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Topical Review

A Genetic Approach to the Study of Neutral Amino Acid Transport in Mammalian Cells in Culture

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A genetic approach has been shown to be a valuable tool in the elucidation of the mechanism of membrane transport and its regulation in microorganisms [2, 45, 57]. During the past several years a similar approach has been extended to a study of amino acid transport in mammalian cells in culture. Methods have been developed for the isolation of mutants of structural and regulatory genes that control amino acid transport. An analysis of these mutants has provided some insight as to the nature of the genetic and metabolic regulation of several of these systems and has suggested a structural and regulatory relationship between some of them. A study of transport mutants also presents the possibility for the unraveling of the individual systems with their overlapping specificities and for the eventual elucidation of the various transport mechanisms involved.

Mutants have been isolated affecting the three major, neutral, amino acid transport systems (A, ASC and L) and a minor one (P). These systems are distinguished from one another based upon relative specificity, Na^+ dependency, pH optimum, and regulation [4, 8, 36, 40, 49]. System A is sodium dependent and transports mainly short, polar or straight chain amino acids such as alanine, glycine, and proline and the nonmetabolizable amino acid analogues, 2-amino isobutyric acid (AIB) and, 2-(methylamino)isobutyric acid. This system is *trans* inhibited, pH dependent, and repressible by A system amino acid. The ASC system is also Na^+ dependent, has a strong preference for serine, cysteine, alanine and threonine and will not transport or be inhibited by N-methylated substrates such as MeAIB. It has a broad pH range, and is not obviously repressible by amino acids. In CHO cells the ASC system has a broad range of substrate specificity [4, 49] and is the major Na^+ transport system in these cells. The P system is a Na^+-dependent system that appears to be specific for proline. So far it has been only described in CHO cells [36]. It is distinct from the imino transport system found in intestinal, renal and choroid plexus brush borders [35, 46, 52] since the latter also transports MeAIB. System L is Na^+ dependent, transports preferentially branched chain and aromatic amino acids and is *trans* stimulated.

Key Words amino acid transport · A system · ASC system · P system · L system, transport mutants · Na^+ K^+ gradient · regulation of transport · insulin connection.

Assay Procedures for Measuring Transport

In most cases, because of the lack of high-affinity specific substrates, the use of specific inhibitors and other exclusionary procedures has been employed by investigators to characterize these systems. We shall only describe those assay procedures used by investigators whose work is reported in this review. However, they do represent the general procedures employed in this field.

A System

We measure the A system in CHO-K1 by determining the fraction of the velocity of proline transport that is inhibited by saturating amounts of MeAIB. The initial, one-minute, velocity of proline transport, in the presence of MeAIB is subtracted from that determined with proline alone [4, 36–38]. (10 mM MeAIB is sufficient to eliminate the uptake of 0.05 M proline through the A system without affect-

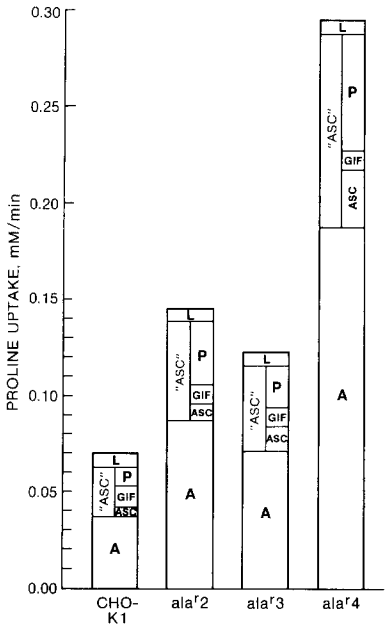

Fig. 1. A comparison of the contribution of the various transport systems to the initial velocity of uptake of 0.05 mM proline by CHO-K1, alaʳ2, alaʳ3 and alaʳ4. A demonstration of the errors involved in the estimation of the ASC system by not taking into account the P system and GIF. Data taken from references 36 and 37.

ing the determination of other Na^+ transport systems.) We chose proline as substrate since the A system is the major transport system for this amino acid [4, 49]. In addition, the mutants we studied were isolated for their ability to take up proline in the presence of inhibitory amounts of a competing amino acid and they were modified in proline transport. Although proline may be metabolized by CHO cells, under conditions of our assay, there was negligible loss of proline due to metabolism. Other investigators have used a similar procedure, as described above, but have employed AIB instead of proline [30, 48, 51]. Uptake of AIB on occasion has been employed directly to measure the A system [11]. In this use of AIB it should be remembered that this analogue is also transported to a minor degree by the ASC and L systems. MeAIB uptake has been used as direct measure of the A system [5, 13]. The advantage of this method is that MeAIB is nonmetabolizable and is apparently specific for the A system although this specificity has recently been questioned [4, 5]. Its disadvantage is that it is a poor substrate and is expensive.

ASC System

Oxender and his colleagues estimated the ASC system in CHO cells by determining that fraction of transport of alanine that is not inhibited by MeAIB and is Na^+ dependent. Transport of alanine in the absence of Na^+ is subtracted from that obtained with alanine in the presence of saturation amount of MeAIB [30, 48, 51]. It is recognized by these investigators that this assay procedure might include other MeAIB resistant, Na^+-dependent transport systems. In fact two additional MeAIB resistant, Na^+-dependent systems have been described; the P [36] and N [29] systems. However, since neither of these transport systems seem to be involved in alanine transport in CHO, the assay procedure, with the limitations specified above, is a reliable method for measuring the ASC system in these cells. We have used proline to measure the ASC systems in CHO cells. To eliminate the P system as a significant factor in determining the ASC system we employed the following methodology. The ASC system is measured by subtracting the velocity of proline transport in the presence of saturating amounts MeAIB and serine from that obtained in just the presence of MeAIB (Fig. 1) [36–38]. Serine is a specific inhibitor of the ASC system under conditions in which the A system is eliminated by MeAIB. Serine can be used as a direct measure of the ASC system since this substrate is relatively specific for this system in CHO cells, accounting for about 94% of its transport [4, 12, 17].

P System

The P system is defined as that velocity of transport of proline that remains when the velocity of transport of proline in the absence of Na^+ is subtracted from that obtained in the presence of proline, and saturating amounts of MeAIB, serine and glutamine. Glutamine removes a small yet undefined component of proline transport called the glutamine inhibitory fraction (GIF). GIF is apparently distinct from the N system. Without taking into account the P system and GIF, in the transport of proline [21], the involvement of the ASC system can be overestimated three to sevenfold at the proline concentrations generally employed (Fig. 1) [36]. We have not ruled out the possibility that the P system, so defined, may contain some yet undiscovered Na^+-dependent systems.

