# Amino Acid Transport in Membrane Vesicles from CHO-K1 and Alanine-Resistant Transport Mutants†

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ABSTRACT: Membrane vesicles were prepared from CHO-K1 and alanine-resistant transport mutants, ala$^r$4 and ala$^r$4-H3.9. Ala$^r$4 is a constitutive mutant of the A system, and ala$^r$4-H3.9, derived from ala$^r$4, may be the result of amplification of a gene coding for an A-system transporter. Under conditions in which the same membrane potential (interior negative) and $Na^+$ gradient were employed, the mutant vesicles show increases in the A system over that of the parental CHO-K1 cell line, paralleling, but not equivalent to, that found in whole cells. L-system and 5′-nucleotidase activities of these vesicles were similar, indicating that the increased A-system activity of the mutant vesicles is not due to the differential enrichment of the A system in these vesicles. The membrane potential was produced by a $K^+$ diffusion gradient (internal > external) in the presence of valinomycin or by the addition of a $Na^+$ salt of a highly permeant anion such as $SCN^-$. Monensin was employed to study the effect of the $Na^+$ gradient on transport and membrane potential. The latter was determined by measuring the uptake of tetraphenylphosphonium ion. A negative membrane potential determines the concentrative ability and the initial velocity of the A system in these vesicles. The concentration of external $Na^+$ has a stimulatory effect on the initial velocity of this system. However, the $Na^+$ gradient (external > internal) has no effect on the initial velocity or the membrane potential when the potential is set by valinomycin and high internal $K^+$. Little if any ASC system could be detected in vesicles from CHO-K1.

Mutants have been isolated from CHO-K1 that have increased levels of transport of amino acids through the A system (Moffett et al., 1983; Moffett & Englesberg, 1984; Englesberg et al., 1986; Englesberg & Moffett, 1986). This system is $Na^+$ dependent, is ubiquitous among the vertebrates, and transports small, straight-chain amino acids such as alanine, glycine, nonmetabolizable amino acid analogues such as α-aminoisobutyric acid (AIB)[1] and α-(methylamino)isobutyric acid (MeAIB), and proline (Bass et al., 1981; Shotwell et al., 1981). Of particular interest to this paper are two alanine-resistant mutants, ala$^r$4 (Moffett & Englesberg, 1984) and ala$^r$4-H3.9 (J. Moffett and E. Englesberg, unpublished experiments). These mutants have 5- and 29-fold increases in their $V_{max}$ of proline transport through the A system, respectively, with little if any change in $K_m$. The increase in $V_{max}$ is not specific for proline transport but for any amino acid transported by the A system. Ala$^r$4 is a constitutive mutant for the A system. Ala$^r$4-H3.9 is a mutant derived from ala$^r$4, subsequent to treatment with hydroxyurea, and selected in a two-step process for resistance to increasingly higher concentrations of alanine and may be the result of gene amplification (J. Moffett and E. Englesberg, unpublished experiments).

We have shown that the A system in CHO-K1 is repressible by the addition of amino acids that are generally but not necessarily transported by this system and that it is also inducible by insulin (Moffett & Englesberg, 1984, 1986; Mendiaz et al., 1986; Englesberg & Moffett, 1986). By genetic analysis of several mutants, we have provided evidence that regulatory genes R1 and R2 jointly control the expression of structural gene(s) for the A-system transporter by negative means (Moffett & Englesberg, 1984, 1986; Englesberg & Moffett, 1986).

Although our evidence supports the conclusion that the mutants described above have an increased number of A-system transporters, we could not exclude the possibility that mutation may have affected changes in the energetics of A-system transport that facilitated these increases in A activity. To distinguish between these two possibilities, we first determined the energetics of A-system transport employing biologically active membrane vesicles prepared from CHO-K1 and the two alanine-resistant, A-system transport, mutants. We show that the initial velocity of transport through the A-system vesicles is contributed by both the external $Na^+$ concentration and the membrane potential. Under conditions in which these factors were the same, we show that vesicles from the mutant cells still maintain increases in A-system transport activity.

## Materials and Methods

*Cells and Cell Culture.* Chinese hamster ovary cells and ala$^r$4, a constitutive mutant for the A system, have been previously described (Moffett & Englesberg, 1984). Ala$^r$4-H3.9 is a mutant of ala$^r$4 isolated in a two-step procedure as resistant to increased concentrations of alanine and may be the result of gene amplification (J. Moffett and E. Englesberg, unpublished experiments). Cells were routinely grown in MEMCHO-4 (Moffett et al., 1983).

*Preparation of Membrane Vesicles.* Membrane vesicles were prepared essentially according to the method of Lever (1977a) and Quinlan and Hochstadt (1976). Briefly, cells grown in roller bottles were rinsed with 0.02% EDTA in PBS, pH 7.4, and incubated in this solution for 30 min to 1 h. Cells were then scraped and collected at 1000*g*, resuspended in Tris

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[1] Abbreviations: AIB, α-aminoisobutyric acid; MeAIB, α-(methylamino)isobutyric acid; $TPP^+$, tetraphenylphosphonium ion; EDTA, ethylenediaminetetraacetic acid; PBS, phosphate-buffered saline; Tris, tris(hydroxymethyl)aminomethane; GIF, glutamine inhibitory fraction.

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buffer (0.01 M Tris-HCl, pH 7.4, 1 mM $MgCl_2$, and 0.25 M sucrose), and centrifuged at 5000*g*. The pellet was resuspended in the same Tris buffer and subjected to nitrogen cavitation for 20 min at 600 psi (4.2 MPa), after which the crude homogenate was collected in a dropwise fashion. Using these conditions, we found 70–90% breakage of the cells and 10–30% breakage of nuclei depending on the preparation. EDTA was then added to the crude homogenate to a final concentration of 1 mM, and the homogenate was then centrifuged at 5000*g*. The supernate was saved and the pellet washed an additional 2 times by centrifugation as described above. The supernates were pooled and subjected to centrifugation at 18000*g* over 1 M sucrose for 35 min. The resulting supernate was centrifuged at 100000*g* for 60 min in a 60 Ti rotor. The pellet was then resuspended in 0.01 M Tris–phosphate, 5 mM $MgCl_2$, and 0.25 M sucrose and frozen at −70 °C. This fraction, which will be referred to as the mixed membrane fraction, contained primarily plasma membrane and endoplasmic reticulum. During the course of this study, several different vesicle preparations were employed for each of the cell lines. All preparations showed similar enrichment data.

