AN ABSTRACT OF THE THESIS OF

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Abstract approved:

Cells of the marine bacterium, Ant-300, accumulate loosely bound amino acids during amino acid uptake. The fraction of amino acid taken up that exists in the loosely bound state depends on the substrate in question. Shock treatment studies indicate that, in general, the initial binding, uptake, and retention of amino acids are sensitive to reduced osmolarity and salt concentration. Cellular components that bind the amino acids arginine and lysine are released from the cells during shock treatment. Charge interactions appear to be responsible for the reversible formation of the ligand-protein complex. It is proposed that these components, presumably proteins, mediate the capture of the amino acids arginine and lysine at the surface of the cell. Chemotaxis provides the cell the opportunity to migrate from areas of low arginine concentration to areas of high arginine concentration. Arginine transport into the cell is mediated by two high affinity components with kinetic constants of $1.2 \times 10^{-8}$ and $4.5 \times 10^{-7}$ M. These mechanisms enable Ant-300 to scavenge nutrients in the marine environment, thereby assuring its own survival and promoting the regeneration of nutrients.
Substrate Capture by a Psychrophilic Marine Bacterium

by

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INTRODUCTION

The success of marine bacteria in the oceanic environment depends to a large extent on their ability to accumulate nutrients against a large concentration gradient. This ability to accumulate nutrients is termed substrate capture. Once an organic substrate is captured by a bacterium, it may be incorporated into cellular material, converted to CO₂, or diverted to metabolite pools.

Marine bacteria are known to accumulate loosely bound substrate during substrate uptake. Since the loosely bound substrate appears to exist outside the cytoplasmic membrane (30), actual transport rates are difficult to estimate. Griffiths et al. (30) have been successful in selectively removing loosely bound substrate following the uptake of glutamic acid by cells of Vibrio marinus MP-1 by osmotically shocking the cells during wash treatment. However, the nature of the interactions between glutamic acid or any other substrate and the components of bacterial capture system are not clearly understood. Essentially nothing is known of the interactions between substrate capture mechanisms and transport systems in marine bacteria.

One must recognize, though, that the conventional techniques used to isolate those components involved in the accumulation of substrates in organisms such as Escherichia coli and Salmonella typhimurium must be altered in order to evaluate the corresponding systems in marine bacteria. For instance, consideration must be made with regard to osmotic and salt requirements of marine bacteria. In view of these complications, an investigation was initiated to determine the substrate capture mechanism(s) of Ant-300, a psychrophilic marine bac-
terium. This in turn, should help explain how marine bacteria scavenge nutrients in the oceanic environment.
LITERATURE REVIEW

Relative to other areas of the world, the open ocean may be considered a "desert" in terms of organic nutrients. Although the dissolved organic carbon (DOC) and particulate organic carbon (PC) in surface waters vary greatly depending upon primary production, the concentration of organic carbon decreases rapidly with depth. Nakajima and Nishizawa (51) found that in the upper 100 meters PC decreases exponentially with depth. Below the surface both DOC and PC appear to be relatively stable in a horizontal as well as vertical direction. Menzel and Ryther (48) have estimated that at depths below 200-300 meters, DOC ranges from 0.35 to 0.70 mg/l and PC occurs in concentrations of 3-10 µg/l.

Since production of organic carbon (OC) in the world's oceans is comparable to that on land (19), mineralization of fixed carbon must occur at significant rates if the levels of OC are to rapidly decrease with depth. Nakajima and Nishizawa (51) calculated that one-third of the PC is grazed by zooplankton while the remainder may be degraded by ultraplankton and bacteria. The indigenous heterotrophic populations responsible for mineralization must therefore be capable of high rates of activity (i.e., during phytoplankton blooms), yet survive when nutrients are sparse.

Unlike spore-forming bacteria in the soil, the success of marine bacteria during low nutrient conditions appears to depend on the ability to colonize existing suspended particulate matter (35). ZoBell (81) demonstrated that the concentrative adsorption of organic matter on solid surfaces in dilute nutrient environments enhances the activity of attached bacteria.
The manner in which marine bacteria bind to surfaces appears to involve the production of extracellular polysaccharides (26, 46). Corpe (21) observed a succession of film-forming organisms during the colonization of submerged slides in seawater. Initially, short chains or micro-colonies became attached followed by stalked and filamentous bacteria. One periphytic isolate has been shown to produce extracellular polyanionic carbohydrate material composed of neutral sugars and uronic acid (20). Protein also appears to be associated with the film. In addition to binding surfaces, the polyanionic capsular material may also participate in the scavenging of dissolved nutrients from seawater.

The term "substrate capture" has been used in reference to the rapid accumulation of nutrients against a concentration gradient (50), a process closely aligned with the active transport of substrates into cells. It is not surprising, therefore, that the intense interest in bacterial transport systems has led to the discovery of a class of proteins located in the cell envelope of gram-negative bacteria which bind ions, sugars, amino acids, and vitamins. To date, osmotic shock-releasable proteins have been isolated which bind sulfate (56), phosphate (47), galactose (7, 14), L-arabinose (34), ribose and maltose (32), leucine, isoleucine, and valine (57), glutamine (75), glutamate and aspartate (78), phenylalanine (74), histidine (5), cystine (12), arginine, lysine and ornithine (62).

Although most of the binding proteins that have been characterized have been isolated from extracts of Escherichia coli, they appear to be present in other organisms as well. Histidine and sulfate binding proteins have been isolated from Salmonella typhimurium (5, 56).
The binding proteins appear to be loosely bound to the cell since mild osmotic shock causes their release into the culture menstruum. Although shock-releasable binding proteins have been assigned a "periplasmic" location (the region between the cytoplasmic membrane and the cell wall), loose association of the proteins with the membrane or cell wall may exist (52).

Kinetic studies have provided evidence for the involvement of substrate-binding proteins in active transport. Osmotic shock causes a substantial decrease in the active transport of those substrates which bind shock-releasable proteins (7, 64, 79). In certain cases, a marked stimulation in transport occurs upon addition of purified binding protein to shocked cells (80). In addition, the dissociation constants ($K_d$) for substrate-protein binding are similar to the $K_m$ values for substrate transport. Piperno and Oxender (57) determined for the LIV-binding protein, which binds leucine, isoleucine and valine, $K_d$ values of 1.1 and 2.2 µM for leucine and isoleucine, respectively. Correspondingly, $K_m$ values for leucine and isoleucine transport in whole cells were 1.1 and 1.2 µM (57). Furthermore, compounds acting as non-competitive inhibitors to the transport of various amino acids, also interfere with the respective amino acid-binding protein reaction (9).

Many of the transport systems involving binding proteins operate at very low substrate concentrations. For instance, arginine specific transport in *E. coli* has a $K_m$ of $2.6 \times 10^{-8}$ M (62), histidine specific transport in *S. typhimurium* exhibits a $K_m$ of $3.0 \times 10^{-8}$ M (5), and galactose (Pmg system) transport has a $K_m$ of $5.0 \times 10^{-7}$ M (65). Kalckar (36) proposed that the galactose-binding protein, which participates in
the high affinity galactose transport system of *E. coli*, acts as a scavenging mechanism during low external galactose concentrations.

Genetic studies have provided the most convincing evidence that binding proteins are the carrier proteins for shock-releasable transport systems and that they are the initial recognition site for the substrate. Ames and Lever (5) have described the involvement of the histidine-binding protein in the high affinity histidine specific transport system in *S. typhimurium*. Three components, the J, K, and P proteins have been identified with the system. The J and K proteins both bind histidine. The strain TA 1771, containing a single mutation in the his J gene, shows no binding activity by the J protein (6). Another single missense spontaneous mutation in the his J gene has been shown to produce a product (J protein) which exhibits normal binding activity toward histidine but functions poorly in histidine transport (40). The mutation also results in altered electrophoretic mobility in the presence of sodium dodecyl sulfate. Reversions allow recovery of transport function and normal mobility. The J protein, therefore, appears to have 2 active sites, one for the initial binding of histidine, and another involved in histidine transport. It is postulated that the second site interacts directly with the P protein which is necessary for both J and K protein mediated histidine transport (40).

Boos (17) has isolated strains of *E. coli* having structural gene mutations in the galactose-binding protein. One mutant, ρβg−, lacks galactose binding activity, but contains antigenically cross-reacting material to the purified binding protein in the shock fluid. The high affinity galactose transport system is also non-functional in
the Pβ g-strain. Peptide maps of normal binding protein and purified cross-reacting material from the mutant demonstrate structural changes in the molecule. A revertant was isolated in which binding activity and Pβ g-transport activity was partially restored. These data provide strong support for the involvement of shock-releasable substrate-binding proteins in active transport.

Several binding proteins, in addition to participating in active transport, are also involved in chemotaxis. Chemotaxis is a response by an organism which results in a directed movement toward a particular nutrient. A bacterium is therefore capable of migrating from an area of no nutrients to an area in which a particular attractant is concentrated. The cellular component which recognizes a specific attractant is termed a "chemoreceptor". The galactose-binding protein serves as the recognition component for galactose chemoreception (32). Chemoreceptors for ribose and maltose also correspond to shock-releasable binding proteins (4, 37).

Adler (1) demonstrated that the functioning of chemoreceptors does not require metabolism or general transport of the compound. It only requires recognition of the attractant by a specific binding protein. Galactose- and maltose-binding proteins each have mutationally separate sites for ligand binding, for coupling to transport, and for interaction with chemoreception (33).

Not all substrates that are transported by permeases or that bind shock-releasable binding proteins elicit a chemotactic response (49). L-glutamine, histidine, isoleucine, leucine, methionine, phenylalanine, tryptophan, tyrosine, and valine are transported by E. coli
yet do not produce a tactic response. To date, only 2 receptors have been identified with amino acids (49). The aspartate chemoreceptor exhibits taxis toward aspartate and glutamate, and the serine chemoreceptor recognizes serine, cysteine, alanine, and glycine.

The mechanism of chemotaxis is still unknown. There is evidence of a conformational change in the galactose-binding protein (15, 16). This change may be transmitted to the flagella (3). Mutants have been isolated which are non-chemotactic toward any substance yet retain normal motility (8). Thus, a "common pathway" appears to link recognition of substrates by receptors to flagellar movement (3). This suggests that a chemotactic response involves at least 3 components.

