Na\textsuperscript{+} and K\textsuperscript{+} Gradients and $\alpha$-Aminoisobutyric Acid Transport in a Marine Pseudomonad*

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SUMMARY

When cells of marine pseudomonad B-16 (ATCC 19855) harvested during the logarithmic phase of growth were aerated in a buffered salt solution containing 0.3 M NaCl, 0.01 M KCl, and 0.05 M MgSO\textsubscript{4} (Complete Salts Solution) immediately prior to analysis, intracellular Na\textsuperscript{+} was found to be 0.09 M and intracellular K\textsuperscript{+} 0.44 M. When the cells were washed with 0.05 M MgSO\textsubscript{4} to deplete them of K\textsuperscript{+} and then were suspended with aeration in the buffered Complete Salts Solution, the intracellular Na\textsuperscript{+} and K\textsuperscript{+} concentrations were initially 0.3 and 0.004 M, respectively. On further incubation, the intracellular K\textsuperscript{+} concentration increased without a lag while the Na\textsuperscript{+} concentration, after a lag decreased until the ion concentrations prevailing in freshly harvested and aerated cells were obtained. The rate of uptake of K\textsuperscript{+} by and expulsion of Na\textsuperscript{+} from the cells was increased by adding ethanol, an oxidizable substrate, to the suspending medium. The $K_m$ and $V_{max}$ for $\alpha$-aminoisobutyric acid transport into freshly harvested and aerated cells of the marine pseudomonad were essentially unchanged when the Na\textsuperscript{+} and K\textsuperscript{+} concentrations in the suspending medium were changed from those present in the Complete Salts Solution to those prevailing inside the cells. Thus the Na\textsuperscript{+} + K\textsuperscript{+} gradients are not required for $\alpha$-aminoisobutyric acid accumulation, a Na\textsuperscript{+}-dependent process. Cells suspended in the Complete Salts Solution from which KCl was omitted lost K\textsuperscript{+} at a slow constant rate which was not enhanced by $\alpha$-aminoisobutyric acid uptake in the addition of NaCN.

Studies with marine pseudomonad B-16 (ATCC 19855) have shown that Na\textsuperscript{+} is required both for the transport of metabolites into the cell (1-3) and to control the porosity of the cytoplasmic membrane (4, 5). Sodium has been found to decrease the $K_m$ for the transport of the nonmetabolizable amino acid analogue, $\alpha$-aminoisobutyric acid, into the cells (3) and to be required for the penetration of this solute through the cytoplasmic membrane (5). Potassium has also been shown to be necessary for trans-

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MATERIALS AND METHODS

Organism—The Gram-negative marine bacterium B-16 (ATCC 19855) has been classified by the Torrey Research Group, Aberdeen, Scotland, as a *Pseudomonas* species type IV (NCMB 19). The organism has been classified by Bauermann et al. (11) as *Alteromonas marinopraesens* strain 214.

Culture Maintenance—The culture was maintained by monthly transfer on slants of a medium containing 0.8\% nutrient broth (Difco); 0.5\% yeast extract (Difco); and 1.5\% agar in a salts solution consisting of 0.22 M NaCl, 0.026 M MgCl\textsubscript{2}, 0.01 M KCl, and 0.1 mm FeSO\textsubscript{4}(NH\textsubscript{4})\textsubscript{2}SO\textsubscript{4}.

Growth of Cells—Using a procedure described previously (12) cells were grown in liquid medium of the same composition as that used for culture maintenance except that agar was omitted. In the final step of this procedure the cells were incubated with shaking for 5 hours before harvesting. To obtain cells in the...
mid-logarithmic phase of growth, cells were also grown to an 
$A_{660} = 0.5$ (Coleman Junior spectrophotometer model 6A), a 
turbidity achieved after about 35-2 hours of incubation. Anal-
yses showed that cells harvested after 35-2 and 5 hours contained 
similar concentrations of Na$^+$ and K$^+$.