L System

We estimated the effect of mutation on the L system by measuring the velocity of proline transport in the absence of Na^+. This represents a minor fraction of proline transport and may include a non-

Table 1. Mutants affecting neutral amino acid transport

Parent mutant		Transport systems[a]				Gene[b]	Reference
		A	ASC	P	L		
CHO	ala^{r}2	2× V_{max}	1.8×	2× V_{max}	0	Regulatory, R1	36, 37
	ala^{r}3	2.3× V_{max}, 1.3× K_m	2.8×	3×	0	Structural	"
	ala^{r}4	5× V_{max}	4.5×	4.5×	0	Regulatory, R1	"
	ala^{r}6	5× V_{max}	3.5×	3.4×	0	Regulatory, R2	Perier & Englesberg (*unpublished data*)
	MeAIB^{r}22	0	6× V_{max}	0	0	Regulatory, Rasc	17 (this paper)
	MeAIB^{r}1143S	0	2.7×	0	0	?	17
	MeAIB^{r}44s	0	3×	0	0	?	"
	MeAIB^{r}47s	0	2.3×	0	0	?	"
	MeAIB^{r}56s	0	2.7×	0	0	?	"
	MeAIB1154e	1.9X		0	0	?	"
	MeAIB1155e	0	1.8×	0	0	?	"
	MeAIB1157e	1.7×		0	0	?	"
	MeAIB1158e	2×	1.8X	0	0	?	"
CHO	ts025C1	0	0	—	39°C 2.7 V_{max}	Leucyl t-RNA synthetase	30
	tsH1	0	0	—	38.5°C 3× V_{max}	Leucyl t-RNA synthetase	39, 48
	tsH1-C11	0	0	—	34°C 2.6× V_{max}	L-system regulatory?	"
	tsH1-D10	0	0	—	34°C 1.8 V_{max}	" "	"
	tsH1-F10	0	0	—	34°C 2.8 V_{max}	" "	"
C11B6[c]	C5F6	—	—	—	0.38 × C11B6	?	41
CHO	CHY-1	1.5× V_{max} 3.5× K_m	0	—	0	Structural?	13
A9	Phe^{r}1	0.19× V_{max} 0.2× K_m	—	—	0.2× V_{max} 0.2× K_m	Structural?	15
	Phe^{r}13	0.41 V_{max}, 0.31 K_m			0.25× V_{max} 0.2× K_m	Structural?	15
A9	5FT^{r}23	0.67 K_m	—	—	0.05× V_{max} 3× K_m	Structural?	53, 54
	5/FT^{r}37	3.7× K_m	—	—		Structural?	"
SVT2	Met^{r}201	0.76× V_{max}, 2× K_m	—	—	—	Structural?	26
	Met^{r}Z1	0.65× V_{max}, 1.8× K_m	—	—	—	Structural?	"
	Met^{r}Z5	0.77× V_{max}, 2.4× K_m	—	—	—	Structural?	"
	Met^{r}C2	0.32 × V_{max}	—	—	—	Regulatory?	"
	Met^{r}301	2× K_m	—	—	—	Regulatory?	"
	Met^{r}10	2× K_m	—	—	—	Regulatory?	"
	GF14 GF17	0.2 V_{max}	—	—	0	Regulatory?	11
	GF18[d]	0.25× V_{max}	—	—	0	Regulatory?	11
MDCK	T1	3-7×	0	—	<	?	5

[a] Comparison with parental culture, 0 = no change in transport; — = not determined. When only V_{max} is given there has been no change in K_m; X = increase in velocity over parental culture.

[b] Criteria employed in this paper in defining structural and regulatory genes—Regulatory = change in V_{max} no change in K_m, recessive; Regulatory? = possible regulatory gene mutation, changes in V_m, no change in K_m. Structural genes = change in K_m, co-dominant; or temperature-sensitive mutant; Structural gene? = possible structural gene mutation, change in K_m or K_m and V_{max}; ? = no clue.

[c] A subclone of C11.

[d] Also 0.55X high affinity glutamate system.

saturable component [36]. The uptake of leucine in the absence of Na^+ has been used to measure the L system [12]. The nonsaturable compound can be eliminated by subtracting the velocity obtained in the presence of saturable amounts of 2-aminobicyclo-(2,2,1)-heptane-2-carboxylic acid (BCH) [30, 39, 48].

Mutant Isolation Procedures

(*See* Table 1 for a listing of the characteristics of the mutants isolated.)

Use of Amino Acids and Amino Acid Analogues-Amino Acid Antagonism and Other Toxic Effects

We screened a number of cell lines, CHO, WI38, A9, 3T3, and SV373 for inhibition of growth as a result of increasing the concentrations of individual amino acids in MEM (Eagle's Minimum Essential Medium [15]) and found that all cell lines tested exhibited similar sensitivities to certain amino acids although there were some notable exceptions [15]. We proposed that this inhibition of growth could be used as a selective tool for isolating mutants affecting specific amino acid transport systems. Inhibition could be the result of amino acid antagonism at the transport level or at the level of the amino acyl *t*-RNA synthetases, or the inhibitory amino acids may be toxic at high intracellular concentrations for a variety of other reasons [14].

Amino acid transport mutants have been isolated from CHO by taking advantage of the unique requirement of this cell line for proline. It was discovered that amino acids and amino acid analogues that are transported by the A system competitively inhibit the transport of proline that is present in the medium and thereby inhibit the growth of this cell

line [9]. Subsequent to EMS mutagenesis, single-step mutants have been isolated that are resistant to alanine and MeAIB. EMS treatment increased the frequency of resistant mutants significantly [17, 36]. Since the inhibition is competitive the concentration of the inhibitory amino acid employed depends on the amount of proline added to the medium, the concentration of the serum and whether or not the serum has been dialyzed. It is also necessary to limit the cell density to obtain clear selection. Pro^+ revertants, that appear at a low frequency under these conditions, can be detected by plating in MEM plus the nonessential amino acids excluding proline plus 4% amino acid free fetal calf serum (FCS). Subsequent to mutagenesis with EMS and allowance for phenotypic expression, we found that the frequency of ala^r pro^- mutants, selected in MEM + 4% FCS + nonessential amino acid containing 0.05 mM proline with the addition of 12 mM alanine, was 5.8×10^{-4}, at least 50× the spontaneous frequency. Mutants isolated by this procedure were stable and had higher rates of proline transport through the A, as well as through the ASC and P systems, thus compensating for the poor uptake of proline by the parental cell line due to the presence of the inhibitory amino acid.

