.

Synthesis of DNA and RNA was observed in cells suspended in media containing mannitol but no Na$^+$ (Fig. 4) and amounted to 50 to 60% of the nucleic acid synthesis carried out by cells incubated in the presence of 0.25 M Na$^+$ and 0.01 M K$^+$. Thus, contrasting the data in Fig. 1 and 2 with those in Fig. 3 and 4, the principal failing of the cells incubated in the medium containing 0.3 M K$^+$ (but no Na$^+$) appears to be their severely diminished capacity to synthesize protein.

To narrow the scope to manageable limits, we therefore began to study the influence of Na$^+$ and K$^+$ on the synthesis and activity of those proteins critical to growth of *V. natriegens* at the expense of mannitol.

**Binding protein as phosphorylating enzyme.** Both membranes from cells grown in mannitol and Na$^+$, and extracts from those membranes bound mannitol in equilibrium dialysis cells. Membranes from the control bacteria grown on SWNB, from cells incubated with mannitol and 0.3 M K$^+$, and from those incubated with mannitol, Na$^+$, K$^+$, and chloramphenicol did not bind labeled mannitol.

Rhodes and Payne (24) used dilute NaOH to extract mannitol-binding protein from crude membranes derived from spheroplasts of mannitol-grown *V. natriegens*. In the current studies, 0.05 M K$_2$CO$_3$ (15) effectively replaced the strong base and extracted binding protein that was more active and stable. Passage of this extract through gel-sieve columns yielded one fraction with an apparent molecular weight very near that of pepsin (34,000) and three other lighter fractions. Only the protein in the first fraction bound mannitol.

### Table 1. Influence of cations on the uptake and distribution of mannitol-1,6-$^{14}$C by resting cells of *V. natriegens* grown on SWNB.

| Fraction | Radioactivity$^a$ of cells suspended in media containing labeled mannitol and |
|----------|----------------------------------------------------------------------------------|
|          | 0.01 M NaCl | 0.25 M KCl | 0.3 M NaCl | 0.01 M KCl |
| Total    | 1,532       | 414        | 176        |
| Cold trichloroacetic acid soluble | 46 | 22 | 10 |
| Alcohol soluble | 180 | 114 | 52 |
| Alcohol ether soluble | 32 | 30 | 26 |
| Hot trichloroacetic acid soluble | 152 | 34 | 12 |
| Residual fraction | 1,122 | 214 | 76 |

$^a$ Counts per minute per milligram of cellular protein after incubation for 4 hr.

![Image](http://aem.asm.org/Downloaded from March 18, 2020 by guest)
FIG. 1. Influence of Na+ concentration on protein synthesis and increase in cell numbers of V. natriegens. The mannitol-minimal medium contained 0.01 M KCl and various concentrations of NaCl. Incubation temperature was 30°C, with constant agitation. Each sample was treated with 1.1 ml of 50% trichloroacetic acid and centrifuged, and the supernatant fluid was discarded. The precipitate from each was suspended in 1 ml of 0.1 M NaOH containing 2% Na2CO3 and 0.33 ml was assayed for protein content. Viable cell counts were obtained by plating samples on SWNA. There was no increase in protein content or cell number in the cultures containing chloramphenicol.

FIG. 2. Influence of Na+ on synthesis of DNA and RNA by cells of V. natriegens. The mannitol-minimal medium contained 0.01 M KCl and various concentrations of NaCl. Incubation temperature was 30°C with constant agitation. Trichloroacetic acid was added to 10-ml samples and incubated for 24 hr at 4°C. The cells were then washed, resuspended in 4 ml of 5% trichloroacetic acid, and placed in a boiling water bath for 30 min. DNA and RNA contents of the hot trichloroacetic acid extracts were then assayed.
(Fig. 5), and specific binding activity was increased 22.5-fold over that of crude membrane. When either adenosine triphosphate (ATP) or phosphoenolpyruvate (PEP), but not adenosine diphosphate (ADP), was added to the binding system, the break-away from equilibrium was reversed. As previously noted (24), paper chromatography of samples taken at various times from these experiments again revealed that \(^{14}C\)-labeled mannitol was recovered unaltered on the binding side of the dialysis cell in the absence of the high-energy phosphates. In the current study, labeled M-1-P that co-chromatographed (31) with authentic cold M-1-P was recovered on chromatograms after addition of the high-energy phosphate donors.

When the binding fraction obtained by \(K_2CO_3\) extraction was further examined, two barely separated protein bands were discerned by gel electrophoresis. Comparing a stained gel with several identically prepared but unstained gels, we found that eluate from the area of these proteins contained both binding and phosphorylating activity. We have not yet been able to isolate these proteins separately and cannot yet determine whether both are required for activity.

With the finding that M-1-P was produced by enzyme located in the membrane, the probability that M-1-PDH would be observed seemed almost certain. Cell-free extracts of mannitol-grown \(V.\ natriegens\) were shown to contain NAD-specific M-1-PDH (Fig. 6), whereas extracts of SWNB-grown cells were inactive. MDH activity was not detected by either method in extracts of cells grown on either mannitol or SWNB.