Preparation of Cell Suspensions—Cells were harvested from 
the medium by centrifugation at 16,000 $\times g_{\text{max}}$ at 4$^\circ$ for 10 min. 
For the preparation of cell suspensions in a salt solution contain-
ing 0.3 m NaCl, 0.01 m KCl, 0.05 m MgSO$\text{}_4$, and 0.05 m Tris-
(hydroxymethyl)aminomethane (Tris) hydrochloride buffer (pH 7.2) 
containing 1 mm phosphate added as H$\text{PO}_4^-$ (designated 
Complete Salts Solution containing 0.3 m NaCl) the cells were 
washed by suspension in and centrifugation from a volume of 
the salt solution equal to the volume of the growth medium. 
The washing procedure was repeated twice more. The final 
suspension was adjusted to give a cell suspension containing 10 
to 15 mg dry weight of cells per ml. For the preparation of cell 
suspensions in a salt solution containing 0.5 m NaCl, 0.01 m 
KCl, 0.05 m MgSO$\text{}_4$, and 0.05 m Tris-HCl buffer (pH 7.2) 
in this procedure the cells were suspended once in a 
volume of 0.05 m MgSO$\text{}_4$ equal to the volume of the growth 
medium. The suspension was centrifuged at 16,000 $\times g_{\text{max}}$ 
for 10 min. The cells were then suspended in the Complete 
Salts Solution containing 0.3 m NaCl, but from which the KCl 
had been omitted.

Preparation of Protoplast Suspensions—Protoplasts of marine 
pseudonadal B-16 were prepared by methods described previ-
ously (12).

Intracellular Na$^+$ and K$^+$—The total Na$^+$ and K$^+$ in packed 
cell preparations was determined. The amount of Na$^+$ and K$^+$ 
associated with the cells was calculated by subtracting from the 
total the amount present in the extracellular fluid. The pro-
cedure used was a modification of that described previously (6).
The volumes of extracellular fluid in the packed cell preparations 
were estimated using both $[^{14}\text{C}]$ulin and $[^{14}\text{C}]$sucrose, employing 
methods which have been reported (13). The modifications 
introduced were as follows. Ten-milliliter volumes of a suspension 
of washed cells (10 to 15 mg per ml dry weight of cells) containing $[^{14}\text{C}]$ulin (0.006%; specific activity 2 mCi per g) 
or $[^{14}\text{C}]$sucrose (3 mm; specific activity 0.033 mCi per pmole) were 
transferred to 50-ml Erlenmeyer flasks and aerated vigorously 
by shaking on a rotary water bath shaker (Gyratory shaker, 
model G-76, New Brunswick Scientific Co.) for 15 min at a 
speed of 250 rpm at 25$^\circ$. Packed cell preparations were 
prepared by centrifuging at 68,500 $\times g_{\text{max}}$ for 10 min.

To measure the change in intracellular Na$^+$ and K$^+$ concent-
tration in cells which had been washed with 0.05 m MgSO$\text{}_4$, a 
series of suspensions of the washed cells were made in the Com-
plete Salts Solution containing 0.3 m NaCl. These suspensions 
were shaken with and without 20 mm ethanol in Erlenmeyer 
flasks on the rotary water bath shaker. Flasks were removed 
at intervals for analysis of intracellular Na$^+$ and K$^+$.

Na$^+$ and K$^+$ Analysis—Samples for analysis were digested 
using the procedure of Sanui and Pace (14) as modified by Gor-
don (15). Packed cell preparations (0.5 to 1.0 g wet weight) 
were transferred with 1.5 ml of distilled water to micro-Kjeldahl 
flasks composed of Vykor glass. A 3-mol volume of concentrated 
HNO$\text{}_3$ (analytical reagent grade) was added and the mixture 
was heated until the HNO$\text{}_3$ had evaporated. The residue was 
dissolved in 3 ml of 60% HClO$\text{}_4$, heated, and evaporated to dry-
ness. During evaporation, HClO$\text{}_4$ vapor was removed with the 
aid of a tube of Vykor glass attached to an aspirator pump. 
The residue in the flask was dissolved in 1 ml of 1 n HCl and 
excess HCl was then removed by evaporation. Distilled water 
(4 to 5 ml) was added to the flask and the sample was again 
evaporated. The residue was finally dissolved in water and 
diluted to volume.