Dextran gradient centrifugation was employed to purify the plasma membrane from the endoplasmic reticulum in the mixed membrane fraction (Quinlan & Hochstadt, 1976). The top two bands in the dextran gradients were pooled and were shown to contain plasma membrane as others had found. The pellets were pooled and contained primarily endoplasmic reticulum.

*Marker Enzyme Analysis.* The enzymes used to monitor the purity of each fraction of the membrane preparation were the following: 5′-nucleotidase (EC 3.13.5), a plasma membrane marker; NADH oxidase (EC 1.6.99.3), an endoplasmic reticulum marker; and succinate–cytochrome *c* reductase (EC 1.3.99.1), an inner mitochondrial wall marker (Lever, 1977a). All assays were performed within 5 days of the vesicle preparation, and the samples were not frozen. Protein was determined according to the method of Lowry et al. (1951).

*Vesicle Transport Studies.* Transport studies in vesicles were performed according to the method of Lever (1977a). Membrane preparations in 0.01 M Tris–phosphate, 5 mM $MgCl_2$, and 0.25 M sucrose were thawed immediately before each experiment. To begin an uptake, a reaction mix containing 0.01 M Tris–phosphate, pH 7.4, 5 mM $MgCl_2$, 0.125 M sucrose, and any other additions, as indicated, was added directly to the membrane preparation. There was 10–300 μg of protein per sample, and A-system activity is linear within this range of protein concentrations. Uptake was terminated by the addition of ice-cold stop buffer (0.8 M NaCl and 0.01 M Tris–phosphate, pH 7.4), and the membranes were collected on a Metricel or nitrocellose (Millipore) filter, 0.45- or 0.22-μm diameter. The sample and filter were then rinsed 4 times with 2 mL of the cold stop buffer. The entire stop procedure required less than 15 s. Filters were then dried at 37 °C and counted by using liquid scintillation. Internal water space was determined by the method of Kletzien et al. (1975) and was found to be 1.4–2.0 and 1.1–1.4 μL of $H_2O$/mg of protein for the mixed vesicle and plasma membrane population, respectively, regardless of the source of vesicle material.

*$TPP^+$ Uptake Measurements.* $TPP^+$ uptake was performed essentially as described by Schuldiner and Kaback (1975) and Lever (1977b). All buffers used for uptake were the same as those used for amino acid transport.

*Determination of Kinetic Parameters for A-System Transport.* $K_m$ and $V_{max}$ for proline transport through the A system were determined by measuring the initial, 10-s uptake of proline, at concentrations of 0.05–5.0 mM, in the presence and absence of 25 mM MeAIB. The velocity in the presence of MeAIB was subtracted from that in the absence of MeAIB (Moffett et al., 1983). The assay was performed in triplicate, and the average values were graphed and kinetic constants and confidence levels were determined by using a least-squares fitting and a jackknife error analysis computer program (Moffett et al., 1983).

Table I: Mixed Vesicle Preparation and Purification of the Plasma Membrane

| cell line | sp act. (nmol $min^{-1}$ $mg^{-1}$) of 5′-nucleotidase[b] | % distribution[a] NADH–Succ oxidase[b] | % distribution[a] cyt *c* reductase[b] |
|---|---|---|---|
| CHO-K1 | | | |
| crude homogenate | 0.27 (100)[c] | 100 | 100 |
| mixed vesicle | 4.40 (71) | 74 | 27 |
| endoplasmic reticulum | 3.48 (8) | 20 | 7 |
| plasma membrane | 11.72 (20) | 5 | 3.5 |
| $ala^r4$ | | | |
| crude homogenate | 0.64 (100) | 100 | 100 |
| mixed vesicle | 3.16 (86) | 86 | 17 |
| endoplasmic reticulum | 0.64 (2.2) | 27 | ND[d] |
| plasma membrane | 6.12 (24) | 2 | ND |
| $ala^r4$-H3.9 | | | |
| crude homogenate | 0.55 (100) | 100 | 100 |
| mixed vesicle | 3.17 (79) | 63 | 21 |
| endoplasmic reticulum | 1.39 (7) | 31 | 6 |
| plasma membrane | 8.11 (18) | 4 | 2 |

[a] Percent distribution equals the amount of total activity remaining divided by the total activity that was originally present in the starting material × 100. [b] Enzyme assays were performed according to Materials and Methods. [c] Numbers in parentheses are the percent distribution of 5′-nucleotidase in each fraction. [d] ND = not detected.

## RESULTS

*Preparation of Membrane Vesicles.* We used three enzymes, specific for different cellular components, to assess the purity of the membrane fractionation procedure (Table I). The data show that the mixed vesicle population is significantly enriched for 5′-nucleotidase as well as NADH oxidase. This indicates, as others have found (Lever, 1977a; Quinlan & Hochstadt, 1976), that the mixed vesicle population is comprised predominantly of plasma membrane and endoplasmic reticulum. In our preparation, we also detected contamination by the mitochondrial membrane (10–30%).

The purified plasma membrane contained increased 5′-nucleotidase and decreased NADH oxidase activity as compared to the mixed vesicle population. The cytochrome *c* reductase activity was reduced in the plasma membrane to below 4% in all the preparations. Similar enrichment data were obtained in the preparation of the mixed vesicle and plasma membrane of the mutants.

*Transport Studies in Mixed Vesicles and Plasma Membrane Fractions.* We used proline to measure transport activity in the mixed vesicle and plasma membrane preparation isolated from CHO-K1. The reaction mixture contained 0.1 mM proline and 100 mM NaCl in the standard uptake buffer described under Materials and Methods. With the mixed vesicle population and the plasma membrane fraction, the velocity was 4–10 and 20–30 times that of the crude homogenate, respectively (data not shown). Figure 1 shows that the proline uptake with the plasma membrane and 100 mM NaCl is linear for only a short time and that steady state is reached in 30–60 s. At steady state, CHO-K1 plasma membrane vesicles concentrated proline 3 times that of the exterior concentration of substrate used in contrast to only 1.3 times by the mixed membrane vesicle. We found that the rate of

