Sodium-stimulated \( \alpha \)-aminoisobutyric acid transport by membrane vesicles from simian virus-transformed mouse cells

(Na\(^+\) specificity/amino acid inhibition/K\(^+\) inhibition/cell transport/biphasic kinetics)

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ABSTRACT Uptake of \( \alpha \)-aminoisobutyric acid, by membrane vesicles derived principally from the plasma membrane and endoplasmic reticulum of mouse 3T3 cells transformed by simian virus 40, is stimulated by sodium chloride. Both in the presence and absence of Na\(^+\) uptake is time-dependent and osmotically sensitive. The Na\(^+\)-stimulated uptake is inhibited by other amino acids. The kinetics of transport of \( \alpha \)-aminoisobutyric acid are shown to be biphasic both in whole cells and in the membrane vesicles. Only the high affinity system is stimulated by sodium in the membrane vesicles. These results demonstrate that observations made on living cells correlate with observations made on isolated membrane vesicles, and indicate that these membrane vesicles have retained the cellular amino acid transport system functionally intact.

It has been established from a number of studies that transport of some nutrients, such as phosphate (1–4), glucose (5), nucleosides (2, 6), amino acids (7–9), and monovalent cations (10–12), is involved in the control of growth of animal cells in tissue culture. The nature of that involvement is not known. By a determination of the molecular mechanisms of transport for these nutrients before, during, and after growth stimulation, we may be able to determine whether transport of essential nutrients across the cell-surface membrane controls growth.

A serious drawback for a determination of the molecular level mechanisms of transport in living cells is that the transported substance is metabolized after interaction with the transport site. This problem can be overcome by a study of the transport system in isolation. A means of doing this was developed by Ka- back (13) who studied nutrient transport by membrane vesicles derived from bacteria. Hochstadt et al. (14–17) have begun to determine mechanisms of transport in membrane vesicles derived from animal cells grown in tissue culture. In addition to the studies reported in this paper, we have completed studies of phosphate uptake by SV3T3 membrane vesicles from simian virus 40-transformed 3T3 cells (SV3T3) (Hamilton and Nilsen-Hamilton, manuscript in preparation).

As a prelude to a comparison of the transport properties of membrane vesicles (MV) derived from normal and transformed cells, we report in this paper our studies of \( \alpha \)-aminoisobutyric acid (AIB) uptake by a membrane-vesicle population derived principally from the cell-surface membrane and endoplasmic reticulum of SV3T3 cells. We describe the effect of Na\(^+\) on transport of AIB by this system. A similar, independent study by Quinlan et al. (18) has recently been reported.

MATERIALS AND METHODS

All solutions used for membrane vesicle preparations were sterilized by filtration or by autoclaving. \( \alpha \)-Amino\(1^{14}\)C]-iso-

Abbreviations: AIB, \( \alpha \)-aminoisobutyric acid; MV, membrane vesicle; STM, 0.25 M sucrose, 5 mM Tris-HCl (pH 7.3), and 0.2 mM MgSO\(_4\); PNS, post nuclear supernatant; HBS, Hank's Balanced Salt Solution; SV3T3, mouse ST3 cells transformed by simian virus 40.
AIB uptake (15-sec rate) 

<table>
<thead>
<tr>
<th>Protein</th>
<th>(Na⁺,K⁺)-ATPase</th>
<th>5'-Nucleotidase (pH 8.5)</th>
<th>β-Galactosidase</th>
<th>NADH oxidase</th>
<th>Cytochrome oxidase</th>
</tr>
</thead>
<tbody>
<tr>
<td>Homogenate</td>
<td>100</td>
<td>100</td>
<td>(48)</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>Post nuclear supernatant</td>
<td>79</td>
<td>94</td>
<td>71</td>
<td>86</td>
<td>78</td>
</tr>
<tr>
<td>Nuclei</td>
<td>17</td>
<td>29</td>
<td>12</td>
<td>14</td>
<td>13</td>
</tr>
<tr>
<td>Pellet (18,000 × g)</td>
<td>26</td>
<td>38</td>
<td>56</td>
<td>43</td>
<td>51</td>
</tr>
<tr>
<td>Membrane vesicles</td>
<td>20</td>
<td>30</td>
<td>29</td>
<td>60</td>
<td>13</td>
</tr>
<tr>
<td>Supernatant (42,000 × g)</td>
<td>32</td>
<td>N.D.</td>
<td>0.6</td>
<td>6</td>
<td>3.9</td>
</tr>
<tr>
<td><strong>Total yield</strong></td>
<td><strong>95</strong></td>
<td><strong>97</strong></td>
<td><strong>97.6</strong></td>
<td><strong>(123)</strong></td>
<td><strong>80.9</strong></td>
</tr>
</tbody>
</table>

Percent yields expressed relative to the homogenate for all enzymes except 5'-nucleotidase which is expressed as percent of the sum of post-nuclear supernatant and nuclear activities. As a result of adenosine binding and loss of the product in the BaSO₄ precipitate, the values of 5'-nucleotidase for the homogenate are lower than the real values (our unpublished observations). N.D. = not detectable.