MeAIB resistant mutants ($MeAIB^r$) are routinely isolated, subsequent to mutagenesis and phenotypic expression, in MEM-containing amino acid-free 4% FCS plus the nonessential amino acids with 0.05 mM proline and 6.5 mM MeAIB [17]. These mutants affected mainly, but not exclusively, the ASC system.

Beginning with an alanine resistant mutant, ala^r4, we have succeeded in isolating mutants that have a 30- to 60-fold increase in A-system activity. This was accomplished by lowering the concentration of proline to 0.03 mM, so as to increase the sensitivity of the culture to alanine, and employing consecutive step-wise selection in increasing concentration of alanine, subsequent to treatment with hydroxyurea [6]. Evidence suggests that the increase in activity over that found with ala^r4 is the result of gene amplification.

Variants of A9 mouse L cells that affected both the L and A systems were isolated as resistant to 20 mM phenylalanine in MEM + 10% FCS. The frequency of occurrence of these variants was increased significantly by mutagenesis and they were stable when grown under nonselective conditions. Phenylalanine which appears to be moderately toxic to all cell lines does reduce the internal concentrations of many amino acids that are transported by the L as well as the A systems. However, the inhibition of growth does not appear to be the result of this effect. Rather, at the concentrations employed, the significant factor that is responsible for the growth inhibition appears to be a direct effect of phenylalanine on protein synthesis [14].

It was originally observed that SVT2 was uniquely sensitive to methionine [15]. Spontaneous mutants were isolated in single-step or multistep procedures that were resistant to 20 to 40 mM methionine in MEM [26]. These mutants were shown to have reduced A-system activity. Mutant frequency could be increased with treatment with EMS. It was originally observed that the methionine resistant mutants had the characteristics of flat revertants, thereby suggesting a relationship between methionine sensitivity and the transformed phenotype. However, when the parental SVT2 was subsequently examined it was found to contain a mixture of transformed and nontransformed cells (flat revertants), and upon recloning the culture and selecting a clone that had the transformed phenotype it was observed that all methionine-resistant mutants isolated maintained the transformed phenotype.

Mutants resistant to 0.4 mM 5-fluorotryptophan, isolated from mouse A9 cells subsequent to mutagenesis, have alterations in the A or both A and L systems [53, 54].

Tritium Suicide Selection

A tritium suicide selection technique has been used successfully in the isolation of transport mutants from mouse lymphocytic cell GF-14, [11, 18] and from CHO cells [13, 41]. In this procedure cells are usually first mutagenized, exposed to a tritiated amino acid at high specific activity, frozen and stored. An amino acid is chosen that is specifically taken up by the transport system under study. The rationale for this procedure is that normal cells will take up more of this amino acid than mutant cells that are deficient in the transport system in question and will be selectively killed by radioactive decay.

Mutants of GF-14 cells, deficient in the A system, were isolated using ^3H AIB. Out of 200 surviving cells that were tested two, GF17 and GF18, were shown to be transport mutants [11, 18]. Similar mutants were also isolated from CHO cells using ^3H proline [13]. In order to obtain high internal concentrations of proline, protein synthesis was allowed to occur during uptake. Since CHO is pro^- there is no interference by proline produced by the cells. The cells were starved for amino acid to minimize *trans* inhibition and incubated in growth medium containing labeled proline. The medium lacked amino acids that would compete with proline

transport through the A system. Also, it contained sufficient amount of L-system amino acids, preventing uptake of the label through that system. Under these conditions significant proline accumulated to reduce survival to less than 1 cell in 10,000. Out of 7000 surviving colonies 20 presumptive transport mutants were identified by a replica plating technique and one, CHY-1, turned out to have a decreased uptake of proline and AIB. Since this procedure would also select for protein synthesis mutants [1, 55] selection was performed at 37°C on the assumption that protein synthesis mutants would be lethal and temperature conditional lethals would be eliminated at this temperature. Since proline will also be taken up by the ASC and P systems the selection is not as stringent as one would like.

Leucyl-tRNA synthetase, temperature-sensitive (ts) mutants of CHO were isolated using a ^{3}H leucine suicide procedure [1, 55]. Oxender and his colleagues [39, 48, 51] discovered that when these cells were incubated at moderate nonpermissive temperatures (37 to 39°C) for several hours there was a two- to threefold increase in the velocity of transport of leucine through the L system over that found at 34°C. Beginning with one such mutant CHO-tsH1, and subsequent to mutagenesis, temperature resistant mutants C11, D10, and F10 were isolated. These mutants have increased levels of L-system activity when grown under both permissive and nonpermissive conditions [48].

C11B6, a subline of C11, was employed in a ^{3}H leucine suicide experiment designed to isolate mutants defective in the L system [41]. An attempt to isolate such mutants with normal CHO-K1 cells was unsuccessful presumably because the L system is shut down in these cells due to regulation and not enough of the isotope is taken up to be effective. Cells were mutagenized with EMS, incubated for several days in MEM supplemented with 3-5 times the normal concentration of essential amino acids to allow for phenotypic expression and exposed to ^{3}H leucine for 3 min in the absence of Na^+. Uptake for the short time period in the absence of Na^+ was designed to decrease the possibility of isolated protein synthesis mutant and mutants of Na^+-dependent systems, respectively. The cells were then frozen to allow for ^{3}H decay. The survivors were mutagenized again and subjected to the same regime as described above. Three cell lines were isolated, C9, D3 and C5F6. The latter mutant was shown to have approximately 0.3 the L system activity of the parental cell line, C11B6, under both "repressed" and "derepressed" conditions, activity equal to that of the "repressed" CHO-K1 tsH1 original parent. The phenotype of this mutant is difficult to interpret without further genetic analysis of both C11B6 and C5F6 to determine whether the phenotype is dominant or recessive.