**Combined assays for phosphorylating capacity and M-1-PDH activity in various fractions.** Since we were unable by equilibrium dialysis to demonstrate binding protein in the membranes of cells incubated with mannitol and high \(K^+\), we at...
FIG. 5. Influence of extracted membrane protein of V. natriegens on equilibrium dialysis of $^{14}$C-labeled mannitol. At the point indicated by the open arrows, ADP, ATP, or PEP was added. Reaction mixture on substrate side (S) contained: NaCl, 250 μmoles; KCl, 10 μmoles; Tris, pH 7.2, 75 μmoles; and $^{14}$C-labeled mannitol, 0.5 μmoles (4.1 × 10⁶ counts per min per μmole). Total volume, 1 ml. Contents of the membrane extract side (M): NaCl, 250 μmoles; KCl, 10 μmoles; Tris, pH 7.2, 75 μmoles; and 0.1 ml Tris containing 100 μg of extracted membrane protein. Total volume, 1 ml. Test systems employed were: ●, protein fraction eluted from acrylamide gel (molecular weight 34,000) after K₂CO₃-extraction from membrane protein, or this fraction with ADP, 7.5 μmoles, added 45 min after break in equilibrium; ○, identical fraction of extract with PEP, 7.5 μmoles, added 45 min after break in equilibrium; and ◀, as in previous system with ATP, 7.5 μmoles, added 45 min after break in equilibrium.

tempted to determine whether these membranes contained enzyme that could synthetize M-1-P. As expected (Table 2), membranes from control cells grown in mannitol with Na⁺ produced M-1-P when incubated with mannitol and ATP or PEP. The product, which co-chromatographed with authentic cold M-1-P, served as substrate for rapid, NAD-linked dehydrogenation by crude, cell-free extracts of mannitol-grown V. natriegens. Despite the provision of the same amount of M-1-P by extracts of membranes from cells grown on mannitol in the presence of Na⁺, the crude extract of cells that incubated with mannitol and high K⁺ displayed less than one-third the M-1-PDH activity exhibited by the extracts of fully-induced cells. We observed that incubation of mannitol and ATP or PEP with extracts of membranes and with cell-free extract of cells exposed to mannitol and to high K⁺ yielded only one-tenth the M-1-PDH activity of the system entirely induced in the presence of Na⁺. The non-induced cells had neither mannitol-phosphorylating capacity nor M-1-PDH activity. Incubation of mannitol and the high-energy phosphates with membranes from cells grown on SWNB yielded no M-1-P, and the extract of these cells displayed no M-1-PDH activity. The limited M-1-PDH activity (Table 2) is consistent with the small amount of protein synthesis and distribution of labeled carbon in cells incubated with mannitol and high K⁺. Significant mannitol-phosphorylating capacity, M-1-PDH activity, and a degree of distribution of labeled products occur only in cells grown in the presence of Na⁺.

DISCUSSION

Typical parameters considered in previous studies designed to reveal the effects of ions on marine bacterial growth were rate and extent of increase...
Table 2. Generation of substrate for mannitol-1-PO₄ dehydrogenase (M-I-PDH) activity by membrane extracts

| Source of M-1-PDH in the reaction mixture                                                                 | Activity with phosphate source<sup>a</sup> |
|----------------------------------------------------------------------------------------------------------|------------------------------------------|
|                                                                                                          | ATP | PEP |
| Cell-free extract of cells grown with Na<sup>+</sup> and mannitol                                        |     |     |
| Complete<sup>b</sup>                                                                                     | 62.9 | 60.0 |
| Complete minus:                                                                                           |     |     |
| Mannitol                                                                                                  | 5.7 | 5.7 |
| Cell-free extract from cells incubated with high K<sup>+</sup>                                           | 2.1 | 2.9 |
| Nicotinamide adenine dinucleotide (NAD)                                                                  | 14.3<sup>c</sup> | 12.9 |
| K₂CO₃-extract of membrane from Na<sup>+</sup>-grown cells                                                  | 2.9 | 4.3 |
| Cell-free extract of seawater-nutrient broth (SWNB)-grown cells incubated with elevated K<sup>+</sup> and mannitol |     |     |
| Complete                                                                                                  | 18.6 | 15.7 |
| Complete minus                                                                                           |     |     |
| Mannitol                                                                                                  | 2.4 | 2.14|
| Cell-free extract from cells incubated with high K<sup>+</sup>                                           | 2.9 | 2.86|
| NAD                                                                                                      | 0.71 | 0.71|
| K₂CO₃-extract of membrane from Na<sup>+</sup>-grown cells                                                  | 1.43 | 1.43|
| Cell-free extract of SWNB-grown cells incubated with elevated K<sup>+</sup> and mannitol                  | 6.0 | 6.4 |
| Complete                                                                                                  |     |     |
| Mannitol                                                                                                  | 2.1 | 2.0 |
| Cell-free extract from cells incubated with high K<sup>+</sup>                                           | 3.1 | 2.8 |
| NAD                                                                                                      | 1.2 | 1.4 |
| K₂CO₃-extract of membrane from resting cells incubated with high K<sup>+</sup> and mannitol                | 2.9 | 3.4 |
| Cell-free extract of SWNB-grown cells                                                                     | 0.4 | 0.45|
| Complete                                                                                                  |     |     |
| Mannitol                                                                                                  | 0.0 | 0.0 |
| Cell-free extract of cells incubated with low K<sup>+</sup>                                              | 0.0 | 0.0 |
| NAD                                                                                                      | 0.0 | 0.0 |
| K₂CO₃-extract of membrane from SWNB cells                                                                  | 0.0 | 0.0 |

<sup>a</sup> Units per minute per milligram of protein. Unit = 0.01 change in optical density at 340 nm.