(β-D-galactoside galactohydrolase; EC 3.2.1.23), a lysosomal enzyme (28), was determined by the method of Ho et al. (29), with the inclusion of 0.1% Triton X-100 for full enzyme expression. Cytochrome oxidase (ferrocyanochrome c: oxygen oxidoreductase; EC 1.9.3.1), which is located on the inner mitochondrial membrane (90), was assayed by the method of Cooperstein and Lazarow (31).

**Assay for Uptake of AIB by Membrane Vesicles.** For each time point, 90 ml aliquots containing from 150 µg to 350 µg of membrane protein in 60 mM sucrose, 10 mM Tris-HCl (pH 7.4 at 37°), 0.1 mM MgCl₂, and 0.1 mM CaCl₂, were preincubated at 37° for 10–20 min. At the end of this period, the reaction was started by the addition of 10 µl of 14C- or 3H-labeled AIB. The reaction was terminated by dilution with 1.4 ml of 0.8 M NaCl, at 37°. Each diluted sample was immediately filtered through a nitrocellulose filter (24 mm diameter and 0.2 µm, average pore size, Schleicher and Schuell, Inc., Keene, N.H.) which had been wetted with sterile water. The filters were then washed twice with the same volume of NaCl. Background adsorption was determined by addition of radioactively labeled AIB to the membrane suspension. This mixture was diluted and filtered immediately and washed with 0.8 M NaCl at 37°. The nitrocellulose filters were dried by heat and the radioactivity was determined in a Beckman liquid-scintillation counter, with a toluene scintillation mixture (Liquifluor, New England Nuclear).

**Assay for Uptake of AIB by SV3T3 Cells.** The uptake assay is based on a method described by Foster and Pardee (32) with cells grown on glass coverslips. The coverslips were prepared by boiling in an aqueous solution of sodium hexametaphosphate (8.8 g/liter) and sodium metasilicate (70.12 g/liter). Coverslips containing confluent cells were rinsed twice with Hank's Balanced Salt Solution (HBS) which contained 20 mM Hepes (4-2-hydroxy-ethyl)-1-piperazineethane-sulfonic acid) at pH 7.0, then preincubated for 25–30 min in the same solution in a CO₂ incubator at 37°. Uptake was in HBS and Hepes at 37° for 2 min. Coverslips were then placed in 1 ml of 0.4 M NaOH and left at 4° overnight to completely dissolve the cells. The solutions were then neutralized with HCl and aliquots removed for determination of radioactivity in a Beckman liquid scintillation counter, with a Liquifluor scintillation mixture which contained toluene and Triton X-100. Protein concentrations were determined for each point, and five coverslips, taken through the same washing procedures, were trypsinized and the cells counted in a Coulter Counter.

**RESULTS**

**Membrane fractionation**

The enzymatic analysis of a typical fractionation of membrane constituents from SV3T3 cells is shown in Table 1. The membrane fraction, MV, was used for the uptake studies described in this paper. This fraction contains 4% of the total mitochondrial activity (range, 1–4%) and 13% of the lysosomal activity (range, 5–14%). Twenty-four percent of the NADH oxidase travels with MV.

AIB uptake activity correlates with the plasma membrane markers, (Na⁺, K⁺)-ATPase and 5'-nucleotidase. It does not correlate with mitochondrial or lysosomal enzymatic activities.

**The kinetics of AIB transport in membrane vesicles**

The uptake of AIB by MV is time dependent (Fig. 1). As evidence that the accumulated [14C]AIB counts represented uptake...
of AIB rather than nonspecific binding to the membrane surface, washing the MV with water, at any time during the time-course, reduced the accumulated \(^{14}\text{C}\) radioactive material back to the background value. By the use of hyperosmotic conditions to shrink the vesicles, we observed a 2-fold reduction in steady-state levels of AIB transport (data not shown), which is further evidence of intravesicular accumulation of AIB.