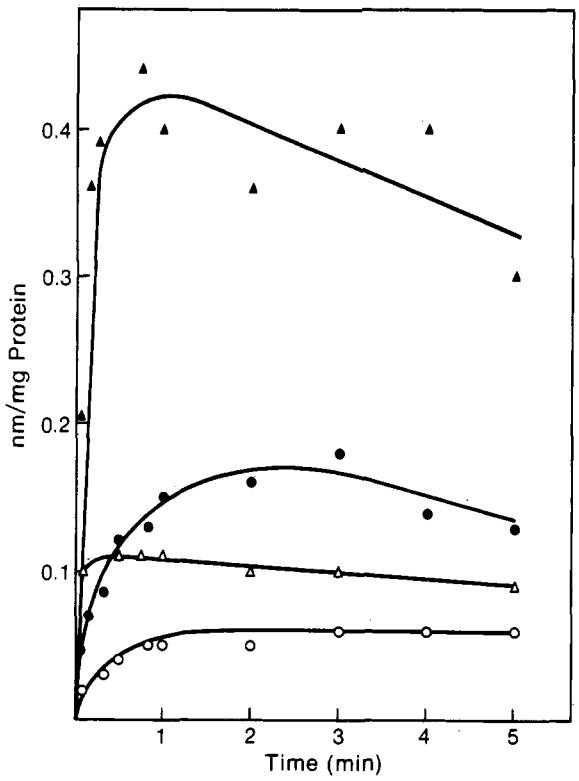


FIGURE 1: Proline uptake in mixed vesicles and plasma membrane vesicles. 110 μg of mixed vesicle (○, ●) protein or 12.5 μg of purified plasma membrane protein (△, ▲) was used for each uptake. The reaction mixture contained 0.1 mM proline, 0.01 mM Tris–phosphate, 0.125 M sucrose, and 5 $MgCl_2$ with 100 mM NaCl (●, ▲) or 100 mM choline chloride (○, △). Average $H_2O$ volume per vesicle was equal to 1.4 μL/mg of protein.

uptake was inhibited by low temperatures (2–4 °C) while temperatures from 30 to 37 °C had no detectable effect. These data agree well with those previously published by other authors using different cell lines (Lever, 1976, 1977a; Hoyoku et al., 1978; Hamilton & Nilson-Hamilton, 1976).

Dinitrophenol had no effect on proline transport at any time after addition of the reaction mixture when the mixed vesicle preparation was used. This indicates that the mitochondrial contamination in this fraction has no effect on the uptake of proline when these vesicles are used (data not given).

*Sodium and pH Dependence of Proline Uptake in Mixed Vesicles.* In order to further characterize proline transport in these vesicles, we determined the relationship between $Na^+$ concentration and proline uptake. There is an increase in the initial velocity and distribution ratio with increasing $Na^+$ concentration. No further significant increases are observed with concentrations of NaCl over 100 mM (Figure 2). Proline and alanine uptake showed a sharp increase in velocity at alkaline pH similar to what is found in the whole cell for proline transport through the A system. Such an increase is not exhibited when uptake through the ASC system is measured with the whole cell. Since alanine is mainly transported by the ASC system in these cells, it would appear that this system may not be active in these vesicles, supporting the evidence to this effect as given below. Leucine uptake was relatively resistant to pH changes from pH 6.5 to 8.0 (Figure 3).

*$Na^+$-Dependent Transport Systems in Vesicles of CHO-K1.* Proline transport in whole cells of CHO-K1 is mediated by the $Na^+$-dependent A, ASC, and P systems and the $Na^+$-independent L system (Bass et al., 1981; Moffett et al., 1983). These systems account for 60–70%, 5–10%, 10–15%, and 5–10% of proline (0.05 mM) transport, respectively. We have

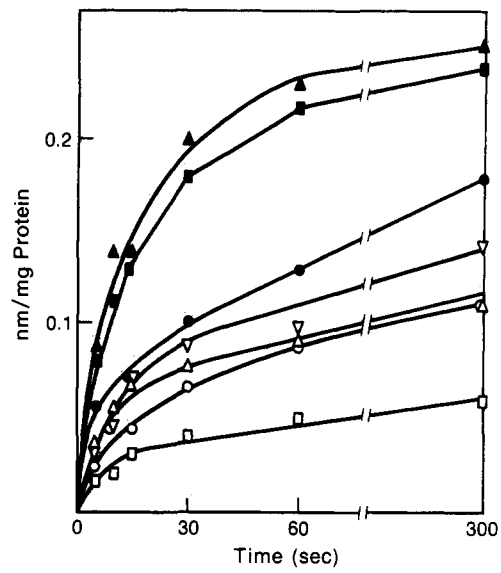


FIGURE 2: Sodium concentration dependence of proline uptake in mixed vesicles from CHO-K1. Uptakes in mixed vesicles (50 μg of protein/sample) with 100 mM choline chloride (□), 5 mM NaCl (○), 10 mM NaCl (△), 25 mM NaCl (▽), 50 mM NaCl (●), 100 mM NaCl (■), or 200 mM NaCl (▲). Choline chloride was added to balance the osmotic effect of NaCl; uptakes were performed as described under Materials and Methods.

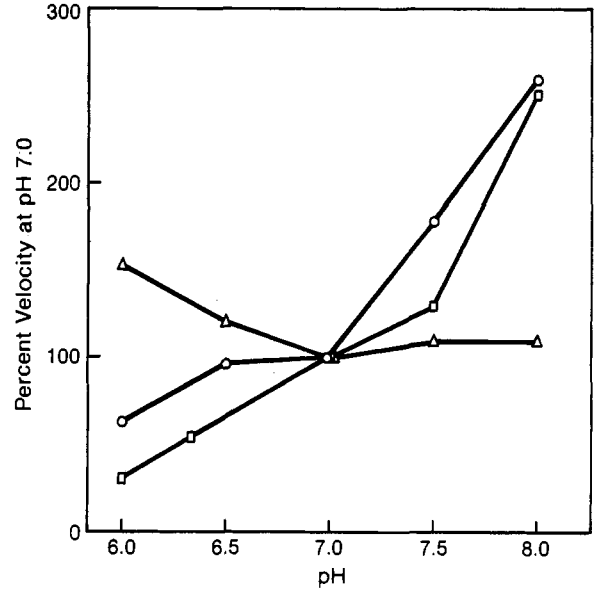


FIGURE 3: pH dependence of proline, alanine, and leucine uptake in mixed vesicle population isolated from CHO-K1. Buffer pH was adjusted with Tris base by using phosphoric acid. The reaction mixture contained the standard mixture with NaCl in assay of 0.1 mM proline and 0.1 mM alanine. Proline and alanine uptakes were also performed in a reaction mixture in which choline chloride was substituted for NaCl. The velocities obtained were subtracted from those obtained in the presence of NaCl. The uptake was measured at a 10-s interval. Leucine uptake (0.02 mM) was measured at a 5-s interval, and choline chloride replaced the NaCl in the reaction mixture. 110 μg of mixed vesicle protein was employed: proline (□); alanine (○); and leucine (△).