<sup>b</sup> Complete reaction mixtures contained: KF, 100 μmoles; KCl, 300 μmoles; MgCl₂, 300 μmoles; 0.1% Triton X-100, 0.01 ml; 0.05 M Tris, pH 9.0, 1.1 ml; mannitol, 7.5 μmoles; adenosine triphosphate (ATP) or phosphoenolpyruvate (PEP), 7.5 μmoles; and 0.3 ml of K₂CO₃ extract of membrane (120 μg protein). After incubation for 15 min at 30°C, NAD (4.5 μmoles) and cell-free extract (750 μg protein) were added. Total volume, 3.0 ml. M-1-PDH activity measured as change in optical density at 340 nm. The K₂CO₃ extract had no M-1-PDH activity.

<sup>c</sup> Activity attributed to high endogenous NAD content and M-1-PDH activity of crude cell-free extract reacting with significant quantity of M-1-P produced in this system.

in cell mass (12, 13, 17, 18). These investigations were neither designed nor expected to locate the biochemical foci of the influences of Na<sup>+</sup> on growth. Subsequently, substrate uptake by resting marine bacterial cells was investigated to determine the involvement of Na<sup>+</sup> and K<sup>+</sup> in the functioning of mechanisms for the penetration of cytoplasmic membranes by organic substrates (4, 22, 30). These studies revealed a number of parallels (as well as some differences) between the influence of Na<sup>+</sup> on substrate transport by marine bacteria and animal cells. However, since they depended on the membrane proteins preexisting in the resting cells chosen for the investigations, such experiments were not likely to suggest additional roles for Na<sup>+</sup>. Our conclusion that Na<sup>+</sup> influences synthesis as well as operation of substrate entry mechanisms (22) now seems well justified, since from the current studies it appears that all protein synthesis is inhibited by the lack of Na<sup>+</sup>. Although the marine bacteria which we have
examined do not grow without Na+, we were impressed by the degree to which elevated concentrations of K+ could replace Na+ in the facilitation of the entry of nonionizing substances without damage to the cells (21). This led us to examine ionic influences on the synthesis of substrate-related binding protein. We reasoned that the synthesis of a membrane-located mechanism for recognition of mannotol would be necessary as a prelude to its utilization as the sole source of carbon and energy for growth. We thus demonstrated the occurrence of mannotol-binding protein (24), which is readily synthesized in media containing Na+ but only minimally in those lacking this cation. The possibility that the complex binding fraction contained (or constituted) enzyme was obscured in our earlier work by the observation that mannotol was recovered unaltered after having been bound. It is obvious now that this was due to the absence of ATP or PEP from the reaction mixtures employed in those original binding experiments (24).

Our demonstration that mannotol-binding (phosphorylating) protein occurred in membranes of *V. natriegens* in significant quantity only after incubation with Na+ present suggests that its controlling influence on membrane protein synthesis may be equally as significant for the marine bacteria as the role of Na+ in the operation of the penetration mechanisms. This argument is based on the assumption that, although the general process of protein synthesis by this bacterium is influenced by Na+, the lack of synthesis of membrane protein (such as substrate entry and phosphorylating enzyme) would impose more severe limitations on the cell than any other failure of protein synthesis. The argument is further consistent with the observation that another agent (K+) can facilitate entry of an acceptable growth substrate (mannitol) but not the synthesis of protein.

Phibbs and Eagon (19) showed that *Pseudomonas aeruginosa* transports mannotol without alteration and traps the substrate by ATP-dependent phosphorylation within the cell. In several other bacteria, on the other hand, phosphorylation is accomplished by membrane-located enzyme (6, 8, 9, 27). Taken together, the observations (i) that complex binding protein from *V. natriegens* membrane catalyzes phosphorylation of mannotol, and (ii) that the M-1-PDH, but not MDH, activity was observed in cell-free extracts of mannotol grown cells now permit us to conclude that mannotol is phosphorylated during the transit of the membrane and that M-1-P is the metabolite catabolized internally during growth.

Although it appears that both ATP:mannitol-1-phosphotransferase and PEP:mannitol phosphotransferase are active in these fractions, determination of the number of component enzymes involved in the phosphorylation of mannotol by *V. natriegens* and the similarities and dissimilarities between this system and phosphorylating enzymes in other bacteria (27) must await the results of additional investigations which are now in progress.

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