Transport of $\alpha$-Aminoisobutyric Acid—Cells were grown to 
mid-log phase ($A_{660} = 0.5$) and washed twice by resuspension 
in and centrifugation from Complete Salts Solution containing 
0.3 m NaCl. A thick cell suspension was prepared such that 
the introduction of 50 to 60 $\mu$l of the suspension into a final 
10-ml volume of incubation medium produced a cell density of 
100 $\mu$g dry weight of cells per ml. Before use in the transport 
studies the thick cell suspension was aerated by shaking on the 
rotary water bath shaker as described previously for intracellular 
Na$^+$ and K$^+$ determinations to ensure that maximum Na$^+$ and 
K$^+$ gradients would prevail.

The basal incubation medium contained 0.05 m Tris-HCl buffer 
(pH 7.2) containing 1 mm phosphate added as H$\text{PO}_4^-$ (designated 
Complete Salts Solution containing 0.5 m NaCl) the same procedure 
was employed using the latter solution to wash and suspend the 
cells. Cells were also washed in a solution containing 0.05 m 
MgSO$\text{}_4$ in this procedure the cells were suspended once in a 
volume of 0.05 m MgSO$\text{}_4$ equal to the volume of the growth 
medium. The suspension was centrifuged at 16,000 $\times g_{\text{max}}$ 
for 10 min. The cells were then suspended in the Complete 
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was heated until the HNO$\text{}_3$ had evaporated. The residue was 
dissolved in 3 ml of 60% HClO$\text{}_4$,
hering cells were placed in glass scintillation vials and dried. For the measurement of $^3$H, 5 ml of scintillation fluid containing 5 g of 2,5-diphenyloxazole per liter of toluene were added to each vial. To determine $^4$K, 10 ml of water were added to each vial and Cerenkov light emission (16) was measured using the tritium IB program of a Nuclear Chicago Isocap/300 liquid scintillation spectrometer.

**RESULTS**

**Intracellular Na$^+$ and K$^+$ Concentrations**—When the cell suspensions were aerated vigorously immediately prior to analysis, the results shown in Table I were obtained. Intracellular K$^+$ concentrations higher than any previously reported for this organism (8) were observed, and Na$^+$ concentrations appreciably lower than those prevailing in the medium were detected. The intracellular fluid volume in the packed cell preparation was measured with the aid of, in one case, $[^3]$Hinulin and in the other, $[^3]$Hsucrose. Previous studies have shown that sucrose occupies 20% of the total available fluid space in a packed cell preparation than inulin suggesting that inulin occupies the space outside of the cell wall of the marine pseudomonad while sucrose occupies the space outside of the cytoplasmic membrane (13). If so, the intracellular fluid volume obtained by subtracting the space occupied by sucrose from the total available fluid space in the packed cell preparation should be a better measure of the volume of fluid in the cells than that obtained with inulin. The results in Table I show that the intracellular K$^+$ concentrations determined using sucrose to measure the extracellular fluid space were higher and the Na$^+$ concentrations lower than when the inulin space was used in previous studies (6, 8). In subsequent experiments in the present study the sucrose space was employed. The inulin space was used in previous studies (6, 8). The intracellular K$^+$ concentrations determined using sucrose to measure the extracellular fluid space were higher and the Na$^+$ concentrations lower than when the inulin space was used. The inulin space was used in previous studies (6, 8). In subsequent experiments in the present study the sucrose space was employed. The results in Table I also show that irrespective of the reagent used to measure extracellular fluid volume the intracellular concentrations of both Na$^+$ and K$^+$ rose when the extracellular Na$^+$ concentration was increased from 0.3 to 0.5 M. The ratio of intracellular to extracellular Na$^+$ remained nearly constant, however, while the K$^+$ ratio went up slightly.