omitted the glutamine inhibitory fraction (GIF) from consideration since it contributes little to the overall $Na^+$-dependent proline transport and has not been sufficiently characterized. The A system is operationally defined as the increment of velocity of amino acid transport that is inhibited by saturating amounts of MeAIB (Bass et al., 1981; Shotwell et al., 1981; Moffett et al., 1983). To determine the extent of transport of proline through the A system in vesicles, we had to determine whether MeAIB inhibited proline transport

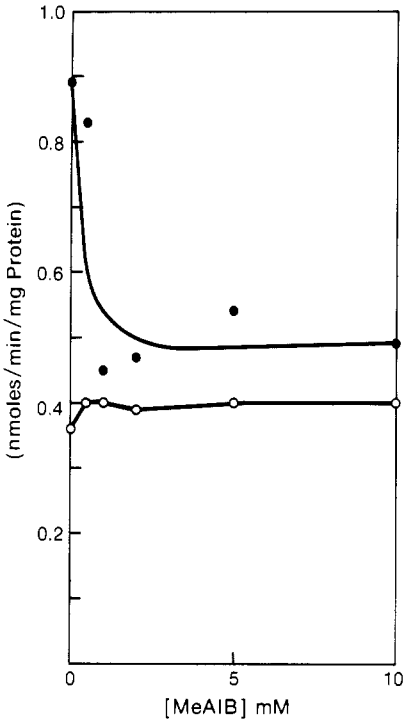


FIGURE 4: Inhibition of proline by MeAIB in mixed vesicles. 50 μg of mixed vesicle protein was added to the reaction mixture containing 0.1 mM proline and increasing concentrations of MeAIB. Uptake was for 5 s. Choline chloride was added to mixtures to balance for osmotic effects in those mixtures with low concentrations of MeAIB. Uptakes are converted to nanomoles per minute per milligram of protein. The reaction mixture contained either NaCl (●) or choline chloride (○).

and the amount of MeAIB that would be required to completely exclude the A system. As shown in Figure 4, 2 mM MeAIB or greater was sufficient to completely block the A system. In this experiment, the A system accounted for 83% of the total $Na^+$-dependent transport of proline. However, this amount is variable from experiment to experiment and in most cases accounts for 95–100% of the total $Na^+$-dependent transport (Table II). Transport in the absence of $Na^+$ measures the L system and a nonsaturable component of transport.

To further explore whether other $Na^+$-dependent transport systems are present in vesicles from CHO-K1, we measured the initial rates of transport of proline, MeAIB, serine, and alanine in the presence and absence of 10 mM MeAIB and in the absence of $Na^+$. In intact cells, alanine and serine are mainly transported 85% and 95%, respectively, by the ASC system (Bass et al., 1981; Shotwell et al., 1981). The results (Table II, Figure 5) show that these two amino acids are predominantly transported by the A system in vesicles since over 90% of the $Na^+$-dependent uptake is inhibited by 10 mM MeAIB. MeAIB, at 10 mM, has no measurable effect on the membrane potential of these vesicles (see below). Such an effect of lowering the membrane potential, if it had occurred, could possibly have led to an overestimation of the A system and would have obscured the activity of the ASC and P transport systems. These results indicate that if the ASC system exists at all in these vesicles, it contributes insignificantly to the total velocity of transport of these amino acids. Since the velocity of proline uptake in the presence of MeAIB is close to, and in some cases equal to, that obtained in the absence of $Na^+$, the results also indicate that the P system is essentially absent from these vesicles prepared from CHO-K1.

*L System.* Leucine uptake and phenylalanine uptake at a concentration of 20 μM are transported entirely by a $Na^+$-independent system which is not affected by the presence of MeAIB (Table II). It appears that both of these amino acids are being transported by a system which is similar to the L system of amino acid transport. The pH profile of leucine transport (Figure 3) also supports this conclusion (Shotwell et al., 1981).

Table II: Uptake of Neutral Amino Acids[a]

| amino acid | additions to reaction | uptake (nmol/mg of protein) for uptake time (s) of 15 | 30 | 60 |
|---|---|---|---|---|
| proline | 100 mM NaCl | 0.14 | 0.16 | 0.20 |
| | 100 mM NaCl + 10 mM MeAIB | 0.05 | 0.05 | 0.05 |
| | 100 mM choline chloride | 0.05 | 0.05 | 0.05 |
| MeAIB | 100 mM NaCl | 0.044 | 0.044 | 0.044 |
| | 100 mM NaCl + 10 mM MeAIB | 0.016 | 0.013 | 0.020 |
| | 100 mM choline chloride | 0.013 | 0.011 | 0.013 |
| alanine | 100 mM NaCl | 0.155 | 0.20 | 0.23 |
| | 100 mM NaCl + 10 mM MeAIB | 0.056 | 0.067 | 0.078 |
| | 100 mM choline chloride | 0.036 | 0.070 | 0.070 |
| serine | 100 mM NaCl | 0.156 | 0.244 | 0.390 |
| | 100 mM NaCl + 10 mM MeAIB | 0.070 | 0.09 | 0.093 |
| | 100 mM choline chloride | 0.05 | 0.07 | 0.07 |
| leucine | 100 mM NaCl | 0.055 | 0.077 | 0.062 |
| | 100 mM NaCl + 10 mM MeAIB | 0.046 | 0.055 | 0.054 |
| | 100 mM choline chloride | 0.058 | 0.079 | 0.062 |
| phenyl-alanine | 100 mM NaCl | 0.067 | 0.067 | 0.060 |
| | 100 mM NaCl + 10 mM MeAIB | 0.067 | 0.067 | 0.060 |
| | 100 mM choline chloride | 0.067 | 0.067 | 0.067 |

[a] The uptakes of each amino acid using various conditions were initiated by adding 0.1 mM (Ala, Pro, Ser, and MeAIB) or 0.02 mM (Leu and Phe) to the vesicle suspension (45 μg of vesicle protein) in reaction buffer containing 0.01 M Tris–phosphate, pH 7.5, 5 mM $MgCl_2$, and 0.125 M sucrose and were performed in duplicate as described under Materials and Methods. All concentrations given are final concentrations. The experiment was repeated twice with similar results.