Binding proteins may also participate in the formation of substrate pools or reservoirs in the periplasmic region of the cell. Langridge et al. (42) estimated that there are as many as $10^4$ binding proteins per cell for sulfate in S. typhimurium. Extensive studies have indicated that binding proteins do not possess enzymatic activity, nor do the substrates undergo chemical change during complex formation (79). In addition, reversible binding of substrate to protein is a common feature of these systems (79).

Griffiths et al. (30) demonstrated the formation of two substrate pools during incubation of V. marinus MP-1 in the presence of glutamic acid. One pool appeared loosely bound to the cell in that it was released by mild osmotic shock. The second pool was subsequently recovered in the soluble fraction following treatment of the cells with cold trichloroacetic acid. The loosely bound glutamate pool may very likely correspond to that which is complexed to periplasmic bind-
ing proteins. The substrate sequestered in the periplasmic space would not be a true pool, however, since binding proteins do not increase the concentration of free solute but rather compete with other transport components for substrate (55).

Due to the vast amount of information available on _E. coli_ and a few other well characterized bacteria, it is not surprising that these organisms have been employed to investigate the mechanisms by which bacteria accumulate nutrients. However, the sequestering of nutrients is of paramount importance to other organisms as well, especially marine bacteria. MacLeod and coworkers (69, 71, 72) have characterized various transport systems in a marine pseudomonad species, but their studies have not dealt directly with substrate capture. Recently, Bell and Mitchell (11) have shown that marine bacteria are capable of chemotaxis toward extracellular products of marine algae. They further demonstrated that in mixed populations derived from natural seawater samples, those bacteria exhibiting taxis toward the algal products were selected over non-chemotactic bacteria. Coral mucus (excreted in copious amounts) also elicits a chemotactic response from the indigenous bacteria of coral reefs (Morita, R.Y., personal communication).

The mechanism(s) by which marine bacteria accumulate substrate is essentially unknown. Do periplasmic binding proteins participate in the scavenging of nutrients in seawater? Are substrate receptors also involved in nutrient transport and chemoreception as appears to be the case in _E. coli_? Is the loosely bound substrate pool found in _V. marinus_ present in other marine bacteria, and if so, what is the nature of this pool? Answers to these questions should provide a better un-
standing of the activities of marine bacteria and their importance in the cycling of nutrients in the marine environment.
MATERIALS AND METHODS

Organism

Ant-300, tentatively identified as a Vibrio species (Baross, J., personal communication), was isolated from the Antarctic Ocean (29). The organism requires a mixture of seawater salts for growth. Additional growth requirements are provided in Vitamin-free casamino acids (Difco). A complex mixture of 18 amino acids as described by MacLeod et al. (45), however, does not permit growth of the organism. The organism's psychrophilic nature is characterized by an optimum temperature for growth of 7 °C and a maximum temperature for growth of 13 °C (29).

Substrate retention

All procedures subsequently described were performed at 5 °C unless otherwise indicated. Cells were cultured in Glucose-Casamino Acid (GCAA) medium composed of glucose, 2.5 g; vitamin-free casamino acids, 0.6 g; sodium nitrate, 0.5 g; Rila Marine Mix (Rila Products, Teaneck, N.J.), 30 g; ferrous sulfate, 0.005 g; distilled water, 1 liter. The pH after autoclaving was 7.8. Cells were harvested when the culture density reached an optical density (OD) of 0.25 at 600 nm by centrifugation in a Sorvall RC-2B centrifuge at 3020 x g and washed 2 times in 4% Rila solution. Rila solution contained the indicated percent of Rila Marine Mix in 10 mM Tris (hydroxymethyl) aminomethane (Tris-HCl) buffer, pH 7.8. Cells were then diluted to approximately $2 \times 10^7$ cells/ml in 4% Rila solution and starved for 16–24 h. Following starvation, 5 ml portions of cell suspension were distributed in
test tubes (18 x 150 mm) containing labelled amino acid ($^{14}$C-UL-alanine, $1.4 \times 10^{-7}$ M, 135 mCi/m mole or $^{14}$C-UL-leucine, $7.4 \times 10^{-8}$ M, 270 mCi/m mole) and the reaction mixture was agitated for 10 min. The reaction was stopped by one of two methods; filtration or acidification followed by filtration. Acidification was carried out by addition of 0.1 N sulfuric acid to the reaction mixture to obtain a final pH of 2.0. The reaction mixture was filtered through 0.45 μ HA filters (Millipore Corp.), which separated the cells from any unreacted substrate. Filters retaining acidified cells were washed with 5 ml of 4% Rila solution. Filters were dried at 70 C, placed in scintillation vials containing 5 ml of Omnifluor (New England Nuclear) cocktail (4 g Omnifluor/liter toluene), and the radioactivity counted in a Nuclear Chicago Mark I scintillation counter.

To determine the effects of salt concentration and osmolarity on substrate uptake, the cells were centrifuged following starvation, suspended in either 1% or 4% Rila solution and equilibrated for 10 min prior to addition of a labelled substrate ($^{14}$C-UL-alanine, 13 nM, 135 mCi/m mole; aspartic acid, 10 nM, 204 mCi/m mole; arginine, 14 nM, 279 mCi/m mole; glutamic acid, 17 nM, 234 mCi/m mole; leucine, 26 nM, 270 mCi/m mole; lysine, 14 nM, 279 mCi/m mole; methionine, 17 nM, 225 mCi/m mole; phenylalanine, 9 nM, 405 mCi/m mole; proline, 15 nM, 225 mCi/m mole; serine, 29 nM, 135 mCi/m mole; threonine, 19 nM, 180 mCi/m mole; tyrosine, 10 nM, 380 mCi/m mole). Subsequent steps were carried out as described previously.
Initial binding of substrate to cells

The following modifications to the above procedure were made to study the effects of various shock treatments on the initial binding of substrate to cells. Cells were harvested from a logarithmic phase culture (OD = 0.55). Following starvation, 10 ml portions of cell suspension were centrifuged at 3020 x g for 10 min and the pellet suspended in the appropriate shock solution at a cell density of approximately 2 x 10^7 cells/ml and equilibrated for 20 min. The various shock treatments were carried out according to the following flow diagram:

```
starved cell suspension
  ↓
  3020 x g; 10 min
  ↓
suspend pellet in shock solution for 20 min
  ↓
  1-R
  ↓
dilute with 7% Rila solution
  ↓
  4% Rila solution
  ↓
S-E
  ↓
  16,300 x g; 10 min
  ↓
suspend pellet in 4% Rila solution
  ↓
S-E
  ↓
  16,300 x g; 10 min
  ↓
suspend pellet in shock solution T-Mg for 20 min
  ↓
  16,300 x g; 10 min
  ↓
suspend pellet in 4% Rila solution
```
Shock solution 1-R consisted of 1% Rila solution. Shock solution S-E consisted of 20% sucrose-1mM ethylenediaminetetraacetic acid (EDTA)-0.033 M Tris-HCl, pH 7.5. Shock solution T-Mg consisted of 1 mM MgCl₂-0.033 M Tris-HCl, pH 8.0. Cells suspended in 4% Rila solution were included as controls. The shocked cell pellets were suspended to original volume in 4% Rila solution and immediately exposed to 0.5 ml of 2 μCi/ml ¹⁴C-UL-amino acid for 60 sec in the presence of chloramphenicol (80 μg/ml), filtered, and washed with 4% Rila solution. The labelled amino acids were present in the following final concentration: arginine, 0.7 μM; aspartic acid, 1.0 μM; glutamic acid, 0.9 μM; lysine, 0.7 μM; phenylalanine, 0.4 μM; and proline, 0.8 μM.

Viability

Cells were cultured in GCAA, harvested when the cell density reached an OD of 0.62, washed once in 4% Rila solution, and suspended to original volume in the various shock solutions described above. After a 20 min equilibration period, shocked cell suspensions were serially diluted in the wash solution and plated on Lib-X agar medium. Lib-X agar medium is composed of yeast extract (Difco), 1.2 g; sodium citrate, 0.3 g; L-glutamic acid, 0.3 g; sodium nitrate, 0.05 g; ferrous sulfate, 0.005 g; Rila Marine Mix, 30 g; Bacto-agar, 15 g; distilled water, 1 liter. The pH after autoclaving was 7.5. Colonies were counted after 6 days.
Protein determination

Cells were treated as in the previous section. Following equilibration, shocked cell solutions 1-R, S-E, and T-Mg were centrifuged at 3,020, 16,300 and 16,300 x g, respectively. The cell pellets were suspended in 0.05 M potassium phosphate buffer, pH 7.4 and lysed by sonication using a Bronwill Biosonik III. The protein associated with the pellet and supernatant fractions was determined by the method of Lowry et al. (44) using bovine serum albumin, fraction 5 (Sigma Chemical Co.) as a standard. Protein in subsequent fractions was determined by this method, also.

Chemotaxis

 Cultures grown in Lib-X broth were harvested in the logarithmic growth phase and examined for motility by phase contrast microscopy. Cultures were subsequently centrifuged at 2500 x g for 10 min and the cells suspended to an OD of 0.05 in 4% Rila solution. After 72 h of starvation, the cells were centrifuged and resuspended in 4% Rila solution to a density of approximately 1 x 10^7 cells/ml. The cell suspension was further incubated for 1-3 h, then applied to a chemotaxis chamber, similar to that described by Adler (2), with a syringe containing a 21 gauge needle. L-arginine (free base) (Sigma Chemical Co.) was diluted to the desired concentration in 4% Rila solution, the pH was adjusted to 7.6, and then drawn into 1 µl disposable micropipettes (Drummond Scientific Co.). At 2 min intervals, the capillaries were inserted into the chamber. The capillaries were removed after the de-
sired period of time, rinsed with 4% Rila solution, and the contents expelled into a screw cap test tube containing 10 ml of rinse solution. The contents were shaken vigorously, diluted, and plated on Lib-X agar medium. All steps were carried out at 5 C.