The BUdR (5-bromodeoxyuridine)—Visible Light Technique

The BUdR technique [44] has been employed in the isolation of mutants of CHO cells that are deficient in the L system [12]. Cells were mutagenized with EMS, grown in a medium containing BUdR and limiting amounts of leucine. The rationale for this procedure is that since leucine is largely transported by the L system, mutants defective in the L system would grow more slowly than the parent cells in such a medium and thus incorporate less BUdR into their DNA. Upon irradiation with visible light cells that have incorporated proportional larger amounts of BUdR would be killed off first, resulting in an enrichment for cells deficient in the L system. To insure that such mutants would be isolated the medium is supplemented with excess L-system amino acids as described above. One mutant, CHY-2, out of seven possible mutants, was chosen for detailed study. This mutant turned out to be pleiotropic, having a deficiency in the L, A and Ly^+ systems.

Selection for Tumorigencity

In vivo selection for tumorigenicity, subsequent to treatment of MDCK cells (dog kidney epithelial cells) with a mutagen, has resulted in the isolation of a clone, MDCK-T_1, with increased velocity of transport of MeAIB. Evidence suggests that these cells may be constitutive for the A system. However, the experiments are complicated by the appearance of biphasic kinetics of MeAIB transport as a result of the mutation or upon starvation of the parental culture [5].

Regulation of the A System

The effect of amino acid starvation on A-system activity has been described in several different cell lines and has been recently reviewed [22, 27, 50]. Our study of this phenomenon in CHO-K1 supports a model (Fig. 2) for the regulation of this system based upon repression via negative control and post-translational modification of the A transporter [37, 38]. As shown in Fig. 2 the model also depicts a mechanism that we have proposed is involved in the joint regulation of the A, ASC and P systems, and it has been amended to include some of our recent findings, as summarized in this paper, regarding the

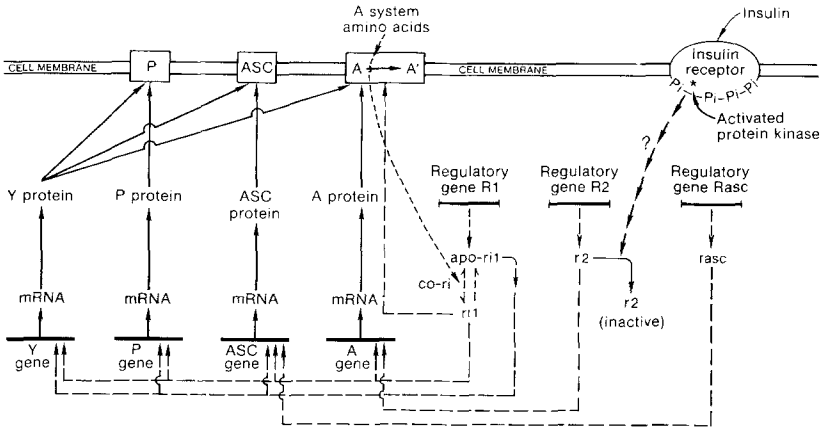

Fig. 2. Model for the regulation of the A system as well as the ASC and P systems and showing the proposed insulin connection [34, 37]

specific regulation of the ASC system [17] and the insulin induction of the A system [34, and E. Englesberg, J. Moffett and F. Perier, *unpublished data*].

We propose that the A system is regulated by at least two regulatory genes, R1 and R2. Gene R1 produces an apo-repressor-inactivator (apo-ri) which is in equilibrium with a repressor-inactivator (ri). In the derepressed state the equilibrium is toward apo-ri. Amino acids that are generally transported by this system for the most part act as both co-repressors (co-r's) and co-inactivators (co-i's) (co-ri's) and by binding to this regulatory protein shift the equilibrium toward the ri form. Although not indicated in Fig. 2 some amino acids can determine the extent of the repressor and activator function of this regulator (*see below*). The ri reacts with a controlling site of gene A, the structural gene for an A-system carrier protein, and prevents further synthesis of the corresponding m-RNA and also modulates the activity of the carrier by converting it into an inactive form. Superimposed upon the regulation by amino acids is the control by insulin. Regulatory gene R2 produces a constitutive repressor r2. R2 shares with ri the regulation of gene A and provides the insulin connection. Evidence suggests that when insulin binds to its receptor and presumably activates the tyrosine specific, cAMP independent protein kinase [10] it regulates the A system directly or indirectly by neutralizing r2. Both apo-ri and ri regulate the expression of the structural genes for an ASC and a P carrier protein and also a gene Y that codes for a protein that is shared by the ASC, P and A system. In addition to regulatory gene R1, regulatory gene Rasc also controls gene ASC by negative means.

Since the genetic analysis of the regulation of the A system has been performed with CHO cells, I shall review the evidence, prior to the introduction of genetic analysis that is in support of this model, with this cell line, although somewhat similar findings have been previously presented with other cells.

Evidence for Transcriptional and Post-Translational Regulation

When CHO cells are starved for amino acids that are generally transported by the A system there is a steady increase in A system velocity which peaks in 15-24 hr at about 3 to 5× the basal level. This increase in activity is a result of an increase in V_{max} (from 0.75 to 4.0 mmol/liter/min) with no accompanying increase in K_m and is inhibited by cycloheximide and actinomycin D. A decrease in amino acid pools that might initially cause *trans* inhibition [7] of the A system has been shown not to be responsible for the large increase in A system activity. Unlike human fibroblasts [19], CHO is similar to rat H4 [27] hepatoma cells in not requiring serum to exhibit this starvation-produced increase in A system activity (SPI) (E. Mendiaz and E. Englesberg, *unpublished data*).

Amino acids that are generally transported by this system inhibit SPI. *Trans*-inhibition is minimized in these experiments by depleting the intracellular amino acid pools by starving the cells in the presence of cycloheximide for 3 h. Cycloheximide inhibits further increases in A system activity and stabilizes the carrier under the conditions of our assay. Nonmetabolizable amino acids such as AIB and MeAIB are very effective inhibitors of SPI, indicating that metabolism is not a necessary requirement for this effect.