Table III: Ion Preference of Proline Uptake

| salt[a] | uptake [nmol (mg of protein)$^{-1}$ min$^{-1}$][b] | salt[a] | uptake [nmol (mg of protein)$^{-1}$ min$^{-1}$][b] |
|---|---|---|---|
| NaCl | 0.171 (100) | KSCN | 0.073 (43) |
| NaSCN | 0.310 (181) | LiCl | 0.078 (46) |
| $NaNO_3$ | 0.240 (140) | RbCl | 0.062 (36) |
| $Na_2SO_4$ | 0.170 (99) | choline chloride | 0.050 (30) |
| KCl | 0.075 (44) | | |

[a] The concentration of each salt was 100 mM. [b] Uptakes were performed with 110 μg of mixed vesicle protein from CHO-K1. Proline concentration was 0.1 mM. Uptake was for 30 s, and the numbers in parentheses are the percentage of uptake of proline in the presence of NaCl. Since the ASC and P systems are essentially absent in vesicle of CHO-K1 cells and since the L system is $Na^+$ independent and accounts for 5–10% of proline transport, the direct measure of proline transport as employed here provides a reliable measure of A-system activity.

*Kinetic Parameters for Proline Transport through the A System.* The Michaelis–Menten parameters for proline uptake through the A system using the mixed membrane vesicles are 1.37 (0.81–1.77) mM and 1.17 (0.81–1.69) nm min$^{-1}$ mg of protein)$^{-1}$ for the $K_m$ and $V_{max}$, respectively. These values are somewhat similar to those reported for proline uptake through the A system in whole cells (Moffett et al., 1983; Moffett & Englesberg, 1984).

*Ion Specificity of Proline Uptake.* Table III shows that, in mixed vesicles made from CHO-K1, anions stimulated the initial velocity of transport in the order $SCN^- > NO_3^- > SO_4^{2-}$, $Cl^-$, probably reflecting their relative permeability to these membranes. The substitution of $K^+$, $Li^+$, or $Rb^+$ for $Na^+$ reduced uptakes to the level found with choline chloride replacing any of the sodium salts.

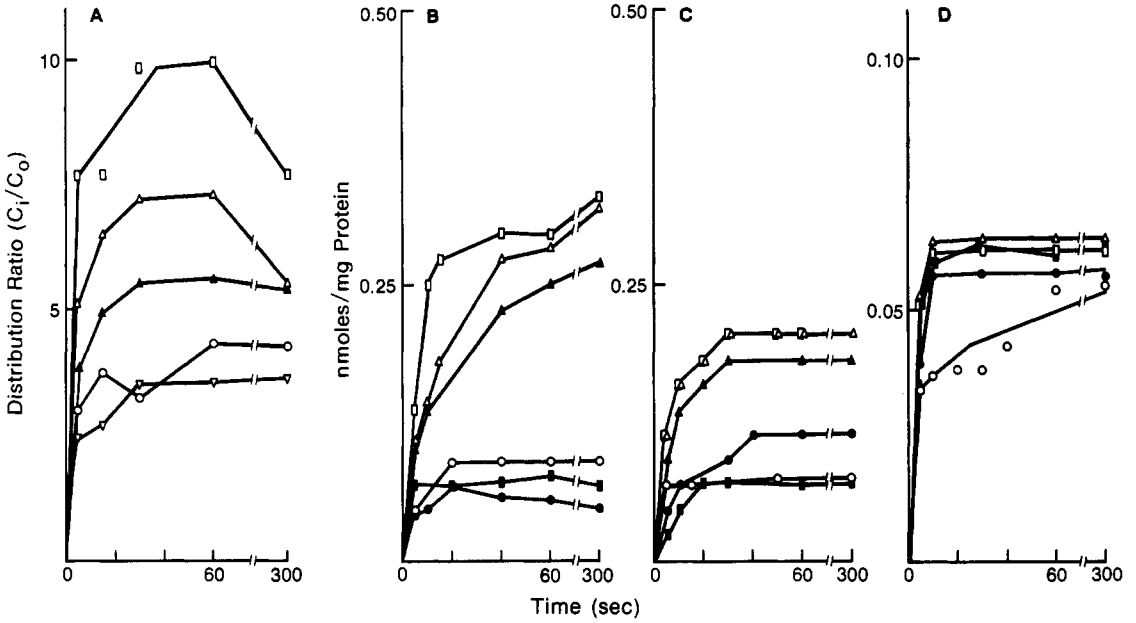


FIGURE 5: Effects of ion gradients on $TPP^+$, proline, alanine, and leucine uptake. Uptakes of the following amino acids were performed in the standard reaction. Mixed vesicle from CHO-K1 containing 50 μg of protein/sample was employed. (A) 0.02 mM $TPP^+$ uptake. (B) 0.1 mM proline uptake. (C) 0.1 mM alanine uptake. (D) 0.02 mM leucine uptake. The following additions were made: 15-min pretreatment with 90 μM valinomycin + 50 mM KCl followed by 10 times dilution into 100 mM NaCl (□) as above + 10 mM MeAIB (■); 15-min pretreatment with nigericin (54 μM) in the presence of 50 mM KCl followed by a 10 times dilution in 100 mM NaCl reaction buffer (○); vesicle not pretreated was added to a reaction mixture with 100 mM NaCl (▲), 100 mM choline chloride (●), or 100 mM NaSCN (△); valinomycin pretreatment without dilution with respect to KCl (▽).

Table IV: Membrane Potential and Effect of Na Chemical Gradient on Proline Uptake in CHO-K1, $Ala^r4$, and $Ala^r4$-H3.9 Mixed Vesicles[a]