**Amino acid transport studies**

Cells were prepared as in "Initial binding of substrate to cells". Following starvation, 5 ml portions were transferred to 25 ml Erlenmeyer flasks containing chloramphenicol (80 μg/ml) and aminooxyacetic acid (8 mM). The flasks were agitated for 3 min at which time glucose (14 mM) was added and the flasks were agitated for an additional 2 min. 14C-UL-arginine was then added to a final concentration of 0.07 μM. At various times, the reaction was stopped by filtration and the filters washed with 5 ml of 4% Rila solution. The filters were treated as described in "Substrate retention". Cells used for studies to determine the rate of arginine accumulation in the absence of glucose were cultured in GCAA without glucose.

Transport kinetics were determined under the conditions outlined by Rosen (62). One min uptake periods were chosen where initial rates were required (see Fig. 3). Substrate concentrations were adjusted so that no more than 10% of that added to the external medium was accumulated by the cells. Cell densities were used in a range over which substrate accumulation increased linearly with cell concentration.

The procedure of Reid *et al.* (59) was used to obtain kinetic constants \( K_m \) and \( V_{max} \) of the transport process. As will be shown subsequently (Fig. 7), the data yield a curvilinear plot when reciprocal
values are plotted according to Lineweaver and Burk (43). It is assumed, therefore, that such a curve results from the operation of 2-saturable components, each of which are described by the usual Michaelis-Menten expression:

\[ V_t = Y_1 + Y_2 = \frac{V_1 S}{K_1+S} + \frac{V_2 S}{K_2+S} \quad (1) \]

where \( Y_1 \) and \( Y_2 \) refer to the velocities of the 2 systems and \( V, K \) and \( S \) have their usual meanings. Initially, values for \( K_1, V_1, K_2 \) and \( V_2 \) were obtained by extrapolating the two linear portions of the plot in Figure 7. The above values were substituted in Equation 1 using arbitrary values for \( S \) and successively adjusted one at a time to obtain a set of values for \( V_t \) which most closely corresponded to the experimental values obtained for \( V_t \).

**Preparation of shock-releasable substrate-binding components**

Cells were cultured in GCAA in Fernbach flasks and in a Microferm fermentor (New Brunswick) to a cell density equivalent to an OD of 0.55. Cells were harvested with a refrigerated Sharples continuous-flow centrifuge, washed once in 4% Rila solution (50 ml/g wet cell paste), and suspended in the shock solutions (25 ml/g wet cell paste) according to the following flow diagram:
Following recovery of the cell-free shock supernatants, 2-mercaptoethanol and streptomycin sulfate were added at final concentrations of 1 mM and 34 µM, respectively. Shock supernatants 1-R and T-Mg were concentrated 100-fold by ultrafiltration using UM-10 membranes (Amicon Corp.). Shock supernatant S-E was saturated (100%) with ammonium sulfate and stored at 0°C overnight. The resulting precipitate was collected by centrifugation at 9150 x g, suspended in distilled water, and dialyzed against either phosphate buffer (pH 8.0) or 10 mM Tris-HCl.
buffer (pH 8.0). Both buffer solutions contained 1 mM 2-mercaptoethanol and 34 µM streptomycin sulfate. Concentrated shock supernatants, if not assayed immediately, were frozen in isopropanol-dry ice, lyophilized at -51 C, and stored at 0 C in a desiccator.

**Binding assay**

Binding assays were carried out in a multichamber equilibrium dialysis apparatus similar to that described by Furlong et al. (28). Prepared shock supernatant (100 µl) was added to one chamber while the corresponding buffer solution (100 µl) containing 14C-labelled amino acid was added to the opposite chamber. Unless otherwise indicated, 14C-UL-arginine and lysine were added to obtain an initial concentration of $4.5 \times 10^{-6}$ M in the buffer chamber. A dialysis membrane (Van Waters and Rogers Scientific), pretreated by steaming in an aqueous solution containing 5% sodium carbonate, separated the 2 chambers. The apparatus was connected to a Power-stir (Eberbach) and rotated for 6-12 h. Following equilibration, 10 µl portions were removed from each chamber and counted in scintillation vials containing 10 ml of Triton X-100 fluor (1 part Triton X-100: 2 parts double strength Omnifluor cocktail).

**Characterization of amino acid-binding components**

**Ammonium sulfate fractionation**

Increasing amounts of ammonium sulfate were added to concentrated or unconcentrated crude shock supernatant T-Mg. At ammonium sulfate saturation values of 20, 55 and 100%, precipitates were recover-
ed and treated as described in the previous section.

Fractionation by centrifugation

Concentrated shock supernatant fractions were fractionated by differential centrifugation. Cells remaining in the fractions were pelleted by centrifugation at 16,300 x g for an additional 20 min. Subsequent centrifugation of the supernatant at 106,000 x g for 60 min separated 'sedimentable material' from 'non-sedimentable material'. Cell envelope fragments were recovered from the shock supernatants by the method of Forsberg et al. (27). Cells were removed as before. Wall fragments were separated from 'non-sedimentable material' by centrifugation at 73,000 x g for 60 min. Centrifugations at 73,000 and 106,000 x g were conducted in a Beckman L2-65 ultracentrifuge with a Type 50 titanium rotor. All centrifugations were carried out at 4 C. The pellets were resuspended in the appropriate buffer prior to assaying for protein and binding activity.

Sephadex G-200 column chromatography

'Non-sedimentable material' (106,000 x g supernatant) was concentrated by ultrafiltration, dialyzed against Tris-HCl buffer (pH 8.0), and applied to a Sephadex G-200 column (200 ml bed volume). The material was eluted with the same buffer at a flow rate of 8 ml/h. Protein was monitored continuously at 280 nm. Fractions were collected, concentrated to a protein concentration of approximately 1 mg/ml, and assayed for binding activity in Tris-HCl buffer.
Ion-exchange chromatography

Celu Ion DEAE (Nutritional Biochemicals Corp.) was prepared and equilibrated with Tris-HCl buffer, pH 7.2. The column length-to-diameter ratio was usually 35:1. Buffer flow rates were controlled by a peristaltic pump at approximately 20 ml/h. Upon completion of column loading, the column was washed with 5 resin bed volumes to remove unadsorbed materials. Adsorbed materials were eluted with 200 ml step gradients of increasing sodium chloride concentrations from 0 to 0.25 M. Column effluent was continuously monitored for protein absorbance at 280 nm during loading, washing, and elution. Subsequent studies on various eluted fractions were carried out following concentration by ultrafiltration and dialysis against Tris-HCl buffer.

Preparative isoelectric focusing

Lyophilized Fraction 3 (100 mg) was reconstituted in distilled water and dialyzed against Tris-HCl buffer, pH 8.0. Carrier ampholines, pH 5-8 (LKB Products, Bromma 1, Sweden) were added to the protein solution, and the mixture was applied to a Sepharose support (18 x 2 x 0.5 cm) prepared according to the method of LaGow and Parkhurst (41). Approximately 0.1 ml of anode and cathode solutions (5% phosphoric acid and 5% ethylenediamine, respectively) were streaked across the gel surface immediately behind and parallel to platinum electrodes at each end of the gel. The gel was covered with wax paper and placed in a refrigerator at 4 C and connected to a Gelman DC power supply. Voltage was applied to obtain 1.2 W (300 V) initially. Following formation of the
pH gradient, the voltage was adjusted to approximately 1 W (400 V). After 12 h, the focussed gel was sliced into sections, diluted in 2 ml of distilled water, and the pH measured with a combination semi-micro electrode (Corning). The slurry was applied to a Sephadex G-25 column (0.5 x 20 cm) equilibrated with Tris-HCl buffer, pH 8.0. The focussed material was separated from the ampholines and running gel by eluting with equilibration buffer. The eluted material was concentrated by ultrafiltration and assayed for binding activity. In the case where Fraction 3 was preincubated with labelled arginine, 1 ml of 10 μCi/ml 14C-UL-arginine (12 μM) was added prior to addition of ampholines. The focussed sections were added directly to scintillation vials containing 15 ml of Triton X-100 cocktail and counted for radioactivity.

**Thin-section preparation**

Shocked cell suspensions were prepared according to the procedure described in "Viability". The cells were prefixed by adding 0.25 ml of 5% glutaraldehyde (Sigma Chemical Co.) to 5 ml of shocked cell suspension. The mixture was immediately centrifuged at 4190 x g for 10 min at 4 C. The cell pellet was resuspended to original volume in the respective shock solutions, each containing 5% glutaraldehyde. After 8 h fixation, at 0 C, the cells were washed 3 times in Kellenberger's Veronal-acetate-sodium chloride buffer, pH 6.1 (22). The cells were pelleted in 400 μl polyethylene micro-tubes (Hruden Laboratory Products, Ann Arbor, Mich.) at 1640 x g in an IEC Clinical centrifuge. The undisturbed pellet was then postfixed with 1% osmium tetroxide in Veronal-acetate-sodium chloride buffer (22) for 5 h at 0 C. Following
3 washes in buffer, the pellet was stained in Veronal-acetate-sodium chloride buffer containing uranyl magnesium acetate (0.5%). The pellet was dehydrated by a graded acetone series (30, 50, 70, 90 and 100%). Prior to the final dehydration step in 100% acetone, the pellet was carefully removed from the micro-tube and placed in a gelatin capsule. The pellet was infiltrated with a mixture (1:1) of Spurrs Embedding Medium (23) and 100% acetone. The pellet was transferred to Beem capsules (no. 00) and embedded in 100% Spurrs Embedding Medium for 8 h at 70 C. All steps prior to the final dehydration were carried out at 0 C. Room temperature was used thereafter.

Thin-sections were cut on a Porter Blum MT-1 ultramicrotome and placed on 300 μ naked copper grids. Grids were stained in 1% uranyl magnesium acetate and 0.2% lead citrate (73) prior to examination in a RCA EMU-3 electron microscope at an accelerating voltage of 50 kv.