These results suggested that at least one component of the SPI occurs at the level of transcription. This was further borne out by following experiments. Cells were incubated in SPI buffer in the presence of cycloheximide and in the presence and absence of test amino acids to allow for the accumulation of m-RNA for the A-system transporter. Subsequently the cycloheximide was exchanged for actinomycin D and system-A activity was measured

Table 2. Comparison of the specificity of the A-system transporter and the apo-ri—the A-system carrier is not directly involved in A-system repression or inactivation[a]

	Inhibition of A system	Activity as		
		co-r	co-i	co-ri
β-alanine	—	+++++	—	+++++
Histidine	++	?	+++++	+++++
Hydroxyproline	++	?	++/+	+++++
Diaminobutyrate	+++++	—	—	—
Phenylalanine	+++++	?	++/+	/+
α-ketoglutarate	++++	?	?	—
Pyroglutamate	++++	?	?	—
Isoleucine	++/+	?	—	—
Valine	++	?	+/+	—
MeAIB, alanine, serine Proline, etc.	+++++	+++++	+++++	+++++

[a] Summary of data presented in Figs. 1-3, in Ref. 38. — = no activity. +++++ = 100%, ++++ = 80%, ++/+ = 50%; ++ = 40%; +/+ = 30%; /+ = 10%. ? = assay not performed

after varying periods of time. Cells incubated in buffer in the presence of cycloheximide showed increasing A-system activity when removed from cycloheximide and incubated in the presence of actinomycin D. Cells incubated in the presence of cycloheximide and A-system amino acids such as alanine, serine, proline and the amino acid analogues MeAIB and β alanine and treated to minimize transinhibition as described above, prevented this subsequent increase in activity while leucine, BCH, compounds not transported by the A system and diaminobutyrate, did not.

When A system-preferred amino acids are added to cells that have undergone SPI, the A system is transinhibited, irreversibly inactivated and repressed. During the first 30 min after the addition of these amino acids there is a rapid decrease in A-system activity. About 75% of this decrease can be reversed by starving the cells for 3 hr in the presence of cycloheximide in order to eliminate *trans*-inhibition. There is a further slow decline in activity after the 30 min, which is irreversible in the sense that depletion of the internal concentrations of the effector does not restore any further activity. Cycloheximide and actinomycin D have no effect on these decreases in activity [37] in contrast to results obtained with chick heart embryo cells [21]. The extent and rate of this irreversible inactivation is concentration dependent [38].

Specificity of the Apo-ri and the Elimination of the Direct Participation of the A System Transporter in the Regulation of the A System

Although most compounds that are transported by the A system, or inhibit transport through the A system, are effective in inhibiting SPI, by preventing the synthesis of m-RNA for the A-system carrier protein and inactivating the A-system carrier protein, there are significant exceptions to this rule (Table 2) [5, 24, 37, 38]. β-alanine, although unable to inhibit proline transport through the A system when present at 1000× the proline concentration, completely prevents the increase in A-system activity during amino acid starvation by inhibiting m-RNA synthesis. Histidine and hydroxyproline have a somewhat similar effect in inhibiting SPI [38]. Diaminobutyrate [37], phenylalanine, alpha ketoglutarate, pyroglutamate, isoleucine, and valine, compounds which inhibit A-system transport, listed in decreasing order of effectiveness, are all equally poor inhibitors of SPI [37]. It is of interest that although diaminobutyrate does not inhibit SPI it prevents alanine from acting as an inhibitor of this process. In essence it acts as anti-co-repressor as does indole-acrylic acid and indole-proprionic acid in the tryptophan operon [43].

This evidence clearly eliminates the A-system carrier as being directly implicated in the amino acid regulation of the A system as proposed by Gazzola et al. [20]. Since the A-system carrier is not involved and these effectors may be transported by diverse systems it is reasonable to propose that these amino acids act intracellularly. This is borne out by the analysis of constitutive mutants (*see below*). Similar conclusions were drawn from findings obtained with other cell lines [23, 24,58, and M. Kilberg, *personal communication*]. White and Christensen [58] have shown, in rat hepatoma cells HTC, that intracellular MeAIB rather than extracellular MeAIB functions as a "co-repressor." Heaton and Gelehrter [24] have shown with the rat hepa-

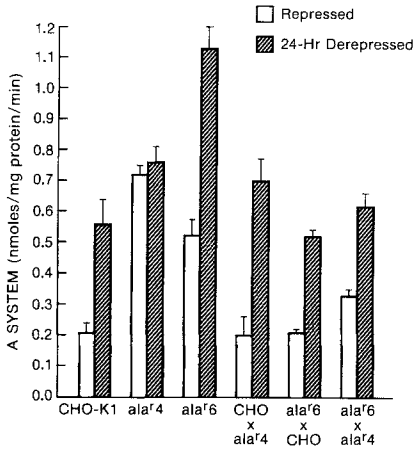

Fig. 3. Cell × cell hybridization showing that the phenotype of ala^{r}6 is recessive to that of CHO-K1 and ala^{r}6 complements ala^{r}4, thus defining regulatory gene R2 (E. Englesberg, J. Moffett and F. Perier, *unpublished data*). Experimental procedures are as described in Table 3, reference 37

toma cell HTC, that threonine, phenylalanine, tryptophan and tyrosine do not block A-system transport but are effective "co-repressors." M. Kilberg (*personal communication*) has found with rat hepatocytes that methionine, S methyl cysteine and ethionine are strong inhibitors of the A system but are ineffective as "co-repressors." Also we have shown that the K_m for MeAIB transport through the A system is two times the concentration of MeAIB required to produce one-half maximal inactivation [38].

So far the information presented, which excludes that available through analysis of mutants, leaves the following questions to be answered. How do amino acids affect both repression and inactivation? Is there another gene required for the inactivation step? Is the structural gene for the A-system carrier controlled by negative means (by a repressor) or by positive means (by an activator). The mutants described below, isolated on the basis of resistance to alanine, provide reasonable answers to these questions and support for the proposed model.

Mechanism of Repression, Negative Control and Separation of *Trans*inhibition from Inactivation