Sodium ions at the external surface of the membrane of eukaryotic cells are often required for active transport (33). The effect of 50 mM NaCl, added simultaneously with AIB to start the uptake reaction (Fig. 1), is to increase the initial rate of uptake and the maximal level reached from 2- to 4-fold. The response to 50 mM NaCl depends on the concentration of AIB used, and is greater at lower AIB concentrations (our unpublished observations). The stimulatory effect of NaCl most likely results from a transient concentration gradient formed across the membrane, because 50 mM NaCl is nonstimulatory when preequilibrated with the membranes before initiation of uptake. Also, with time, the initial stimulation, seen when NaCl is added with AIB, decreases to the level found when the vesicles are equilibrated with Na\(^+\) prior to addition of label.

When there is a requirement of eukaryotic active transport systems for monovalent cations at the external surface of the cell, that requirement is generally specific for Na\(^+\) (33, 34). We have shown (Table 2) that stimulation of AIB transport into SV3T3 membrane vesicles is also specific for Na\(^+\) when compared with the other chlorides of alkali metal ions, NH\(_4\)^+ and choline chloride. In addition, this experiment provides evidence against transient osmotic or ionic strength effects which might cause stimulation of AIB uptake by the membrane vesicles.

The specific Na\(^+\)-stimulated uptake of AIB is evidence that this uptake is a mediated one. Further evidence for mediated uptake by the membrane vesicles was provided by studies which showed inhibition of AIB uptake by other amino acids (Table 3). No significant inhibition of uptake by these amino acids was observed in the absence of NaCl.

To compare the parameters, \(K_m\) and \(V_{max}\), obtained for membrane vesicles with those for cells, we determined the effect of AIB concentration on the initial rates of uptake in SV3T3 cells grown on glass coverslips (Materials and Methods). The use of AIB, an amino acid analog which is not metabolized, in initial studies such as these, has the advantage that cells can be more directly compared with membrane-vesicle studies to verify that the transport system has been isolated functionally intact. The kinetics of AIB uptake by SV3T3 cells are biphasic (Fig. 2).

Similar studies were made with the membrane vesicles. Comparisons of the uncorrected, apparent values of \(K_m\) and \(V_{max}\) are shown in Table 4. Biphasic uptake kinetics were obtained for cells and membrane vesicles. Good agreement is found for \(K_m\) values, from cells and membrane vesicles, determined in the low concentration range of 0.1 mM to 2 mM.

![Fig. 2. Lineweaver-Burk plot of AIB uptake by SV3T3 cells. Initial rate determinations (Materials and Methods) were made in the concentration range 0.1–100 mM AIB. In the inset is shown the straight line obtained by least-squares analysis in the concentration range 0.1–2.5 mM. The line of the main figure begins to curve off at 2.5 mM and then at approximately 5 mM it straightens to a new line of steeper slope. The values of \(K_m\) and \(V_{max}\) obtained by extrapolation, have not been corrected for the contribution to uptake from each of the other systems. We have designated them the uncorrected apparent \(K_m\) and \(V_{max}\) values. They are meaningful only for comparisons. These values are given in Table 4. The average protein per coverslip was 353 \(\mu\)g (167 estimations) and the average number of cells per coverslip was \(5.12 \times 10^6\).](image-url)
Table 4. Summary of uncorrected apparent $K_m$ and $V_{max}$ values obtained from SV3T3 cells and membrane vesicles

<table>
<thead>
<tr>
<th>System</th>
<th>AIB concentration range (mM)</th>
<th>$K_m$ (mM)</th>
<th>$V_{max}$ (nmol/min per mg of protein)</th>
<th>Na$^+$ concentration (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SV3T3 cells</td>
<td>0.1–3.3</td>
<td>4.4</td>
<td>18</td>
<td>140</td>
</tr>
<tr>
<td></td>
<td>20–100</td>
<td>26</td>
<td>31</td>
<td>140</td>
</tr>
<tr>
<td>Membrane vesicles</td>
<td>0.1–2.0</td>
<td>15</td>
<td>2.1</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>0.1–2.0</td>
<td>3.4</td>
<td>0.99</td>
<td>25</td>
</tr>
<tr>
<td></td>
<td>0.1–2.0</td>
<td>2.6</td>
<td>1.0</td>
<td>50</td>
</tr>
<tr>
<td></td>
<td>0.1–2.0</td>
<td>1.1</td>
<td>0.51</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>20–70</td>
<td>143</td>
<td>155</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>20–70</td>
<td>153</td>
<td>155</td>
<td>50</td>
</tr>
</tbody>
</table>

The Lineweaver-Burk plots used to derive these values are Figs. 2, 3, and 4.