The 73,000 x g pellet described in "Characterization of amino acid-binding components" was washed twice in shock solution T-Mg and suspended in 0.4 M potassium phosphate buffer (pH 7.6) containing 5% glutaraldehyde. Following fixation, glutaraldehyde was removed by 3 rinses in phosphate buffer and 2 rinses in Veronal-acetate-sodium chloride buffer. The pellet was postfixed with oxmium tetroxide, stained, dehydrated, and embedded as above.
RESULTS

Substrate retention

Transport studies on marine bacteria are complicated by the formation of several substrate pools during substrate uptake (30). Retention of these pools by the cells depends on the treatment used to terminate the transport reaction. Table 1 shows the effects of several different wash treatments on the retention of alanine and leucine by Ant-300.

Cells washed with an isotonic salt solution (4% Rila) following filtration retain the greatest amount of substrate. A 4% Rila solution contains the basic salt mixture of seawater with a salinity of 34 parts per thousand (°/oo).

Since acid treatment is routinely employed to terminate substrate uptake reactions in heterotrophic activity studies, it was of interest to determine the difference in amount of substrate accumulated by cells prior to acid treatment, and that which is retained after treatment. Cells washed with 1% Rila solution experience a rapid change in both external osmotic pressure and seawater ion concentration, a treatment comparable to the shock treatment used by Griffiths et al. (30) to remove loosely bound glutamic acid from V. marinus MP-1. It is apparent that both acid and 1% Rila cause the release of significant amounts of accumulated substrate.

A survey was subsequently conducted to determine whether loosely bound substrate accumulates during the transport of other amino acids.
TABLE 1. EFFECTS OF VARIOUS WASH TREATMENTS ON SUBSTRATE RETENTION

<table>
<thead>
<tr>
<th>Substrate</th>
<th>4% Rila</th>
<th>1% Rila</th>
<th>Acid</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alanine</td>
<td>59</td>
<td>45</td>
<td>28</td>
</tr>
<tr>
<td>Leucine</td>
<td>31</td>
<td>26</td>
<td>15</td>
</tr>
</tbody>
</table>

1 pmoles of substrate associated with the cells following a 10 min uptake period. Cells were cultured in GCAA, harvested, and washed in 4% Rila solution. Following starvation, the cells were exposed to $^{14}$C-labelled alanine or leucine. The reaction was stopped by either filtration, after which the cells were washed with 4% or 1% Rila solution, or acidification followed by filtration.
Column 1 of Table 2 presents the percentage of substrate accumulated by the cells during exposure to various amino acids that is subsequently retained following 1% Rila wash treatment. The percentages are based on the amount of substrate retained by cells washed with 4% Rila solution. The data indicate that the fraction of substrate associated with cells that is loosely bound, varies with each amino acid. For instance, leucine, aspartic acid, and lysine are not readily released by the wash treatment. In contrast, nearly all of the glutamic acid associated with cells seem to exist in the loosely bound state.

Cells exposed to 1% Rila solution also exhibit a reduced capacity to take up amino acids. Uptake in this case refers to the cell's ability to accumulate substrate. A comparison of the values in Columns 1 and 2 of Table 2 reveals that, in general, less substrate is detected in cells exposed to amino acids in the presence of 1% Rila than in the presence of 4% Rila. It is interesting that uptake and retention of aspartic acid are relatively unaffected by reduced salt concentration and osmolarity.

While it appears that reduced osmolarity and salt concentration inhibit substrate uptake in Ant-300, the actual lesion may exist at the level of substrate capture. Since substrate capture involves the initial binding of a substrate to the cell, a study was conducted to determine whether cells, previously suspended in 1% Rila solution, initially bind as much substrate as cells maintained in 4% Rila solution. As shown in Column 1 of Table 3, the binding mechanism(s) for the various amino acids is generally sensitive to a decrease in osmolarity and salt concentration. Binding of aspartic acid, like uptake and retention of
**TABLE 2. EFFECTS OF REDUCED OSMOLARITY AND SALT CONCENTRATION ON UPTAKE AND RETENTION OF VARIOUS SUBSTRATES**

<table>
<thead>
<tr>
<th>Substrate</th>
<th>%* substrate retained following wash (1% Rila)</th>
<th>%* activity remaining following uptake and wash (1% Rila)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alanine</td>
<td>74</td>
<td>7</td>
</tr>
<tr>
<td>Aspartic acid</td>
<td>82</td>
<td>80</td>
</tr>
<tr>
<td>Arginine</td>
<td>68</td>
<td>11</td>
</tr>
<tr>
<td>Glutamic acid</td>
<td>8</td>
<td>1</td>
</tr>
<tr>
<td>Leucine</td>
<td>85</td>
<td>18</td>
</tr>
<tr>
<td>Lysine</td>
<td>90</td>
<td>5</td>
</tr>
<tr>
<td>Methionine</td>
<td>54</td>
<td>40</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>49</td>
<td>4</td>
</tr>
<tr>
<td>Proline</td>
<td>50</td>
<td>16</td>
</tr>
<tr>
<td>Serine</td>
<td>70</td>
<td>30</td>
</tr>
<tr>
<td>Threonine</td>
<td>36</td>
<td>36</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>48</td>
<td>14</td>
</tr>
</tbody>
</table>

1 Starved cells were exposed to $^{14}$C-amino acids in the presence of 4% Rila solution for 10 min, filtered, and washed with 1% Rila solution.

2 Starved cells were exposed to $^{14}$C-amino acids, in the presence of 1% Rila solution for 10 min, filtered, and washed with 1% Rila solution.

* Percentages are based on the amount of substrate retained by cells exposed to substrate and washed in 4% Rila solution, which was arbitrarily assigned a value of 100.
TABLE 3. EFFECTS OF VARIOUS SHOCK TREATMENTS ON THE INITIAL BINDING OF SUBSTRATES TO CELLS

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Shock treatment 1-R</th>
<th>Shock treatment S-E</th>
<th>Shock treatment T-Mg</th>
</tr>
</thead>
<tbody>
<tr>
<td>Arginine</td>
<td>34</td>
<td>20</td>
<td>9</td>
</tr>
<tr>
<td>Aspartic acid</td>
<td>92</td>
<td>57</td>
<td>2</td>
</tr>
<tr>
<td>Glutamic acid</td>
<td>5</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>Lysine</td>
<td>42</td>
<td>20</td>
<td>8</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>29</td>
<td>-</td>
<td>2</td>
</tr>
<tr>
<td>Proline</td>
<td>-</td>
<td>76</td>
<td>3</td>
</tr>
</tbody>
</table>

1 Cells were shocked as described in Materials and Methods. Following shock treatment, cells were suspended in 4% Rila solution, exposed to substrate for 60 sec in the presence of chloramphenicol, then filtered and washed with 4% Rila.

2 The amount of substrate accumulated by shocked cells is presented as a percentage, based on the amount accumulated by unshocked cells, which was arbitrarily assigned a value of 100.
the amino acid, is minimally affected by the shock treatment. The same
treatment, however, nearly completely inhibits glutamic acid binding.
The binding mechanism(s) for the other amino acids tested is somewhat
less sensitive to the treatment than the mechanism mediating the binding
of glutamic acid.

The importance of seawater salts in substrate capture was exa-
mined in cells suspended in a sucrose solution of comparable osmolarity
as normal seawater (35 °/oo salinity). EDTA was added at 1 mM to che-
late traces of divalent cations. The data indicate that the temporary
complete removal of seawater salts (shock treatment S-E) causes irre-
versible inhibition of the binding of amino acids to cells (Column 2 of
Table 3). The charged amino acids were noticeably affected by this
treatment. If cells exposed to shock treatment S-E are subsequently
exposed to a decrease in osmotic pressure (shock treatment T-Mg), nearly
complete inhibition of binding of all amino acids is observed (Column
3 of Table 3).

The preceding results may be summarized as follows: 1) the ions
as well as the osmolarity of seawater are necessary for optimum binding
of amino acids to cells of Ant-300, 2) varying degrees of binding inter-
ference are imposed by alterations in salt concentration and osmolarity,
3) the binding of each amino acid is basically unique as demonstrated by
responses to salt concentration and osmolarity.

The effects of the shock treatments were also determined on
other properties of the cell. Table 4 shows the effect of shock treat-
ment on viability. Shock treatment 1-R causes no decrease in cell via-
bility. In fact, a slight increase in viability is observed. Shock
TABLE 4. VIABILITY OF SHOCKED CELLS

<table>
<thead>
<tr>
<th>Shock treatment</th>
<th>Viable cells/ml</th>
<th>% viability</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (4% Rila)</td>
<td>2.8 x 10^7</td>
<td>100</td>
</tr>
<tr>
<td>1-R</td>
<td>7.0 x 10^7</td>
<td>250</td>
</tr>
<tr>
<td>S-E</td>
<td>6.5 x 10^6</td>
<td>25</td>
</tr>
<tr>
<td>T-Mg</td>
<td>6.0 x 10^5</td>
<td>2</td>
</tr>
</tbody>
</table>

1 Shooked cells were prepared as described in Materials and Methods.

2 The fraction of the population remaining viable is presented as a percentage, based on the number of viable cells present in the 4% Rila solution, which is arbitrarily assigned a value of 100.
treatment S-E causes a 75% decrease in viability, thus corroborating the requirement for seawater salts by marine bacteria. The greatest loss in viability results from shock treatment T-Mg.

Ultrastructural changes resulting from the various shock treatments were examined by electron microscopy of thin-section preparations. Figure 1 shows a cross section of a normal cell. A double-track arrangement delineating the cytoplasmic (inner) and outer membrane is typical of most gram-negative bacterial cell envelopes. The periplasmic region comprises the electron transparent zone between the inner and outer membranes. Figure 2 shows a section of cells shocked in 1% Rila solution. As expected from the viability study, the cells appear intact. In addition, no abnormal features are associated with the cell envelope. Shock treatment S-E causes a separation and ballooning effect between the inner and outer membranes as shown in Figure 3. No significant breakage of the outer membrane is observed, however. Subsequent osmotic shock in the presence of low Mg$^{++}$ ion concentration (shock treatment T-Mg) results in breakage of the outer membrane as illustrated in Figure 4. Further examination reveals that the outer membrane fragments fold back on themselves, separate from the cells, and in some cases form closed vesicles (Fig. 4).