Alanine-resistant mutants, ala^{r}2 and ala^{r}4, are partial and full constitutive of the A system. Ala^{r}2 has about 2× the basal repressed level of the parental cell line. This increase is a result of twofold increase in V_{max} of proline transport through the A system. There is no change in K_m. Upon being subject to SPI, the A-system activity of this mutant reaches that of the parental culture when the latter is subjected to the same conditions. Ala^{r}4 has A-system activity equal to that of the repressed parental cell line and is not further derepressible. This mutant has a V_{max} 5× that of the repressed parental level with no significant change in K_m. Another important characteristic of ala^{r}4 is that, although it is subject to *trans*inhibition, it is resistant to the irreversible inactivation. This presents the first proof that *trans*inhibition is distinct from irreversible inactivation. These results suggest that the mutants have an increase in the number of A-system transporters when grown under repressed conditions. When these mutants were crossed to the parental culture the hybrids were found to have the phenotype of the repressed parental culture (Fig. 3). Segregants with the phenotype similar to ala^{r}4 were isolated from the hybrids at a high frequency, demonstrating that the recessive allele was present in the hybrid clones. Since ala^{r}4 was isolated in a single step at a relatively high frequency and both the constitutivity and the resistance to inactivation segregated simultaneously, it seems reasonable to conclude that both characteristics are the result of a single mutational event. It is on the basis of this evidence that we have proposed that the product of gene R1 produces an apo-repressor-inactivator (apo-ri) rather than just an aporepressor. Ala^{r}2 and ala^{r}4 were also crossed to one another and failed to show complementation, indicating that the mutations involved occurred in the same gene [38]. A reasonable conclusion that one can draw from these experiments is that the increased activity of the mutants is due to mutation in a regulatory gene (gene R1). If the mutations were in a structural gene for the A-system transporter one would expect that the mutant phenotype would be co-dominant and that there would likely be a change in the K_m for the A system in the mutants. On the basis of these results, together with the evidence described above that a regulatory component of this system was at the transcriptional level and that amino acid effectors probably functioned intracellularly without being metabolized, we postulated that the parental allele of gene R1 produced a potential repressor, inactivator, an apo-repressor-inactivator (apo-ri) that is in equilibrium with a repressor-inactivator (ri) and that amino acid effectors function by shifting the equilibrium to the ri form. In ala^{r}4 the apo-ri can no longer be converted into an active ri, so this mutant is fully constitutive and does not exhibit irreversible inactivation of the A system in the presence of a co-ri. In some recent studies with ala^{r}2 we have shown that the rate of derepression of the A system is twice and the inactivation rate is equal to that of the parental

culture. To explain the phenotype of ala^{r}2 we proposed that the mutation in this mutant has not only reduced the affinity of apo-ri to the co-ri, since this mutant is partially constitutive in the presence of co-ri's, but it has also shifted the equilibrium of this regulatory protein toward the apo-ri, resulting in less ri then present in the parental culture in the absence of co-ri. Moffett and Englesberg [38] have also been able to show that certain amino acids can mediate the transformation of the apo-ri so that the resulting compound differs in the extent to which it acts as repressor and inactivator. For example, although alanine, serine, proline and MeAIB can convert the apo-r to ri, the apo-ri is converted by β-alanine and tryptophan into *r* (no inactivator activity) and hydroxy proline converts it to *r* and reduced inactivator activity (Table 2).

The data I have presented has enabled us to answer the questions posed above. Amino acids affect both repression and the irreversible inactivation of the A transporter by reacting with the apo-ri produced by gene R1 and converting this product into a ri. We have shown that just one gene is involved in this mechanism and that this system is regulated by internalized amino acid effectors and appears to work by negative control.

Structural and Regulatory Connection Between the A, ASC and P Systems

So far we have dealt with the evidence dealing with the regulation of the A system. The mutants that we have analyzed, however, are pleiotropic, affecting not only the A system but also the ASC and P systems. The increases in these other systems were comparable to the increases in the A system, although they do not appear to be coordinated (Fig. 1). The increase in activity of the ASC and P in ala^{r}2 and ala^{r}4 is recessive as has been shown for the A, and where measured, the complete phenotype segregates as a unit. On the basis of the fact that all three mutants are pleiotropic and the mutation frequency is quite high, it is reasonable to conclude that each is a the result of a single mutational event. What is the nature of the change that affected three transport systems? We tested the possibility that the increase in activity on the part of ala^{r}2 and ala^{r}4 might be the result of a mutation affecting the activity of the (Na^+,K^+)-ATPase that would result in an increased Na^+ gradient, on the assumption that the Na^+ gradient contributed significantly to the velocity of transport through the three systems involved. To our surprise we found that the Na^+ content of the cells harvested from MEMCHO and subsequently prepared for measuring transport had significantly higher Na^+ content (Table 6, Ref. 37, and *see below*) than the parental culture. Indeed that Na^+ content of the cells paralleled the increase in the A system, although it was not directly proportional. There was no discernable pattern with regard to the K^+ concentrations. It is obvious from these results that the increase in velocity of the mutants cannot be explained on the basis of an increased Na^+ or K^+ gradient. We have proposed that pleiotropic effect of mutants ala^{r}2 and ala^{r}4 is due to the joint regulation of the structural genes for the P and ASC system by regulation gene R1. Since both the ASC and P systems do not appear to be regulated by amino acid starvation, we have proposed that both the apo-ri and the ri modulate by negative control the structural genes for ASC and P systems transporters so that in the conversion of the apo-ri to ri the regulation of these genes is not affected. Mutation in gene R1 as in ala^{r}2 and ala^{r}4 would reduce the binding of the proteins to the controlling site of these genes, thus allowing further expression of the respective genes involved.

Ala^{r}3 also produced a pleiotropic effect, affecting both A, ASC and P systems. However, this mutant differs from ala^{r}2 and ala^{r}4 in that the increased activity is co-dominant. Also the kinetic parameters showed increases in both V_{max} and K_m. These attributes are ones that are expected of mutations in a structural gene. To explain the pleiotropic effect we postulated that this mutant was the result of a mutation in a gene Y that coded for a protein that was shared by both the A, ASC and P systems. The histidine transport system in *Salmonella typhimurium* is an example of systems sharing a common subunit [2].

Gene R2 and the Proposed Insulin Connection

Another ala^r mutant, ala^{r}6, has recently been characterized (F. Perier, and E. Englesberg, *unpublished results*). This mutant has 4-5 times the V_{max} of the A system as compared to that of the repressed parental culture. The mutant is further derepressible by amino acid starvation, resulting in an increase equivalent to that shown by the parental culture upon derepression. The phenotype of ala^{r}6 is recessive to the parental phenotype, demonstrating that the mutation is in a regulatory gene. When ala^{r}6 was crossed with ala^{r}4 oua^r HPRT$^-$ we found that the mutants complemented one another (Fig. 3). These results indicate that the mutation in ala^{r}6 occurred in a different regulatory gene, gene R2, which produces a repressor r2 that does not re-