**Establishment of Na$^+$ and K$^+$ Gradients**—When cells of marine pseudomonad B-16 are washed with 0.05 M MgSO$_4$ they lose their intracellular K$^+$ (7, 8). When such K$^+$-depleted cells were suspended with shaking in Complete Salt Solution containing 0.3 M NaCl but no KCl, the internal Na$^+$ concentration was found to be 0.3 M, the same as the external, over the whole period of incubation (Fig. 1). If K$^+$ at 0.01 M was added to the suspending medium, however, the intracellular K$^+$ concentration rose steadily from zero and leveled off at about 0.9 M, while the Na$^+$ concentration, after a lag, decreased to about 0.125 M. The addition of ethanol, an oxidizable substrate, increased the rate and extent of entry of K$^+$ and exit of Na$^+$. Since the uptake curves for K$^+$ are parabolic while the exit curves for Na$^+$ are sigmoid, the entry of K$^+$ and exit of Na$^+$ are evidently not tightly coupled.

**Effect of Na$^+$ and K$^+$ Gradients on Transport**—The active transport of α-aminoisobutyric acid and d-fucose into cells of marine pseudomonad B-16 is a Na$^+$-dependent process (2). If the accumulation of α-aminoisobutyric acid by cells of marine pseudomonad B-16 is dependent upon the maintenance of Na$^+$ + K$^+$ gradients then abolition of these gradients should decrease the rate of entry of α-aminoisobutyric acid and prevent accumulation. To test this possibility, the uptake of α-amino$[^3]$C-isobutyric acid by the cells was compared when the cells were suspended in either a normal Complete Salt Solution containing Na$^+$ at 0.3 M and K$^+$ at 0.01 M or a Complete Salt Solution modified to contain 0.09 M Na$^+$ and 0.44 M K$^+$, the concentrations of Na$^+$ and K$^+$ which one would expect from Table I to be present inside the cells. The results (Fig. 2) show that the rate of uptake of α-amino$[^3]$C-isobutyric acid by cells suspended in the two salt solutions was the same. Using a value of 1.6 μl per mg dry weight of cells as the intracellular fluid volume (a value representing the average of values determined in previous experiments, Table I and Fig. 1), the change in the intracellular α-aminoisobutyric acid concentration with incubation time, also shown in Fig. 2, indicates that accumulation of α-aminoisobutyric acid by the cells against a gradient did in fact take place and that the rate of accumulation was not affected by the differences in the Na$^+$ and K$^+$ concentrations in the two suspending media. In this experiment the uptake time was measured from the time of addition of the cells to the salt solution containing the radioactive amino acid and uptake by the

**Table I**

<table>
<thead>
<tr>
<th>Extracellular concentration (M)</th>
<th>Intracellular concentration (M)</th>
<th>L/E ratios</th>
</tr>
</thead>
<tbody>
<tr>
<td>Na$^+$</td>
<td>K$^+$</td>
<td>Na$^+$</td>
</tr>
<tr>
<td>302 ± 0.1</td>
<td>10.5 ± 0.0</td>
<td>90.2 ± 6.0</td>
</tr>
<tr>
<td>509 ± 0.0</td>
<td>11.8 ± 0.0</td>
<td>129 ± 8.3</td>
</tr>
<tr>
<td>307 ± 0.0</td>
<td>10.6 ± 0.0</td>
<td>129 ± 8.3</td>
</tr>
<tr>
<td>509 ± 3.0</td>
<td>11.8 ± 0.0</td>
<td>187 ± 14.0</td>
</tr>
</tbody>
</table>

*Ratios* of the intracellular to the extracellular ion concentrations.

![Fig. 1. Uptake of K$^+$ and expulsion of Na$^+$ by K$^+$-depleted cells of marine pseudomonad B-16.](http://www.jbc.org/)

Cells were incubated under conditions of aeration in the presence or absence of 20 μl ethanol in Complete Salt Solution (containing, unless otherwise indicated, 0.30 M Na$^+$ and 0.01 M K$^+$). Curve 1, change in intracellular K$^+$ concentration in the presence of ethanol; Curve 2, as in Curve 1 but without ethanol; Curve 3, internal Na$^+$ concentration when K$^+$ was omitted from suspending medium; Curve 4, change in intracellular Na$^+$ concentration in the absence of ethanol; Curve 5, as in Curve 4 but with ethanol present.
cells was determined at half-minute intervals for a total of 3 min. The results in Fig. 1 show that changes in the internal ion concentrations of the cells take place quite slowly. It thus seems unlikely that any gross changes in internal Na\(^+\) and K\(^+\) concentrations would have occurred during the time of measurement of the uptake of α-aminoisobutyric acid.