The amount of protein released from the cells by shock treatment is presented in Table 5. A negligible quantity is released by cells exposed to 1% Rila solution, confirming ultrastructural studies which indicate the cells remain intact. In spite of localized effects on the cell envelope, cells suspended in sucrose-EDTA appear to remain intact since only 3% of the cell protein is released into the shock solution.
Figure 1. Electron micrograph of a thin-section preparation of a normal cell.
Figure 2. Electron micrograph of a thin-section preparation of cells exposed to 1-R shock treatment.
Figure 3. Electron micrograph of a thin-section preparation of cells exposed to S–E shock treatment.
Figure 4. Electron micrograph of a thin-section preparation of cells exposed to T-Mg shock treatment.
TABLE 5. PROTEIN RELEASED FROM CELLS AS A RESULT OF SHOCK TREATMENT

<table>
<thead>
<tr>
<th>Shock treatment</th>
<th>% of total cell protein released</th>
</tr>
</thead>
<tbody>
<tr>
<td>1-R</td>
<td>&lt;1</td>
</tr>
<tr>
<td>S-E</td>
<td>3</td>
</tr>
<tr>
<td>T-Mg</td>
<td>27</td>
</tr>
</tbody>
</table>

Shocked cell preparations were prepared as in Table 4.
Those cells transferred from sucrose-EDTA to low concentrations of magnesium chloride in Tris-HCl buffer (shock solution T-Mg), however, release large quantities of cell protein into the shock solution, indicating there is damage to the cytoplasmic membrane as well as the outer membrane.

**Release of amino acid-binding components from shocked cells**

A shock treatment similar to T-Mg treatment has been shown to release periplasmic proteins from cells of *E. coli* (54). Some of these proteins are thought to participate in substrate capture (7). Since shock treatments S-E and T-Mg inhibit Ant-300's capacity to bind amino acids, produce observable changes in the periplasmic region of the cell envelope, and cause the release of cellular protein into the shock fluid, an investigation was conducted to determine whether substrate-binding components are released from shocked cells.

The supernatant fluid of shocked cell suspensions were assayed for glutamic acid, aspartic acid, arginine, and lysine-binding activity by equilibrium dialysis. The supernatant of 1% Rila shocked cell suspensions contain no detectable binding activity for any of the amino acids tested. Cells exposed to shock treatment S-E release arginine- and lysine-binding components into the shock fluid, as shown in Table 6. Gradients with ratios less than 1 indicate poor equilibration of the amino acid across the dialysis membrane. Ratios of 1.05 or greater are an indication of binding activity (80). Shock treatment T-Mg also causes the release of binding components for arginine and lysine from cells as shown in Table 7.
TABLE 6. BINDING OF AMINO ACIDS TO COMPONENTS RELEASED FROM CELLS BY S-E SHOCK TREATMENT

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Gradient</th>
<th>pmoles bound/ml</th>
<th>specific activity²</th>
</tr>
</thead>
<tbody>
<tr>
<td>Arginine</td>
<td>1.26</td>
<td>1052</td>
<td>568</td>
</tr>
<tr>
<td>Aspartic acid</td>
<td>0.92</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Glutamic acid</td>
<td>0.95</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Lysine</td>
<td>1.22</td>
<td>958</td>
<td>518</td>
</tr>
</tbody>
</table>

¹Crude shock supernatant (1.5 l) was recovered from 15 l of culture according to flow diagram 2 and saturated with ammonium sulfate. The resulting precipitate was suspended in distilled water and dialyzed against Tris-HCl buffer (pH 7.2). The reconstituted precipitate was assayed for amino acid-binding activity in the same buffer. ¹⁴C-UL-arginine and glutamic acid were added at an initial concentration of 4.5 and 5.3 μM, respectively (1.25 μCi/ml). ¹⁴C-UL lysine was added at an initial concentration of 7.2 μM (2.0 μCi/ml) and ¹⁴C-UL-aspartic acid was present at 12.0 μM (2.5 μCi/ml).

²Protein concentration was 1.86 mg/ml. Specific activity is expressed as units of binding activity/mg protein. One unit of binding activity is 1 pmoles arginine bound/ml.
TABLE 7. BINDING OF AMINO ACIDS TO COMPONENTS RELEASED FROM CELLS BY T-Mg SHOCK TREATMENT\(^1\)

<table>
<thead>
<tr>
<th>Substrate(^2)</th>
<th>Gradient</th>
<th>pmoles bound/ml</th>
<th>Specific activity(^3)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Arginine</td>
<td>1.42</td>
<td>1410</td>
<td>186</td>
</tr>
<tr>
<td>Aspartic acid</td>
<td>0.82</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Glutamic acid</td>
<td>0.85</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Lysine</td>
<td>1.29</td>
<td>1434</td>
<td>189</td>
</tr>
</tbody>
</table>

\(^1\)Crude shock supernatant (500 ml) was recovered from 11 l of culture according to flow diagram 2, concentrated by ultrafiltration to 51 ml, and dialyzed against Tris-HCl buffer (pH 7.2). Binding assays were carried out in the same buffer.

\(^2\)Substrate concentrations used are described in Table 6.

\(^3\)Protein concentration was 7.6 mg/ml.
Arginine transport

Three different mechanisms appear to mediate the transport of substrates in bacterial cells (38). One mechanism involves shock-releasable substrate-binding proteins. In view of the data indicating shock-releasable arginine-binding activity, it was of interest to characterize arginine transport in Ant-300.

Carbon starved cells retain considerable endogenous energy for the transport of arginine (Fig. 5). Cells readily take up arginine in the absence of a supplementary energy source. In fact, the addition of glucose, which is metabolized by Ant-300 (29), causes no significant stimulation of arginine uptake.

A steady state level of arginine is not attained during arginine uptake, indicating that the substrate is rapidly metabolized by the cells (Fig. 5). The addition of aminooxy acetic acid (AOA), an effective competitor for the enzyme arginine decarboxylase in *E. coli* (79), has no effect on the accumulation of arginine in Ant-300 (not shown). The rapid metabolism of arginine in *E. coli* appears to affect only the steady state level of arginine in the cell and not the initial rate of arginine transport (79). It will be assumed, therefore, that arginine uptake in Figure 5 represents the initial rate of arginine transport in Ant-300.

In *E. coli*, shock sensitive transport systems are inhibited by arsenate, which depletes cells of ATP and other high energy compounds, whereas shock resistant transport systems are inhibited by the ionophore, 2-4 dinitrophenol (DNP), which dissipates a membrane potential as well as uncouples oxidative phosphorylation (13). The following study was
Figure 5. Uptake of L-arginine. Cells starved in 4% Rila solution for 18 h were exposed to $^{14}$C-UL-arginine (0.07 μM), •; and in the presence of glucose (14 mM), ★; for increased periods of time.
conducted to determine the form of energy required for arginine accumulation in Ant-300. Figure 6 illustrates the effects of the metabolic inhibitors DNP, sodium arsenate, sodium amytal, and potassium cyanide on arginine uptake. Neither DNP nor sodium arsenate significantly inhibit the uptake reaction. The electron transport inhibitors, amytal and cyanide, cause the greatest inhibition of transport.

The determination of the affinity constant, $K_m$, and the maximum rate of substrate transport, $V_{max}$, are based on a linear relationship between accumulation rate and external substrate concentration. The Lineweaver-Burk plot illustrated in Figure 7, however, describes a biphasic slope over a range of arginine concentrations from 0.035-0.70 μM. These results suggest that over this concentration range, two saturable components mediate arginine uptake; one exhibiting a $K_m$ of $1.5 \times 10^{-8}$ M and a $V_{max}$ of 12 pmoles/min/5 x $10^7$ cells, and another having a $K_m$ of $4.5 \times 10^{-7}$ M and a $V_{max}$ of 30 pmoles/min/5 x $10^7$ cells. Thus, arginine uptake in Ant-300 occurs through two high affinity systems.

Competition studies were carried out to determine the specificity of the arginine uptake systems of Ant-300. Table 8 shows the inhibition of arginine uptake by L-lysine and L-ornithine at two different arginine concentrations. The degree of inhibition is greater at an arginine concentration of 0.035 μM than at 0.35 μM. The varying degree of inhibition at different arginine concentrations supports the contention that two transport systems participate in arginine uptake; the lower affinity system exhibiting a higher specificity for arginine than the higher affinity system. Both systems, however, appear to be rela-
Figure 6. Effects of metabolic inhibitors on L-arginine uptake. Cells were treated as in "Materials and Methods". DNP (2 mM), □; sodium arsenate (200 μM), ○; potassium cyanide (20 mM), ★; or sodium amytal (10 mM), ▼; was added to the reaction mixture 5 min prior to the addition of ¹⁴C-labelled arginine. A control was included in which cells were exposed to arginine in the absence of inhibitor, ●.
pmoles accumulated / 5 x 10^7 cells
Figure 7. Double reciprocal plots of initial rates of transport at various extracellular arginine concentrations. Uptake was carried out as described in "Materials and Methods". The points represent the experimental data. The $K_m$ and $V_{max}$ were calculated from substituting experimental data in Equation 1.
$V$ (pmoles / min / $5 \times 10^7$ cells)


<table>
<thead>
<tr>
<th>Arginine concentration</th>
<th>Competitor</th>
<th>pmoles transported&lt;sup&gt;2&lt;/sup&gt;</th>
<th>% inhibition&lt;sup&gt;3&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.035 μM</td>
<td>none</td>
<td>10.8</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>L-lysine (0.72 μM)</td>
<td>8.4</td>
<td>23</td>
</tr>
<tr>
<td></td>
<td>L-ornithine (0.72 μM)</td>
<td>7.2</td>
<td>34</td>
</tr>
<tr>
<td>0.36 μM</td>
<td>none</td>
<td>20.5</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>L-lysine (7.2 μM)</td>
<td>18.5</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>L-ornithine (7.2 μM)</td>
<td>16.2</td>
<td>21</td>
</tr>
</tbody>
</table>

<sup>1</sup>Cell suspensions were prepared as in Figure 5. Immediately upon addition of labelled arginine, unlabelled L-lysine or L-ornithine was added at the final concentrations indicated in parenthesis. The reaction mixture was treated as in Figure 3.

<sup>2</sup>pmoles transported per 5 x 10<sup>7</sup> cells.