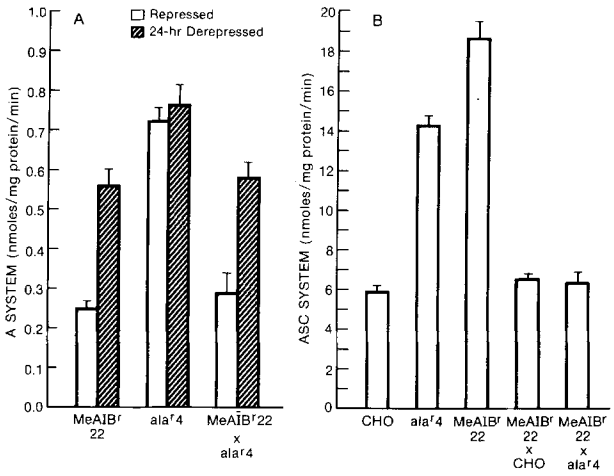

Fig. 4. Cell × cell hybridization showing the phenotype of MeAIB^{r}22 is recessive to that of CHO-K1 and MeAIB^{r}22 complements ala^{r}4, thus defining regulatory gene Rasc. Experimental procedures are as described in Table 3, reference 37

spond to amino acid effectors and in conjunction with ri regulates gene A by negative control (Fig. 3). What is the function of this regulatory gene that affects the A system and is apparently not affected by starvation for A system-preferred amino acids? We have shown that CHO-K1 cells can be grown in a modified F-12 defined medium. Insulin is required for optimum growth of this cell line. However, growth can occur in the absence of insulin with prolonged incubation. Insulin induces the A system in CHO-K1 that are grown in a defined medium M-F12 in the absence of insulin. This induction is inhibited by cycloheximide and actinomycin D, indicating that the induction is at the transcriptional level [34]. Although ala^{r}6 still requires insulin for growth, when this mutant is grown in M-F12 in the presence or absence of insulin there is no insulin effect demonstrable on the A system. These results have led us to propose that insulin induces the A system by inactivating r2, and in ala^{r}6 a mutation has resulted in the inactivation of r2, thus negating the insulin effect.

Regulation of the ASC System

The ASC system is apparently not affected by amino acid starvation. However, as we have shown above, this system is affected by some of the ala^r mutants. On the basis of the analysis of these mutants we have postulated that the structural gene, gene ASC, is regulated by both apo-ri and the ri, products of gene R1. Evidence from analysis of MeAIB^{r}22 has identified another regulatory gene, gene Rasc, that produces a constitutive repressor that regulates gene ASC. Mutant MeAIB^{r}22 produces a six-fold increase in V_{max} with a negligible increase in K_m for the ASC system as measured by serine transport and has no significant effect on the A system. Hybrids isolated in HAT ouabain medium from a cross between MeAIB^{r}22 and CHO-K1 oua^r HPRT$^-$ showed that the mutant phenotype was recessive, implicating a regulatory gene mutation [17]. Further analysis of hybrids from a cross between MeAIB^{r}22 and ala^{r}4 oua^r HPRT$^-$ showed that the mutants complemented one another, i.e., they had the normal A and ASC activity (Fig. 4) and also the MeAIB resistance pattern (*data not shown*) of the parental CHO-K1 cell line. These results indicated that the regulatory mutations involved were in different genes (gene R1 and gene Rasc).

Regulation of the L System

An examination of amino acid transport in a mutant of CHO cells, tsH1, that has a temperature sensitive (ts) leucyl t-RNA synthetase activity, revealed that at a marginally permissable growth temperature of 38°C, with limited leucine in the medium, there was an increase in L-system activity attributable to an increase in V_{max} of this system [39, 51]. This increase is specific for the L system and is inhibited by higher concentrations of leucine. It was shown that cycloheximide but not initially actinomycin D inhibited this increase in activity, suggesting that the control is at the level of translation. An analysis of the intracellular amino acid pools revealed that the increase in activity was not the result of *trans* stimulation. Although other ts amino acyl-tRNA synthetase mutants for other amino acids transported by the L system show a similar effect, mutants of the leucyl synthetase were most effective in increasing L-system activity (D. Oxender, *personal communication*). It is of interest that ts mutants of synthetases for some amino acids transported by systems other than the L also showed increased rates of transport of these amino acids at nonpermissive temperatures. Additional work is required to determine whether systems other than the L system may be affected. Experiments have previously shown that starvation for amino acids that derepress the A system do not increase the response of cells to L system amino acids [22, 36]. However, growing CHO cells in very low limiting concentrations of a single L-system amino acid, for example at 10 μM leucine, does effect an increase in L-system activity [48, 51 and D. Oxender, *personal communication*].

Temperature-resistant revertants of CHO tsH1

have been isolated that have increased L-system activity as a result of an increase in the V_{max} of this system. The mutation does not involve the leucyl-t RNA synthetase since the activity of this enzyme remains temperature sensitive and the increase in V_{max} of the mutants is present at both permissive and nonpermissive temperatures. This evidence suggests that the mutation is probably in some regulatory gene and results in constitutive synthesis of the L-system transporter.

The results with the ts synthetases are difficult to interpret. Since so many different synthetases and the corresponding charged t-RNA's may be involved, one would imagine that the effect on the L system might be the result of a general inhibition of protein synthesis or the synthesis of a specific polypeptide. But this does not seem to be a reasonable explanation since starvation for a number of A-system amino acids has no apparent effect on the L system and also that the ts leucyl synthetase seems to be more effective than other ts synthetases in deregulating the L system. The possibility that specific charged t-RNA's or the ratio of these charged to uncharged t-RNA's may be involved in this phenomenon cannot at this point be eliminated. In conjunction with this regulation, the analysis of the "revertants" suggests that another element present in the parental CHO cells acts as a negative regulator, "repressor" of this system. Since the revertants are immune to the regulation imposed by the original ts mutation, and this mutation is still present in the revertants, it is reasonable to assume that whatever signal is generated by the synthetases, this signal might work by converting the potential "repressor" into a functioning one, a "repressor" that works on the level of translation. There are other possibilities and an analysis of the dominance relationship of these mutant alleles would be helpful in verifying the type of mutation and the nature of the regulatory mechanism that is involved. This regulation of the L system, which appears to be at the translational level, is in contrast to the transcriptional and post-translational regulation of the A system.

Using a hybrid between CHO ts025C1 containing a temperature-sensitive leucyl t-RNA synthetase and normal human leukocytes, it has been possible to identify a gene on human chromosome 20 that codes for a component that produces increased L-system activity in the hybrid [30]. The hybrid has the maximum derepressed level of the L system as exhibited at moderate nonpermissive temperatures by the ts parent and is not further derepressible. Kinetic studies indicate that the hybrid has both an increase in K_m and V_{max} for the L system. Since both human and hamster L-system activity is probably being measured it is difficult to interpret these results. One possible explanation is that human chromosome 20 may contain a gene that codes for an L-system transporter that is immune to the regulatory elements of the Chinese hamster.