AIB. Lack of agreement is found between $K_m$ values determined in the high concentration range 5–100 mM AIB. This could result from a higher contribution of simple diffusion to uptake by the membrane vesicles. At high AIB concentrations the uptake does not respond to NaCl (Table 4 and Fig. 3, top and bottom). To test the possibility that 50 mM NaCl was insufficient to cause stimulation at the higher concentrations of AIB, the effect of concentrations greater than 50 mM NaCl, on the initial rate of uptake of 50 mM AIB, was measured. No significant effect was observed up to 300 mM NaCl. However, a pronounced inhibition of uptake was obtained with KCl. At KCl concentrations of 100 and 200 mM added with 50 mM AIB, 50 and 86% inhibition of AIB uptake was observed. These results suggest that very little simple diffusion occurs through the membrane vesicles even at 50 mM AIB. However, the possibility that KCl is destroying membrane integrity has not been ruled out.

We determined the effect of Na$^+$ on the uncorrected apparent $K_m$ and $V_{max}$ values for uptake of AIB by the membrane vesicles, in the range of AIB concentration where uptake was sensitive to Na$^+$. We varied the choline/Na$^+$ ratio, and we measured uptake at a constant 100 mM final salt concentration. In the range 25 mM NaCl to 100 mM NaCl, the uncorrected apparent $K_m$ value changed from 3.4 mM to 1.1 mM AIB, and the uncorrected apparent $V_{max}$ value was decreased by one-half (Table 4 and Fig. 4).

**DISCUSSION**

Our main finding is that AIB uptake by SV3T3 membrane vesicles is specifically stimulated by Na$^+$, only if sodium chloride is added at the start of the uptake reaction. This implies that the energy of a Na$^+$-electrochemical gradient is used to drive AIB uptake. These findings are in agreement with those of Quinlan et al. (18). Both reports describe the Na$^+$-dependent amino acid uptake into membrane vesicles derived from tissue-culture cells. However, one report (35) described the phenomenon in membrane vesicles derived from Ehrlich Ascites cells and another (36) in membrane vesicles from enterocyte brush border membranes.

We have further shown that the Na$^+$-stimulation is accompanied by alterations in apparent $K_m$ values. This would indicate an increase in the affinity of the carrier for AIB in the presence of Na$^+$ according to the models of Heinz and others (33, 34).

Our results indicate that AIB is take up by the SV3T3 cells and membrane vesicles by two kinetically distinguishable components: one with a low apparent affinity for AIB which is Na$^+$ independent, and the other with a high apparent affinity for AIB which is Na$^+$ dependent. The advantage of membrane-vesicle studies of uptake is that the two systems can be studied independently in the presence and absence of Na$^+$. 

![Fig. 3. Lineweaver-Burk plots of uptake of high concentrations of AIB in the absence and presence of 50 mM NaCl. Uptake, for 15 sec, was as described in Materials and Methods. For each estimation, 335 μg of membrane protein was used. The concentration of [3H]AIB was varied from 20 to 70 mM. The [3H]AIB cpm per experiment were 3.24 × 10^5. Initial rates have the units: nmol/min per mg of protein. Each point is the mean of duplicate determinations. Linear regression analyses were used to obtain the lines of best fit. Uncorrected values of apparent $K_m$ and $V_{max}$ are given in Table 4.](image)

![Fig. 4. The effect of NaCl on $K_m$ and $K_{max}$ of SV3T3 membrane vesicles. Uptake, for 15 sec, was as described in Materials and Methods. For each estimation, 335 μg of membrane protein was used. The concentration of [3H]AIB was varied from 0.1 mM to 2 mM. The [3H]AIB cpm per experiment were 4.19 × 10^5. Initial rates have the units: nmol/min per mg of protein. Each point is the mean of triplicate determinations. The lines of best fit were derived by least-squares analysis. The two points in parentheses were not included in the least-squares analysis. O−O, 100 mM choline chloride final concentration added with [3H]AIB. O−O, 25 mM NaCl/75 mM choline chloride. ▲−▲, 50 mM NaCl/50 mM choline chloride. O−O, 100 mM NaCl. Values of apparent $K_m$ and $V_{max}$ are given in Table 4.](image)
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