<sup>3</sup>Percent inhibition is based on the amount of arginine accumulated in the absence of competitors which is arbitrarily assigned a value of 0.
tively specific for arginine in that a 20-fold molar excess of lysine or ornithine produces, at best, only 34% inhibition of arginine accumulation.

Chemotaxis

The involvement of chemotaxis in the capture of arginine was studied in Ant-300. During rapid growth, cells appear non-motile. Starvation in 4% Rila solution, however, seems to induce cell motility. The number of motile cells in a population increases over a 72 h starvation period. After longer periods of starvation, there is a decrease in the number of motile cells.

Non-motile cells taken directly from a logarithmic phase culture do not actively migrate towards arginine after a 1 h period of exposure to the attractant. Arginine does, however, elicit a chemotactic response from cells starved for 72 h (Fig. 8). As shown in Figure 9, the arginine concentration at which the maximum response to attractant occurs is at $10^{-4}$ M. The 'threshold concentration', the lowest concentration of attractant that gives an accumulation in the capillary greater than that obtained in the absence of attractant, is between $10^{-5}$ and $10^{-6}$ M.

Characterization of components binding arginine and lysine

Shock supernatant T-Mg was fractionated by ammonium sulfate precipitation. When assayed in phosphate buffer, arginine-binding activity is observed in the 20-55% (Fraction 2) and the 55-100% (Fraction 3) ammonium sulfate fractions, the latter fraction displaying the highest
Figure 8. Time course of entry of cells into capillaries containing $10^{-4}$ M arginine, o; and no attractant, •.
number of cells in capillary ($\times 10^3$)

min

0 15 30 45 60 90 120

0 4 8 12 16
Figure 9. Concentration-response curve for L-arginine.
Cells were exposed to attractant at 5 C for 1 h.
arginine concentration in capillary (M)

number of cells in capillary ($\times 10^3$)
specific activity (Table 9). Lysine-binding activity is observed in the 0-20% ammonium sulfate fraction (Fraction 1) as well as in Fractions 2 and 3.

Binding of arginine and lysine by the various ammonium sulfate fractions was also determined in the presence of Tris-HCl buffer. As shown in Table 10, binding activity for both amino acids occurs in all 3 fractions. It is also apparent that greater arginine- and lysine-binding activities are obtained in Tris-HCl buffer than in phosphate buffer.

Fraction 3 was further fractionated by differential centrifugation. As shown in Table 11, arginine-binding activity is displaced from the supernatant fraction with increased gravitational force. Binding activity appears to be associated with material sedimenting between 35,000 and 106,000 x g, as well as with large material sedimenting at 16,000 and 35,000 x g. The bulk of the binding activity, however, remains in solution as forces as great as 106,000 x g.

Membrane fragments released from cells by T-Mg shock treatment were isolated by the method of Forsberg et al. (27). Arginine-binding activity is associated with material sedimenting between 35,000 and 73,000 x g (Table 12). Thin-sections prepared from the 73,000 x g pellet reveal the presence of membrane fragments and vesicles (Fig. 10). Thus, arginine-binding activity appears to be associated with membrane fragments, presumably the outer double-track layer which separates from the cells during shock treatment as shown in Figure 4. The major component(s) binding arginine, however, are "non sedimentable" by centrifugation.
TABLE 9. ARGinine- AND LYSINE-BINDING ACTIVITIES IN VARIOUS AMMONIUM SULFATE FRACTIONS OF T-Mg SHOCK SUPERNATANT (PHOSPHATE BUFFER)\textsuperscript{1}

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Protein (mg/ml)</th>
<th>Arginine, pmoles bound/ml</th>
<th>Lysine, pmoles bound/ml</th>
<th>Specific Activity Arginine</th>
<th>Specific Activity Lysine</th>
</tr>
</thead>
<tbody>
<tr>
<td>0-20%</td>
<td>NM\textsuperscript{2}</td>
<td>0</td>
<td>100</td>
<td>NM</td>
<td>NM</td>
</tr>
<tr>
<td>20-55%</td>
<td>10.0</td>
<td>110</td>
<td>282</td>
<td>11</td>
<td>28</td>
</tr>
<tr>
<td>55-100%</td>
<td>5.9</td>
<td>387</td>
<td>271</td>
<td>66</td>
<td>46</td>
</tr>
</tbody>
</table>

\textsuperscript{1}Crude T-Mg shock supernatant (1:1) was recovered from shocked cell suspensions according to flow diagram 2, concentrated to 96 ml by ultrafiltration, and fractionated by ammonium sulfate precipitation. Precipitates were suspended in distilled water and dialyzed against 0.05 M potassium phosphate buffer (pH 7.6). Binding assays were carried out in the same buffer.

\textsuperscript{2}Not measured.
<table>
<thead>
<tr>
<th>Fraction</th>
<th>Protein (mg/ml)</th>
<th>pmoles bound/ml</th>
<th>Specific activity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Arginine</td>
<td>Lysine</td>
</tr>
<tr>
<td>0–20%</td>
<td>3.7</td>
<td>337</td>
<td>121</td>
</tr>
<tr>
<td>20–55%</td>
<td>7.5</td>
<td>637</td>
<td>503</td>
</tr>
<tr>
<td>55–100%</td>
<td>5.8</td>
<td>534</td>
<td>1137</td>
</tr>
</tbody>
</table>

1 Crude T-Mg shock supernatant (600 ml) was recovered from shocked cell suspensions according to flow diagram 2. Precipitates were suspended in distilled water and dialyzed against Tris-HCl buffer (pH 8.0). Binding assays were carried out in the same buffer.
### TABLE 11. DIFFERENTIAL CENTRIFUGATION OF FRACTION 3: ARGININE-BINDING ACTIVITY IN VARIOUS FRACTIONS

<table>
<thead>
<tr>
<th>Fraction</th>
<th>pmoles bound/ml</th>
<th>specific activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>crude Fraction 3</td>
<td>1830</td>
<td></td>
</tr>
<tr>
<td>16,000 x g supernatant</td>
<td>1480</td>
<td></td>
</tr>
<tr>
<td>35,000 x g supernatant</td>
<td>1210</td>
<td></td>
</tr>
<tr>
<td>106,000 x g supernatant</td>
<td>1120</td>
<td>280</td>
</tr>
<tr>
<td>106,000 x g pellet</td>
<td>1180</td>
<td>98</td>
</tr>
</tbody>
</table>

Lyophilized Fraction 3 (97 mg) was reconstituted in 11 ml of distilled water and dialyzed against Tris-HCl buffer, pH 8.0. Following centrifugation, 0.1 ml of supernatant was assayed for arginine-binding activity in the same buffer. The 106,000 x g pellet was suspended in buffer prior to assaying for binding activity.
### TABLE 12. DIFFERENTIAL CENTRIFUGATION OF CRUDE T-Mg SHOCK SUPERNATANT: ARGinine-BINDING ACTIVITY IN VARIOUS FRACTIONS

<table>
<thead>
<tr>
<th>Fraction</th>
<th>pmoles/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>16,000 x g</td>
<td></td>
</tr>
<tr>
<td>supernatant</td>
<td>365</td>
</tr>
<tr>
<td>pellet</td>
<td>208</td>
</tr>
<tr>
<td>35,000 x g</td>
<td></td>
</tr>
<tr>
<td>supernatant</td>
<td>389</td>
</tr>
<tr>
<td>pellet</td>
<td>182</td>
</tr>
<tr>
<td>73,000 x g</td>
<td></td>
</tr>
<tr>
<td>supernatant</td>
<td>312</td>
</tr>
<tr>
<td>pellet</td>
<td>499</td>
</tr>
</tbody>
</table>

1Lyophilized T-Mg shock supernatant (50 mg) was reconstituted in 10 ml of distilled water and dialyzed against phosphate buffer. Following centrifugation, the pellet was suspended in 0.5 ml of buffer. Supernatants and resuspended pellets were assayed for arginine-binding activity using an initial concentration of 9 μM 14C-UL-arginine (279 mCi/m mole) in the buffer chamber.
Figure 10. Electron micrograph of a thin-section preparation of membrane fragments recovered from the shock fluid following exposure of cells to T-Mg shock treatment.
The supernatant fraction obtained from the 106,000 x g centrifugation step, described above, was applied to a Sephadex G-200 column to determine the nature and size of the non-sedimentable component(s) binding arginine and lysine. Figure 11 shows that both arginine- and lysine-binding activity follow the 280 nm peak upon elution with Tris-HCl buffer. The broad peaks of binding activity, however, make molecular weight estimations difficult.

If binding of arginine and lysine to components in the shock supernatants are non-specific, and depend mainly on the net charge of the various proteins present, then binding activity should vary with hydrogen ion concentration. Figure 12 demonstrates that between pH 7.3 and 7.9, arginine-binding activity in Fraction 3 is relatively constant. However, binding activity decreases with pH below pH 7.3 and increases with pH above pH 7.9.

Fraction 3 was further characterized by preparative isoelectric focussing on Sepharose gels (41). A large fraction of the material applied to the gel migrates and precipitates between pH 5.1 and 6.0. However, no arginine-binding activity is associated with the material precipitating in this pH range. Likewise, preincubation of Fraction 3 with \(^{14}\)C-labelled arginine prior to isoelectric focussing yields no significant amounts of radioactivity associated with the focussed material.