| | | CHO-K1 | | | ala$^r$4 | | | ala$^r$4-H3.9 | | |
|---|---|---|---|---|---|---|---|---|---|---|
| additions[b] | salts (100 mM) | $TPP^+$[d] ($C_i/C_o$) | proline[c] (nmol/mg of protein) 10 s | 60 s | $TPP^+$ ($C_i/C_o$) | proline (nmol/mg of protein) 10 s | 60 s | $TPP^+$ ($C_i/C_o$) | proline (nmol/mg of protein) 10 s | 60 s |
| (1) valinomycin | NaCl | 5.4 | 0.13 | 0.29 (2.03)[e] | 5.6 | 0.34 | 0.43 (4.1) | 5.74 | 0.81 | 1.1 (7.7) |
| (2) valinomycin | choline chloride | 5.4 | 0.06 | 0.068 (0.5) | 6.1 | 0.08 | 0.074 (0.52) | 4.34 | 0.08 | 0.19 (1.4) |
| (3) valinomycin | NaCl + KCl | 2.6 | 0.10 | 0.094 (0.7) | 3.6 | 0.11 | 0.071 (0.5) | 2.40 | 0.19 | 0.30 (2.1) |
| (4) valinomycin + monensin | NaCl | 5.1 | 0.17 | 0.18 (1.2) | 5.4 | 0.27 | 0.17 (1.2) | 5.85 | 0.75 | 0.58 (3.0) |
| (5) valinomycin + monensin | choline chloride | 5.1 | 0.06 | 0.056 (0.4) | 6.1 | 0.09 | 0.04 (0.28) | 5.14 | 0.11 | 0.23 (1.6) |
| (6) valinomycin + nigericin | NaCl | 3.0 | 0.07 | 0.082 (0.58) | 3.1 | 0.09 | 0.068 (0.48) | 2.62 | 0.17 | 0.22 (1.5) |
| (7) valinomycin + nigericin | choline chloride | 2.5 | 0.07 | 0.09 (0.64) | 2.8 | 0.11 | 0.035 (0.25) | 2.22 | 0.10 | 0.12 (0.82) |

[a] To start the uptake, reaction mixture was added to the ionophore-treated vesicles and diluted 10-fold to produce a $K^+$ gradient and an inside negative potential. [b] The vesicles were preincubated with 50 mM KCl and valinomycin or with 50 mM KCl, valinomycin, and nigericin for 15 min. Monensin was added with the reaction mixture. Concentrations of the ionophores were 90 μM valinomycin, 27 μM nigericin, and 14 μM monensin. [c] The final concentration of proline was 0.1 mM. Since we have shown that most of the $Na^+$-dependent uptake of proline is inhibited by MeAIB and uptake with choline chloride substituted for $Na^+$ is the same for all vesicles regardless of the source (mutant or wild type), the increases in proline uptake in mutant vesicles reflect increases of proline uptake through the A system. [d] $TPP^+$ uptakes were performed by using a 60-s time period and with type EH filters from Millipore as described under Materials and Methods. [e] The numbers of parentheses for the 60-s proline uptake refer to the distribution ratio based on 1.4 μL of $H_2O$/mg of protein.

*Nature of the Effect of $Na^+$ and $K^+$ Ion Gradients on A-System Uptake in Vesicles.* We studied the effect of ion gradients on transport of amino acids by using ionophores which are specific for either sodium or potassium. Also, to measure the contribution of the different ionophores and conditions on the membrane potential, we followed $TPP^+$ uptake under the same conditions. $TPP^+$ and its derivatives have been shown to give an indication of the membrane potential in both whole cells and membrane vesicles (Schuldiner & Kaback, 1975; Lever, 1977b,c). $TPP^+$ steady-state uptake is reached in less than 30 s (Figure 5). A system transport for proline, alanine, and leucine was determined by measuring the uptake in the presence and absence of MeAIB as previously described. When vesicles were preloaded with $K^+$ in the presence of the ionophore valinomycin, a $K^+$-specific ionophore (Pressman, 1976), and then diluted into a reaction mixture containing labeled proline or alanine and $Na^+$ (100 mM NaCl), a 10-fold dilution of $K^+$ was achieved, and there was a marked increase in A-system activity as compared to that obtained when vesicles were subjected to only a $Na^+$ gradient as produced with NaSCN or NaCl (Figure 5). Moreover, the velocity and steady state of proline transport were greater when the $Na^+$ gradient was established with NaSCN than with NaCl as we have shown in Table III. These increases in A-system activity correlated with increases in negative membrane potential (internal negative), as measured by $TPP^+$ uptake. When valinomycin- and KCl-preloaded cells are diluted with a reaction mixture containing choline chloride instead of NaCl, there is no effect on membrane potential as measured by the $TPP^+$ distribution ratio, but there is a marked decrease in proline uptake, demonstrating the requirement for $Na^+$ (compare rows 1 and 2, Table IV).

To further explore the role of $Na^+$ and its chemical gradient had on A-system activity, we used the ionophore monensin, an ionophore which catalyzes a $Na^+/H^+$ exchange with a stoichiometry of 1:1 and therefore dissipates the sodium

chemical gradient (Pressman, 1976). Monensin had no effect on $TPP^+$ uptake when a negative interior potential was provided by pretreatment with valinomycin and KCl followed by dilution with respect to KCl (compare row 1 with rows 4 and 5, Table IV). In these experiments, the monensin was added with the reaction mixture. Since $TPP^+$ uptake was not affected, the $Na^+$ chemical gradient does not appear to be involved in the generation of the membrane potential in these vesicles under these conditions. Also, the initial velocity of proline transport was not affected, but the distribution ratio was decreased. When choline chloride replaced NaCl in the reaction mixture, there was a decrease in both the initial rate and the distribution ratio. Further experiments show that when monensin is preincubated with the vesicles in the presence of 100 mM NaCl the results are the same; i.e., the initial rate is not affected, but the distribution is diminished (data not given).

Nigericin, a $K^+$ ionophore, in contrast to valinomycin, carries out a nonelectrogenic $K^+/H^+$ exchange (Pressman, 1976). When vesicles were preincubated with valinomycin, nigericin, and 50 mM KCl, followed by the addition of 100 mM NaCl, and a 10-fold dilution of KCl was obtained, a decrease in the initial velocity and distribution ratio for proline uptake through the A system occurred (compare row 1 with row 6, Table IV). A similar response was found when vesicles pretreated with valinomycin and 50 mM KCl were diluted into the reaction mixture containing an equal concentration of KCl (Table IV, row 3) or when vesicle pretreated with nigericin and KCl was diluted into NaCl (Figure 5). $TPP^+$ uptake under either of these conditions is decreased when compared to valinomycin pretreatment alone. Since the $Na^+$ chemical gradient was maintained, these data indicate that the electropotential, generated by $K^+$, is needed for maximum A-system activity at both the initial rate and the steady state.