Initial rates of uptake of α-aminoisobutyric acid were determined at various concentrations of the amino acid for cells suspended in the two salt solutions. The results for cells suspended in the normal Complete Salts Solution are shown in Fig. 3 and for cells suspended in the Complete Salts Solution containing Na\(^+\) and K\(^+\) at the concentrations found previously to prevail inside the cells, in Fig. 4. It is evident that the transport kinetic data are not affected by the difference in concentrations of Na\(^+\) and K\(^+\) in the two suspending media. Lineweaver-Burk plots of the results obtained show that K\(_m\) and V\(_{max}\) for α-aminoisobutyric acid transport were the same in the two systems. Thus abolition of the Na\(^+\) + K\(^+\) gradients did not affect the kinetics of transport of α-aminoisobutyric acid into cells of the marine pseudomond.

Loss of K\(^+\) from Cells—When cells of the marine pseudomond were suspended in Complete Salts Solution containing 0.3 M NaCl but from which the KCl had been omitted the cells lost K\(^+\) at a slow steady rate (Fig. 5). This rate of loss was not enhanced by adding NaCN to the suspending medium.

In yeast and mammalian cells the uptake of basic and neutral amino acids is accompanied by a loss of K\(^+\) (17–19). The results in Fig. 5 show that the uptake of α-aminoisobutyric acid by the marine pseudomond did not lead to the loss of K\(^+\) from the cells.

Effect of Ouabain—Ouabain has been shown to be a potent

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**Fig. 2.** Effect on uptake of α-aminoisobutyric acid (AIB) by the marine pseudomond B-16 of changing the Na\(^+\) and K\(^+\) concentrations in the suspending medium from those present in the basal incubation medium (Na\(^+\); 0.3 M; K\(^+\); 0.01 M) to the concentrations (Na\(^+\); 0.09 M; K\(^+\); 0.44 M) normally found present inside the cells.

**Fig. 3.** Effect of α-aminoisobutyric acid (AIB) concentration on initial rates of uptake of the amino acid analogue by marine pseudomond B-16 when cells were suspended in the basal incubation medium containing normal concentrations of Na\(^+\) and K\(^+\) (Na\(^+\); 0.3 M; K\(^+\); 0.01 M). The inset shows a Lineweaver-Burk plot of the results obtained.

**Fig. 4.** Effect of α-aminoisobutyric acid (AIB) concentration on initial rates of uptake of the compound by marine pseudomond B-16 when cells were resuspended in the basal incubation medium containing concentrations of Na\(^+\) and K\(^+\) (Na\(^+\); 0.09 M; K\(^+\); 0.44 M) equal to those normally found present inside the cells. The inset shows a Lineweaver-Burk plot of the results obtained.

**Fig. 5.** Effect of α-aminoisobutyric acid (AIB) uptake and the addition of NaCN on the loss of K\(^+\) from cells of marine pseudomond B-16 suspended in Complete Salts containing 0.3 M NaCl but no KCl. Change in intracellular K\(^+\) concentration: Curve 1, with no addition; Curve 2, with 1.6 \(\times 10^{-5}\) M α-aminoisobutyric acid present; Curve 3, with \(10^{-2}\) M NaCN present. Curve 4 shows the uptake of α-aminoisobutyric acid under conditions identical to those used for Curve 2, except that the K\(^+\) present in the cells was unlabeled.
inhibitor of Na+-dependent transport in animal cells (9, 20). Previous studies have shown that ouabain does not affect Na+-dependent a-aminoisobutyric acid transport into intact cells of marine pseudomonad B-16 (2). This marine pseudomonad has been shown to have four separable cell wall layers lying outside the cytoplasmic membrane (21). Since such layers could conceivably prevent the penetration of ouabain to the level of the cytoplasmic membrane thus masking the effect of this inhibitor, protoplasts of the organism devoid of cell wall material were prepared. Such protoplasts have been shown to transport a-aminoisobutyric acid by a Na+-dependent process (12). In the present study, the effect of ouabain on the transport of a-aminoisobutyric acid by the protoplasts was examined. Ouabain at a concentration of 10-3 M was found not to be inhibitory (Fig. 6).