Fraction 3 was applied to a DEAE anion exchange column equilibrated with Tris-HCl buffer and eluted with a step gradient of increasing sodium chloride concentration. Table 13 displays the binding activities associated with the various fractions. Arginine binding acti-
Figure 11. Elution profile of Fraction 3 from Sephadex G-200. The 106,000 x g supernatant (81 mg protein in 6 ml) was dialyzed against Tris-HCl buffer, pH 8.0 and applied to a Sephadex G-200 column (2.5 x 35 cm; bed volume = 200 ml). The protein was eluted and separated into 6 fractions. Each fraction was concentrated by ultrafiltration, then assayed for protein and binding activity. pmoles arginine bound/ml, •; pmoles of lysine bound/ml, ■; specific activity of arginine-binding component, ○; specific activity of lysine-binding component, □. Binding assays were carried out in Tris-HCl buffer. Solid line indicates absorbance at 280 nm.
absorbance (280 nm)

[Graph showing absorbance and specific activity over fraction numbers 1 to 6.]
Figure 12. Effect of hydrogen ion concentration on arginine-binding activity. Two mg amounts of lyophilized Fraction 3 were reconstituted in distilled water to 0.4 ml volumes and dialyzed against Tris-HCl buffer solutions ranging in pH from 6.3 to 8.7. Binding assays were carried out in the same buffer at the respective hydrogen ion concentration. The binding ratio, $\bullet$, was determined as follows: DPM in protein chamber/DPM in buffer chamber. Arginine-binding activity, $\circ$. 
TABLE 13. BINDING ACTIVITY IN FRACTIONS ELUTED FROM A DEAE COLUMN

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Amino acid</th>
<th>Activity</th>
<th>Ratio</th>
<th>Specific activity</th>
<th>Protein (mg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fraction 3</td>
<td>Arginine</td>
<td>1810</td>
<td>1.54</td>
<td>430</td>
<td>4.2</td>
</tr>
<tr>
<td></td>
<td>Lysine</td>
<td>2160</td>
<td>1.40</td>
<td>515</td>
<td></td>
</tr>
<tr>
<td>Wash</td>
<td>Arginine</td>
<td>938</td>
<td>1.25</td>
<td>198</td>
<td>4.6</td>
</tr>
<tr>
<td></td>
<td>Lysine</td>
<td>847</td>
<td>1.14</td>
<td>157</td>
<td></td>
</tr>
<tr>
<td>0.05 M NaCl</td>
<td>Arginine</td>
<td>197</td>
<td>1.05</td>
<td>246</td>
<td>0.83</td>
</tr>
<tr>
<td></td>
<td>Lysine</td>
<td>33</td>
<td>1.01</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>0.10 M NaCl</td>
<td>Arginine</td>
<td>38</td>
<td>1.01</td>
<td>-</td>
<td>0.45</td>
</tr>
<tr>
<td></td>
<td>Lysine</td>
<td>54</td>
<td>1.01</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>0.15 M NaCl</td>
<td>Arginine</td>
<td>23</td>
<td>1.01</td>
<td>-</td>
<td>0.54</td>
</tr>
<tr>
<td></td>
<td>Lysine</td>
<td>0</td>
<td>0.99</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>0.20 M NaCl</td>
<td>Arginine</td>
<td>177</td>
<td>1.05</td>
<td>131</td>
<td>1.35</td>
</tr>
<tr>
<td></td>
<td>Lysine</td>
<td>151</td>
<td>1.03</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>0.25 M NaCl</td>
<td>Arginine</td>
<td>129</td>
<td>1.03</td>
<td>-</td>
<td>0.65</td>
</tr>
<tr>
<td></td>
<td>Lysine</td>
<td>102</td>
<td>1.02</td>
<td>-</td>
<td></td>
</tr>
</tbody>
</table>

1. 0.6 g of lyophilized Fraction 3 was reconstituted with distilled water to 26 ml (109 mg protein) and dialyzed against Tris-HCl buffer, pH 7.2. The dialyzed protein solution was applied to a DEAE cellulose column (bed volume = 35 ml) and washed with several bed volumes of Tris-HCl buffer. Fractions were eluted with a step gradient of increasing sodium chloride concentration. The various fractions were concentrated to approximately 7 ml by ultrafiltration and dialyzed against Tris-HCl buffer. The salt-free fractions were then assayed for protein and binding activity.

2. pmoles bound/ml.

3. See Figure 12.

4. Specific activities were only calculated for binding ratios of 1.05 or greater.
vity is associated with the 0.05 M, 0.20 M and 0.25 M sodium chloride fractions. Components binding lysine are also present in the latter 2 fractions. Yields of 30 and 15% for arginine- and lysine-binding activities, respectively, were obtained from the column. The unbound fraction contains the bulk of the binding activity for both arginine and lysine.

Shock supernatant S-E also contains a component that elutes from a DEAE column at a sodium chloride concentration between 0.20 - 0.25 M that binds both arginine and lysine (not shown).

The fraction eluted from the DEAE column by 0.05 M NaCl was further characterized with respect to arginine-binding activity. Table 14 shows the effects of arginine concentration on binding activity. In general, the amount of ligand bound increases with arginine concentration. However, the ratio of bound to unbound substrate remains relatively constant.

Ion concentration also affects ligand-protein binding. The data in Table 15 indicate that optimum arginine binding in the 0.05 M NaCl fraction occurs in the presence of 0.01 M NaCl. Sodium chloride concentrations greater than 0.01 M completely inhibit binding. Arginine binding in the other fractions eluted from the DEAE column, including the active fraction of shock supernatant S-E, was inhibited by 0.1 M sodium chloride.
TABLE 14. EFFECTS OF ARGinine CONCENTRATION ON BINDING ACTIVITY

<table>
<thead>
<tr>
<th>Arginine concentration</th>
<th>Activity²</th>
<th>Ratio³</th>
</tr>
</thead>
<tbody>
<tr>
<td>$4.6 \times 10^{-6}$ M</td>
<td>63</td>
<td>1.04</td>
</tr>
<tr>
<td>$9.0 \times 10^{-6}$ M</td>
<td>101</td>
<td>1.03</td>
</tr>
<tr>
<td>$1.8 \times 10^{-5}$ M</td>
<td>122</td>
<td>1.02</td>
</tr>
<tr>
<td>$4.6 \times 10^{-5}$ M</td>
<td>272</td>
<td>1.02</td>
</tr>
</tbody>
</table>

¹The fraction eluted from the DEAE column by 0.05 M NaCl was concentrated by ultrafiltration, dialyzed against Tris-HCl buffer, pH 7.2, then exposed to a range of arginine concentrations. Binding activity was determined in Tris-HCl buffer, pH 7.2.

²See Table 13.

³See Figure 12.
TABLE 15. EFFECTS OF ION CONCENTRATION ON BINDING ACTIVITY

<table>
<thead>
<tr>
<th>Sodium chloride concentration</th>
<th>Activity</th>
<th>Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>141</td>
<td>1.04</td>
</tr>
<tr>
<td>0.010 M</td>
<td>267</td>
<td>1.07</td>
</tr>
<tr>
<td>0.025 M</td>
<td>0</td>
<td>0.99</td>
</tr>
<tr>
<td>0.050 M</td>
<td>15</td>
<td>1.00</td>
</tr>
<tr>
<td>0.075 M</td>
<td>4</td>
<td>1.00</td>
</tr>
<tr>
<td>0.100 M</td>
<td>47</td>
<td>1.01</td>
</tr>
</tbody>
</table>

1The fraction eluted from the DEAE column by 0.05 M NaCl was assayed for arginine-binding activity in the presence of varying concentrations of NaCl. 10 λ of a concentrated NaCl solution was added to each side of the dialysis chamber containing the buffer and protein solution to obtain the appropriate NaCl concentration in a final volume of 100 λ.
Amino acid uptake by ANT-300 appears to involve the formation of a loosely bound amino acid pool that is sensitive to reduced osmolarity and salt concentration (shock treatment 1-R). The proportion of substrate accumulated by cells, that exists in the loosely bound state, depends upon the amino acid in question. Essentially all of the glutamic acid accumulated over a 10 min period appears loosely bound. Strikingly similar observations were made with regard to glutamic acid pools formed during glutamic acid uptake in V. marinus (R.P. Griffiths, personal communication).

Besides releasing loosely bound substrate, shock treatment 1-R interferes with the initial binding (1 min exposure period) and accumulation (10 min exposure period) of substrates in Ant-300. It is quite possible, therefore, that the cellular mechanism(s) responsible for the two latter functions is closely coupled to, if not identical to, the mechanism(s) retaining the loosely bound substrate pool.

Since the initial binding, accumulation, and retention of various amino acids respond differently to changes in salt concentration and osmolarity, it is likely that the cell employs more than one mechanism to sequester substrates. For instance, aspartic acid and glutamic acid, although similar in structure, appear to be accumulated by different mechanisms; one which is sensitive and another which is relatively resistant to reduced salt concentration and osmolarity.

Kepes (38) has proposed that several basically different systems mediate the active transport of substrates in bacteria. In E. coli,
glutamic acid, aspartic acid, lysine, and arginine are transported by systems involving shock-releasable substrate-binding proteins (62, 63, 77), while proline is transported by a system involving a membrane-bound permease (39). Presumably, the different associations that these transport components retain with regard to the cell envelope determine their response to shock treatment.

The influence of salt concentration and osmolarity on the initial binding of substrates by Ant-300 may provide a basis for distinguishing different classes of capture mechanisms. The initial binding of the charged amino acids arginine, lysine, and glutamic acid are quite dependent upon the presence of seawater ions (Column 2, Table 3). This does not appear to be the case with regard to the initial binding of proline. The binding of proline is, however, sensitive to rapid changes in osmotic pressure (Column 3, Table 3).

Salt-sensitive binding components may be more exposed to the external environment than binding components which function in the absence of seawater salts. The former could conceivably retain a periplasmic location similar to the shock-sensitive binding proteins in E. coli. Periplasmically located binding components in Ant-300 may, therefore, require a stable ionic environment for structural and functional stability.

Binding components which function in the absence of seawater salts, such as those binding proline, may be associated with membranous structures. Components stabilized by the membrane might be more sensitive to changes in osmotic pressure than to changes in specific ion concentrations.
Due to their osmotic and salt requirements, marine bacteria such as Ant-300 cannot tolerate the shock treatments developed to selectively release periplasmically-located, substrate-binding proteins from *E. coli* and other enteric bacteria. Sequential suspension of Ant-300 cells in shock solutions S-E and T-Mg, a treatment similar to that developed by Neu and Heppel (53) which causes no significant envelope damage or loss of viability in *E. coli*, causes disruption of the cell envelope and loss of viability in Ant-300. Selective release of substrate-binding proteins from Ant-300, therefore, cannot be accomplished by exposure to Neu and Heppel shock treatment.