We have proposed that the ASC and P systems are essentially not functional in the vesicles prepared from CHO-K1. The strongest evidence supporting this conclusion with regard to the ASC systems is based upon the almost complete inhibition of alanine and serine transport by MeAIB (Table II). Since we have established that an interior negative membrane potential is required for maximum $Na^+$-dependent uptake of alanine or proline, we examined the possibility that the high concentration of MeAIB used to measure the A system might have caused a depolarization of the membrane which resulted in the inhibition of $Na^+$-dependent uptake. To test this hypothesis, we measured the uptake of $TPP^+$ in the presence and absence of MeAIB using NaSCN to establish the $Na^+$ chemical gradient and an interior negative potential. We could not detect any significant change in the potential as measured by $TPP^+$ uptake as a result of the presence of 10 mM MeAIB in the reaction mixture. Similar results were obtained with the addition of 0.1 mM proline and of 0.1 mM proline and 10 mM MeAIB (data not shown). It would seem that the effect on the potential, as a result of uptake of these amino acids, is minimal and is not detectable by our procedure and therefore presents no problem in our estimation of the A and ASC systems in these vesicles.

*Comparison of Amino Acid Uptake in Mutant and CHO-K1 Vesicles.* We found that the mutants had increases in A-system activity over that found in CHO-K1 that paralleled those found with intact cells whether the A system was measured by proline uptake or directly with MeAIB (Table V). The higher A-system uptake in the mutants is not due to differential enrichment of the A system in mutant vesicles as compared to those of the parental CHO-K1 cells since the activity of 5′-nucleotidase, a plasma membrane marker, was found to be similar in the mutant and CHO-K1 vesicles, with that of CHO-K1 being slightly higher than those of either of the mutants. Also, the values for leucine uptake in the vesicles were identical for the mutants and CHO-K1 as in the whole cells (Moffett et al., 1983; J. Moffett and E. Englesberg, unpublished data).

Table V: Amino Acid Uptake in Mixed Vesicles and Plasma Membrane from CHO-K1, Ala$^r$4, and Ala$^r$4-3.9

| | uptake [nmol (mg of vesicle protein)$^{-1}$ min$^{-1}$][a] | | | |
|---|---|---|---|---|
| | proline[b] | MeAIB[c] | leucine[d] | 5′-nucleotidase[e] |
| CHO-K1 | | | | |
| mixed vesicle | 0.35 ± 0.02 | 0.29 ± 0.05 | 0.34 ± 0.06 | 4.41 |
| plasma membrane | 0.84 ± 0.19 | 0.60 ± 0.24 | 2.16 ± 0.47 | 11.72 |
| ala$^r$4 | | | | |
| mixed vesicle | 0.084 ± 0.18 | 0.48 ± 0.04 | 0.28 ± 0.015 | 4.48 |
| plasma membrane | 1.44 ± 0.19 | 1.68 ± 0.54 | 2.16 ± 0.39 | 6.12 |
| ala$^r$4-H3.9 | | | | |
| mixed vesicle | 6.84 ± 0.36 | 5.34 ± 0.23 | 0.31 ± 0.04 | 3.72 |
| plasma membrane | 9.12 ± 1.10 | 7.44 ± 1.18 | 2.16 ± 0.6 | 8.11 |

[a] Uptakes were performed as described under Materials and Methods and in the legend of Table II with the exception that NaSCN was substituted for NaCl. The amount of vesicle protein used in the determinations was 100–200 μg for the mixed vesicle uptakes, depending on the vesicle source, and 10–25 μg for the purified plasma membrane. [b] Uptake of proline is the uptake through the A system (proline uptake minus proline uptake in the presence of 10 mM MeAIB). The concentration of proline was 0.1 mM, and the uptake was determined by using a 10-s time period. [c] Uptake of MeAIB was the same as that given for proline. [d] Leucine uptake was performed in the presence of 100 mM choline chloride in place of NaSCN. The concentration of leucine was 0.02 mM. A 5-s uptake was used. [e] 5′-Nucleotidase activity was expressed as nanomoles per milligram of protein per minute.

In addition to the increase in A-system activity, we detected a significant increase in proline uptake through other $Na^+$-dependent systems in vesicles of ala$^r$4-H3.9 from experiments similar to that described in Table II with CHO-K1. As we have shown in that table, there was little if any transport through such a system with CHO-K1. To ascertain as to whether our procedure for assaying the A system in ala$^r$4-H3.9 was reliable, we performed an experiment similar to that described in Figure 4 for CHO-K1, and we obtained similar results; i.e., 2 mM MeAIB or greater completely saturated the A system (data not given). Experiments with ala$^r$4 showed similar but smaller increases in other $Na^+$-dependent transport systems that paralleled that found with the intact cells, but results varied so that on occasion no $Na^+$-dependent transport other than the A system was detectable. Because of the small size of the increase of velocity in vesicles of ala$^r$4-H3.9 [<0.16 nmol min$^{-1}$ (mg of protein)$^{-1}$ with KCSN], we did not attempt to break it down into the individual transport systems that might be responsible for the increase.

Although the A-system activity was higher in the mutants than in the intact cells, the overall increase in activity was less than anticipated. For example, in whole cells, ala$^r$4 and ala$^r$4-H3.9 have an initial rate of proline (0.10 mM) uptake through the A system of 5 and 41 times that found with CHO-K1, respectively (Moffett & Englesberg, 1984; J. Moffett and E. Englesberg, unpublished experiments), while

ala$^r$4 has about 2 times and ala$^r$4-H3.9 about 19 times more A-system activity than CHO-K1 in mixed vesicles. The latter presumably were measured under conditions in which the same electrochemical gradients were maintained (Table IV). In other experiments with mixed membrane vesicles of ala$^r$4-H3.9, A-system activity varied from 10 to 20 times that of CHO-K1. When plasma membrane vesicles are further purified from the mixed vesicles, increases in A-system activity of ala$^r$4 and ala$^r$4-H3.9 are 2 and 11 times that of CHO-K1, respectively. Normalizing these values with regard to 5′-nucleotidase activity produces, at the most, a 20% increase in the relative amounts of A-system activity in the mutants as compared to CHO-K1.

With vesicles in which we show the membrane potential to be the same, as measured by $TPP^+$ uptake, and in which the same external $Na^+$ concentration was employed, increases in A-system uptake by the mutant vesicle preparations were similar to that described above (Table IV). Of particular interest is the finding that ala$^r$4 and ala$^r$4-H3.9 also exhibited increases in distribution ratios from 2 to 4 times that of CHO-K1, respectively.