### DISCUSSION

The reasons for the failure to detect a downhill Na+ gradient into cells of this marine pseudomonad on two previous occasions have become clear from the results of the present investigation. In the first study (6) the cells had been depleted of K+ by washing with a MgSO4 solution, and contained only 0.02 m K+ at the time of analysis. In the second study (8), the cells were washed with a Complete Salts Solution, but contained only 0.18 m K+ at the time of analysis. The results in Fig. 1 show that at either of these intracellular K+ concentrations, the intracellular Na+ concentration was not appreciably lower than the Na+ concentration prevailing in the medium. It would thus appear that in the course of preparing the cells for analysis, the intracellular K+ concentration dropped to a level which would no longer sustain a Na+ gradient.

Loss of K+ from the cells was not enhanced by NaN3. Thus the loss which occurs during harvesting and washing would seem to be due to the failure of a metabolically driven entry reaction to balance the loss of K+ from the cells by an exit reaction proceeding at a constant rate. Evidence has been obtained that is consistent with the conclusion that K+ is actively transported into cells of this organism by a carrier which serves as an electron transfer intermediate in a chain transferring electrons from specific electron donors to oxygen. Thus energy coupling for the K+ pump in this marine pseudomonad appears similar to that proposed for amino acid and sugar transport in Escherichia coli (22) and differs from the mechanism believed to be operative in animal cells. In the latter, the pump is believed to be an adenosine triphosphatase activated by a combination of Na+ and K+ and inhibited by ouabain (20, 23). No such adenosine triphosphatase has been detected in the marine pseudomonad (24). In other bacteria a Na+ + K+-activated ATPase has either not been detected (25) or is present in only trace amounts (26).

The K+ and Na+ movements into and out of K+-depleted cells of the marine pseudomonad are strikingly similar to those observed by Schultz and Solomon for E. coli when stationary phase cells of the organism were resuspended in fresh medium (27). Only after an appreciable accumulation of K+ has occurred does the intracellular Na+ concentration begin to decrease. In the marine pseudomonad, this extrusion of Na+ by the cell is best explained as being a response to a need to preserve electroneutrality when the energy-coupled transport of K+ creates an internal milieu which is electropositive.

Halophilic and moderately halophilic bacteria appear to have higher internal K+ concentrations than non-halophiles (28), perhaps because the intracellular K+ concentration increases as the osmolality of the suspending medium increases (29). A downhill Na+ gradient into cells of various species of bacteria has also been reported (28).

The fact that cells of this marine pseudomonad can maintain Na+ + K+ gradients helps to explain why protoplasts of this organism are stable at least for a limited period when suspended in Complete Salts Solution containing 0.3 m Na+ (12).

There is presently much debate concerning the role of Na+ and K+ gradients as energy sources for organic solute accumulation in animal cells (for reviews see Refs. 9 and 10). Although in the case of some types of cells and tissues there is sufficient potential energy in the Na+ and K+ gradients to drive organic solute accumulation, evidence has been presented indicating that in animal cells accumulation or reversal of the gradients does not occur (10, 32, 33). In these cells, however, high external K+ does reduce transport. Since K+ can inhibit Na+ activation of transport in animal cells competitively (9), it is not clear whether the reduction of transport by high external K+ is due to a smaller Na+ gradient or to interference with the role of Na+ (10). With the marine pseudomonad, abolition of the Na+ and K+ gradients by placing the same concentrations of these ions outside as inside the cell had no effect on transport kinetics. Thus, for these cells it is clear that the Na+ and K+ gradients provide none of the energy required for the accumulation of a-aminoisobutyric acid.

Since the uptake of certain amino acids by ascites cells and yeast is accompanied by a loss of K+ (17-19) it has been proposed that one of the ways a K+ gradient could be coupled to transport is through the need for K+ to form a complex with the amino acid carrier on the inside surface of the cell to bring the carrier back to the outside surface (32). Since there was no exchange of K+ for a-aminoisobutyric acid in the marine pseudomonad such a mechanism would not apply in these cells.

### REFERENCES


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