The physiological effects experienced by Ant-300 from shock treatment S-E most closely resemble those reported for *E. coli* following Neu and Heppel shock treatment. Cells of Ant-300 release approximately 3% of their protein and, although their envelopes are distorted by this treatment, 25% of the cells remain viable. Also, as mentioned above, the ion effects on the initial accumulation of arginine, lysine, glutamic acid, and proline in Ant-300 are similar to the osmotic effects on the initial rate of transport of the same amino acids in *E. coli* produced by Neu and Heppel shock treatment.

Both shock treatments S-E and T-Mg cause the release of components from Ant-300 that bind arginine and lysine. These components, however, do not resemble the shock-releasable substrate-binding proteins believed to participate in the translocation of compounds across the cytoplasmic membrane of *E. coli* and other Gram-negative bacteria. Instead, they appear to be proteins which, for the most part, interact in
in a "non-specific" manner with basic amino acids. The following data support this idea. 1) Components binding arginine and lysine precipitate over a wide range of ammonium sulfate concentrations. 2) Binding activity is associated with components exhibiting a wide distribution of sizes as judged by elution from a Sephadex G-200 column. 3) Components displaying a low degree of binding elute from an anion exchange column over a relatively wide range of sodium chloride concentrations.

The following data indicate that charge interactions may be responsible for the observed binding between arginine and cellular protein. 1) Except between pH 7-8, arginine-binding activity increases with pH. As a rule, substrate binding proteins are relatively stable over a wide pH range (64). It is likely, therefore, that as the proteins released from Ant-300 become more negatively charged (with increasing pH), their affinity for arginine increases. 2) A significant fraction of the components released from Ant-300 by shock treatment possess a pI between pH 5.1 and 6.0, indicating that many of the components exhibit a net negative charge under normal assay conditions. 3) Arginine-binding activity is sensitive to ionic strength. Although 0.01 M sodium chloride enhances binding activity of the arginine-specific-binding fraction eluted from the DEAE column, slightly higher concentrations completely inhibit activity. Other fractions displaying arginine- and lysine-binding activities in the absence of sodium chloride, also lose activity upon addition of the salt.

The fact that lysine-binding activity is concommitantly found in nearly all fractions displaying arginine-binding activity further sup-
ports the contention that binding is "non-specific". The possibility that arginine and lysine share a common transport-related binding protein does not seem likely in view of the minimal interference by lysine on the initial rate of arginine accumulation, shown in Table 8. A common binding protein (LAO protein) has been demonstrated for arginine, lysine, and ornithine in E. coli which exhibits a pI of 5.1 (62). The periplasmically located LAO protein, however, does not appear to participate in transport. Electrostatic interactions between the negatively charged protein and the basic amino acids may be responsible for the observed binding activity. Those fractions recovered from the supernatant of S-E and T-Mg shocked cell suspensions may contain proteins similar in nature to the LAO protein. Assuming then that these proteins are located in the cell envelope, it is quite possible that substrates are accumulated near the cell surface and not necessarily transported across the membrane.

The components binding arginine recovered from the various DEAE fractions are quite sensitive to sodium chloride. The arginine-specific binding component is inactivated at sodium chloride concentrations greater than 0.01 M. The sodium chloride concentration in seawater, however, is approximately 0.3 M. Thus, if arginine-binding components exist in the cell envelope and participate in scavenging arginine from seawater, then the bacterial cell envelope must provide protection against salt inactivation. Envelope components isolated from other marine bacteria also display sensitivity to specific ions. The optimum sodium concentration for L-alanine uptake in isolated membrane vesicles of a marine
pseudomonad is 0.075 M, whereas, in whole cells the optimum concentration for uptake is 0.2 M (70).

The loss of arginine-binding activity following isoelectric focussing may be due to precipitation of binding components at low pH. Early attempts to isolate substrate-binding proteins by the method of Willis et al. (76) involved precipitating crude shock fluid at pH 4.5 prior to applying to a cation exchange column. Binding activities for arginine and lysine were lost during this step, also. Apparently, precipitation at low pH irreversibly destroys arginine-binding capacity of the shock releasable components. Isoelectric focussing also seems to cause dissociation of ligand-component binding since shock fluid fractions, preincubated with labelled arginine do not retain the radioactivity in the subsequently focussed bands. It is possible that the ampholines used to establish the pH gradient interfere with amino acid-protein binding.

Arginine accumulation in Ant-300 exhibits some features of transport systems involving binding proteins. One similarity is that accumulation proceeds via high affinity components. Ant-300 also exhibits chemotaxis towards arginine. As mentioned before, some chemoreceptors in E. coli have been identified as corresponding binding proteins. If a similar mechanism exists in Ant-300, then the chemoreceptors may also function as binding proteins.

The synthesis of arginine-binding proteins in Ant-300 may depend on the culture conditions. Motility, for instance, is not observed in rapidly growing cells. But, upon suspension in 4% Rila solution lacking an organic carbon source, cells develop the capacity to migrate toward
and into a capillary containing arginine. Synthesis of chemoreceptors, which as mentioned above, can correspond to substrate-binding proteins, may also be induced under these conditions. Rahmanian et al. (58), in fact, found that the production of the LIV protein in E. coli is repressed by the presence of leucine in the growth medium. Thus, it may be necessary to culture the cells in the absence of certain nutrients or use starved cells to investigate the importance of substrate binding proteins in substrate capture.

The response of ANT-300 to metabolic inhibitors appears unique. Neither arsenate at concentrations which inhibit shock sensitive transport systems nor DNP at concentrations which inhibit shock resistant systems (13) has a significant effect on the rate of arginine accumulation. It is possible that these inhibitors are not able to penetrate to their respective sites of action within the cell. If this is the case, ANT-300 may retain sufficient amounts of ATP to drive arginine transport during exposure to arsenate which normally dissipates intracellular pools of high energy compounds (13). Similarly, exogenously supplied DNP may not interact with the energized "common factor" which is thought to be an intermediate between respiration and transport, and respiration and ATP production (31). An investigation is currently in progress to determine whether arsenate and DNP treated cells are depleted of intracellular ATP. Additional studies may be required to determine whether ATP is necessary for arginine transport in ANT-300.

The inhibitory effects of amytal and cyanide on arginine accumulation in ANT-300 indicate that arginine transport is, to some extent, coupled to electron transport. Fan et al. (25) also found that an argi-
nine-specific transport system of *Pseudomonas putida* is inhibited by cyanide. Correspondingly, Sprott and MacLeod (68) found that transport of L-alanine and α-aminobutyric acid in membrane vesicles of a marine pseudomonad are stimulated by electron donors but not by ATP. Whether electron donors also stimulate arginine transport in ANT-300 remains to be determined. It is interesting, though, that a significant amount of arginine accumulates in the presence of electron transport inhibitors.

Inhibitor-resistant arginine accumulation may correspond to that which is complexed to binding proteins at the surface of the cell. The fraction of arginine taken up by cells that is released by 1% Rila shock (32%) is, in fact, similar to the fraction of total substrate accumulated that is resistant to amytal (32%), the most effective transport inhibitor. Chromatographic analyses are currently in progress to determine the labelled compounds released from cells by 1% Rila shock treatment, as well as that which accumulates in cells in the presence of electron transport inhibitors. If these studies reveal that the label is retained as arginine in both cases, and that electron transport is the sole driving force for arginine accumulation, then these findings would support the idea that cells have the capacity to accumulate arginine near the cell surface without the input of metabolic energy. Binding proteins are likely to be the cellular components which mediate the surface accumulation of substrates. Just how these substrates are transported once they are bound to the cell surface, remains to be determined.

The efficient mineralization of organic matter in the marine environment must depend to a large extent on the scavenging ability of the indigenous heterotrophic population. Bacteria probably rely on a combi-
nation of mechanisms to capture the various substrates. The high affinity arginine capture component in Ant-300 represents one method by which bacteria are able to accumulate a substrate against a large concentration gradient. The cells may retain similar or quite different mechanisms to concentrate other amino acids. Although free amino acid concentrations in the ocean are, at present, difficult to determine, Clark et al. (18) estimate that the free arginine concentration ranges from $2\cdot10^{-8}$ M in California coastal waters. Such concentrations are within the functional range of ANT-300's high affinity capture system.

Chemotaxis enables marine bacteria like ANT-300 to actively migrate toward areas in which potential nutrients are concentrated. Although the concentrations of dissolved free attractants in the open ocean are less than the 'threshold' concentrations necessary to elicit a chemotactic response, chemotaxis may be important in directing bacteria to suspended particulate matter which has been eluded to have a concentra-
tive effect on nutrients in seawater (10, 60, 61). It is known that a wide range of microbial activities are stimulated in the presence of ag-
gregates. The high concentrations of ornithine that have been found associ-
ed with particulate matter (24) indicates that this is a site of arginine degradation.

A third characteristic displayed by ANT-300 is the ability to accumulate quantities of substrates under starvation conditions. Unlike many starved cells, ANT-300 retains the capacity to transport and accumu-
late arginine without a supplementary energy source, a characteristic which may be important in the marine environment. Coupling electron transport to the transport of substrates like arginine in Ant-300 may be
the most efficient means of obtaining transport energy in an oxidative environment as the open ocean. Future investigations may determine whether mechanisms such as these facilitate the activities and survival of bacteria in the marine environment.
SUMMARY

Substrate capture by the marine bacterium, Ant-300, appears to involve binding components located near the cell surface. Charge interactions are apparently responsible for the reversible formation of a ligand-binding component complex, a reaction which may not require the direct input of metabolic energy. The accumulation of the amino acid arginine proceeds via two high affinity components exhibiting transport constants \((K_m)\) of \(1.5 \times 10^{-8}\) and \(4.5 \times 10^{-7}\) M. A significant portion of arginine accumulation is resistant to exogenously added metabolic inhibitors. The energy source for arginine transport remains to be determined. Chemotaxis provides starved cells with an additional means of capturing arginine, that is concentrated on detrital particles or in decaying plant or animal debris. It appears then, the marine bacterium, Ant-300 is well equipped to scavenge nutrients such as arginine in the ocean. By such means, the organism assures its own survival, and in addition, promotes the efficient mineralization of these compounds.
BIBLIOGRAPHY


23. Ibid. p. 71-72.