The mutants and CHO-K1 also behave in a similar manner in responses to changes in the membrane potential and $Na^+$ gradient. When the $K^+$ diffusion potential, produced by valinomycin and high internal $K^+$ concentration, is lowered by adding nigericin or by a high external $K^+$ concentration to reduce the $K^+$ gradient, the uptake of $TPP^+$ and the initial rates of proline transport and the distribution ratio decline in a similar manner (Table IV). This indicates that the mutants, as well as CHO-K1, require a negative potential for normal A-system activity. When monensin is added to the reaction mixture, the initial velocity of proline transport appears not to be affected in the mutant, as it was not affected in CHO-K1, and the distribution ratio is reduced to half but is still twice that found for CHO-K1.

## Discussion

The ability to study amino acid transport in membrane vesicles from mammalian cells provides a means to directly manipulate the energetics of the systems involved. The results in this paper show that differences in electrical potential and high concentration of external $Na^+$ are needed for maximum A-system uptake in membrane vesicles. In this study, a negative interior potential was produced by KCl and the electrogenic ionophore valinomycin or by using the permeant anion $SCN^-$. We have shown that although increasing the external $Na^+$ concentration produces increases in the initial velocity and concentrating ability of the A system, the $Na^+$ gradient (external > internal) has a minimal effect, if any, on the velocity of transport through the A system under conditions in which a membrane potential is provided by valinomycin and KCl. The $Na^+$ chemical gradient has no effect on the membrane potential under these conditions as measured by $TPP^+$ accumulation. Similar results were obtained by Lever (1977b,c) with 3T3 cells. The fact that high internal concentrations of $Na^+$, obtained by using monensin, appeared to have no effect on the initial rate of transport while decreasing the concentrating ability of the transporter suggests that high internal $Na^+$ concentration inhibits the ability of the A system to translocate substrate across the lipid bilayer, from outside to inside, when the amino acid is present at high internal concentration.

The vesicles had similar properties with regard to the A system, as found for whole cells, in terms of sensitivity of proline transport to inhibition by MeAIB, the $K_m$ for proline transport, and pH and temperature dependence. However, one major difference in $Na^+$-dependent uptake in whole cells and vesicles is that the ASC system does not appear to be present in the latter or if it is, it is functioning at a very low rate. We base our conclusions on the following findings: (1) The uptake of alanine and serine, substrates that are transported 85% and 95%, respectively, by the ASC system in whole cells, is almost totally inhibited by MeAIB, a specific inhibitor of the A system, and also MeAIB, at concentrations employed in this study, does not affect the membrane potential of the vesicles. (2) The pH dependence for alanine uptake is similar to that expected for the A rather than for the ASC system, as exhibited in intact cells. (3) The maximum initial rate of transport of alanine and serine in vesicles is similar to that found for proline, whereas in whole cells, the velocity of uptake of these amino acids is 4–8 times greater than that found for proline (Bass et al., 1981). Whether the fractionation procedure caused a major loss or inactivation of the ASC transporter protein, or whether some cytoplasmic component is required for ASC activity, cannot be determined by the data given in this report and will require further investigation.

The fact that the uptake of externally given proline (0.1 mM) and MeAIB (10 mM) does not significantly affect the membrane potential, as measured by the distribution of $TPP^+$, as mentioned above and shown under Results, is not surprising. The initial velocity of uptake through the A system is measured usually at 10 s. At this time interval, we calculate that the amount of MeAIB transported into the vesicles, on the basis of the kinetic parameters established with intact cells (Moffett et al., 1983), is 0.1 mM. (The amount of proline transported is much smaller.) The membrane potential (interior negative) established with 100 mM NaSCN or NaCl is maximal within 10 s (Figure 5). Presumably, the concentration of $Na^+$ (positive charge) contributed by the cotransport of $Na^+$ with proline plus MeAIB in 10 s is insignificant as compared to the interior negative charge established by the anion and the dissipation of the potential by leakage.

L-system activity was monitored by leucine transport, and it appears to be affected like L-system activity in intact cells. Leucine uptake does not require $Na^+$; it is not inhibited by MeAIB, but it is stimulated by low pH, another characteristic associated with the L system (Shotwell et al., 1981).

We have isolated mutants with increased A-system activity. Our work with these mutants has led us to propose that some of them are constitutive for the A system, resulting from a mutation in a regulatory gene, gene R1 or R2 (Moffett & Englesberg, 1984; Englesberg & Moffett, 1986; Englesberg et al., 1986) and causing the production of additional transporter molecules. From one of these constitutive mutants, ala$^r$4, we have isolated what may be a gene amplification mutant, ala$^r$4-H3.9, that affects further increases in A-system activity presumably as a result of the production of additional A-system transporters. To test an alternative hypothesis that the increases in A-system activity we have observed in these mutants might be due to mutations affecting the energetics of the A system rather than increases in the concentration of the A-system transporter, we compared the amino acid transport of ala$^r$4 and ala$^r$4-H3.9 with that of the parental cell line CHO-K1. We have shown that when the electrical potential and the external $Na^+$ concentrations are the same, vesicles prepared from mutants ala$^r$4 and ala$^r$4-3.9 exhibit higher initial velocities of transport of proline and MeAIB paralleling the velocities found in whole cells.

This increase that we have found in the number of A-system transporters in the mutants would be expected to produce an increase in the initial velocity, but not necessarily an increase

in the distribution ratio. However, we have found that both of these parameters are increased in the mutants. Similar results have been obtained in the whole cells when uptake of MeAIB, a nonmetabolizable analogue, is determined at steady state (J. Moffett and E. Englesberg, unpublished data). To explain this phenomenon, we propose that there is a leak of A-system substrates from both cells and vesicles which is not associated with the A-system transporter. This suggests that the amount of A-system ligand concentrated by the cells is below the concentration permitted by the energy of the system. Thus, in the mutants, the increases in the distribution ratio for A-system transport over that obtained with the wild type are probably the result of increases in the amount of transporter.

Although the velocities of transport through the A system in vesicles of the mutants, as compared to those of CHO-K1, reflected the increases shown with whole cells, the increases we found were only 25–50% of the expected values. There are a number of possible explanations for this discrepancy. Uptake may not be linear at 10 s, the time interval generally employed in measuring the A system in these vesicles. We do know that uptake through the A system is not linear beyond 15 s. The divergence from linearity is probably due to leakiness of the vesicles, as postulated above, and the resultant rapid dissipation of the membrane potential and, also, possibly to transinhibition (Moffett & Englesberg, 1984). Whether, because of their position, the additional transporter molecules placed in the membrane in the mutants are as stable as that of CHO-K1 should also be considered. We, however, cannot exclude the possibility that in addition to the increase in the number of transporters both mutants may have undergone some change affecting the energetics of the A system that has been uncoupled in the vesicles.

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