Structure and Mechanism of Transport

Kinetic studies indicate that the mutants of A9 cells isolated as resistant to 20 mM phenylalanine in MEM have changes in both a high and low affinity system that transports phenylalanine [15]. The high affinity system for phenylalanine, presumably the L system, showed a fourfold reduction of V_{max} with a change in K_m that may not be significant. The low-affinity transport system showed a several-fold reduction in both K_m and V_m. This system was not identified at the time, but on the basis of our findings that phenylalanine is transported through the A system in A9 and in CHO [4] it is reasonable to conclude that this low-affinity system is the A system. A similar connection between the A and L system was observed with a 5FT resistant mutant of A9 [53, 54]. A mutation affecting both the A system and a high-affinity glutamate system has been reported in mouse lymphocyte cells [11]. The mechanism involved in these pleiotropic effects is unknown. An examination of the intracellular amino acid pools in these mutants would be helpful in determining whether a mutation affecting one system (A) might indirectly affect another (L) by reducing *trans* stimulation.

Ala^{r}3, the result of a structural gene mutation, has increased activity of the A, ASC and P systems. We have interpreted this result to mean that all three systems may share a common subunit [37].

The high intracellular concentration of Na^+, combined with increased velocities of transport through the A system by ala^{r}2, ala^{r}3 and ala^{r}4, raises the question as to the influence of the Na^+ chemical gradient on the initial velocity of transport in this system. For example, ala^{r}4 has an intracellular Na^+ content close to the concentration of Na^+ in the uptake buffer used in measuring transport velocity [37]. This intracellular concentration is 2.7× that found in the parental culture, yet the velocity of transport through the A system by ala^{r}4 is 5× that of the parental culture. We have proposed that the increase in the Na^+ content of the mutants is the result of their increased velocity of transport of amino acids through Na^+-dependent A, as well perhaps by the ASC and P systems and the cotransport of Na^+ with the amino acids. There is no doubt that Na^+ in the *cis* position is required for transport by the A system in CHO-K1 [4, 49] and in other cell lines [50]. The effect of Na^+ in the *trans* position on influx velocity through the A system is not too

clear. Petronini et al. [42] has shown that varying the intracellular Na^+ content of SV40 3T3 cells within "physiological range" had little effect on proline transport (presumably through the A system). White and Christensen [58] showed that amino acid starved hepatoma HTC cells, treated with ouabain so that the intracellular Na^+ content increased from 25 to 108 mM, had a 50% reduction in V_{max} and 1.6-fold increase in K_m for MeAIB transport. These workers proposed that in the presence of excess intracellular Na^+ the mobility of the carrier is lower than that of the empty carrier, thus resulting in a *trans* inhibition of the A system by Na^+. However, since ouabain treatment also produces a large decrease in intracellular K^+ [3, 16, *see also* results given below] one cannot be sure that the effect observed on A-system transport may not be due to the change in the intracellular K^+ concentration. A crucial role of intracellular K^+ as a determinant of ouabain toxicity is borne out by the finding that the tolerance to ouabain of a particular ouabain-resistant mutant is due to a ouabain induction of a K^+-ATPase transport system, a system that is Na^+ independent and which apparently only restores the K^+ gradiant [16]. There is other evidence that the Na^+ electrochemical gradient is not solely responsible for A-system amino acid transport and that a membrane potential contributed by a K^+ gradient may energize this system [25, 56]. It should be noted that we have been assuming that the total Na^+ that we have measured in CHO cells resides in the cytoplasm and is not sequestered in the nucleus or other unidentified intracellular compartments. This possibility appears not to be a factor in experiments performed by Heinz et al. [25]. Perhaps the most convincing evidence that the Na^+ chemical gradient is important as an energy source for A-system transport comes from experiments with membrane vesicles [31-33]. These experiments have also shown that the K^+ gradient in vesicles can generate an electrical potential that can drive this system.

We have considered the possibility that the increased Na^+ content of the mutant cells may have triggered an increase in the activity of the (Na^+,K^+)-ATPase, resulting in a hyperpolarization of the plasma membrane thus providing the energy source for the A system in the mutants. We tested this possibility by treating CHO-K1 and ala^r4 with ouabain (1.0 mM), a potent inhibitor of the (Na^+, K^+)-ATPase, and measuring the initial velocity (1 min) of MeAIB (0.1 mM) uptake and intracellular Na^+ and K^+ concentrations. Rb^+ was employed to estimate the K^+ levels. Cells were equilibriated with $^{22}Na^+$ and $^{86}Rb^+$ for 24 h during growth in MEM-CHO [37]. We found that MeAIB uptake by CHO-K1 and ala^r4 was reduced by 25% (0.032 to 0.024 mM) and 17% (0.12 to 0.10 mM), respectively, in the first 20 min of exposure to ouabain. Na^+ and Rb^+ concentrations were affected as expected. Since the differences in velocity of transport of MeAIB between mutant and parental culture were maintained during this treatment, it is reasonable to conclude that the increased velocity on the part of the mutant containing a high concentration of Na^+ is not energized by an increased velocity of the (Na^+,K^+)-ATPase [47].

As mentioned above, experiments with membrane vesicles have demonstrated that Na^+ and the membrane potential both contribute to energizing the A system [31–33]. We have arrived at a similar conclusion with experiments with CHO-K1 (Moffett and Englesberg, *unpublished results*). High concentrations of external Na^+ may convert the transporter into a form that can now respond to the existing membrane potential [33]. The apparent response to a Na^+ gradient may result from an inhibition of the translocation of the transporter from inside to outside by excess internal Na^+ [58]. If we except this hypothesis, the results we have obtained with ala^r mutants may be explained by proposing that there is an increase in membrane potential indirectly brought about by the cells reacting to the increased concentration of Na^+. As an alternative possibility, the increased number of A-system transporters in ala^r2 and ala^r4, that results in increased velocity of A-system transport, may mask the Na^+ effect. We have also considered the possibility that the mutants may be resistant to the *trans*-effect of Na^+. However, this seems unlikely, at least with ala^r2 and ala^r4, since evidence indicates that the increase in A-system velocity in these mutants is the result of regulatory gene mutations.

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