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Arginine and Lysine Transport in Sugarcane Cell Suspension Cultures*

A. Maretzki and M. Thom

ABSTRACT: The initial rate of transport of extracellular arginine into sugarcane cells grown in suspension culture was shown to be at least four times as great as that of lysine but the system approached saturation at an exogenous arginine concentration of about 10 μM, whereas lysine transport remained linear until the initial lysine concentration in the medium exceeded 100 μM.

The rate of lysine transport was found to be much more susceptible to the presence of arginine in the medium than was arginine transport to exogenous lysine. Preincubation of sugarcane cells with cycloheximide severely reduced the velocity of lysine uptake but the inhibition was not additive to that obtained with arginine. Under similar conditions, the interference of cycloheximide with arginine uptake was much less.

Difference of arginine and lysine in transport site affinity and in the mutual inhibition by these amino acids of their transport rates, as well as differences in response to cycloheximide treatment, suggest the existence of multiple transport sites for basic amino acids in the sugarcane cell.

Selective permeability of living cells to concentrate important nutrients against a concentration gradient has become a well-established fact in animal tissues as well as in microorganisms. The facilitation of movement of organic substances across membrane barriers by specific and active transport systems appears to be an essential feature common to all biological systems.

The precise mechanism responsible for the accumulation of amino acids in higher plant cells has not been reported, although similar processes can be tacitly assumed to function in higher plants as they do in other living organisms. Amino acids appear to be assimilated and transported by related but discrete systems and involve the mediation of agents fitting the description of permeases as originally conceived by Cohen and Monod (1957).

The concept of multiple amino acid transport systems was first proposed by Cohen and Rickenberg (1956) who measured the reversible, interdependent concentration by *Escherichia coli* of several exogenously supplied neutral amino acids and their effect on cell growth. Overlap of transport-mediating systems for neutral amino acids was demonstrated by Oxender and Christensen (1963) in Ehrlich ascites tumor cells and two sites were proposed to account for the accumulation of all amino acids in these cells. A relatively nonspecific transport system with affinity for both basic as well as a wide range of other amino acids has been shown to exist in old cultures of *Neurospora crassa* (Pall, 1969). However, most transport systems for basic amino acids appear to have specificity for these amino acids as a group (*e.g.*, Roess and DeBusk, 1968) or to have high specificity for either lysine or arginine. Specific transport systems for arginine have been reported in *Saccharomyces cerevisiae* (Grenson *et al.*, 1966) and *Escherichia coli* W (Wilson and Holden, 1969). Specific lysine transport systems were shown to exist in *Saccharomyces cerevisiae* (Grenson, 1966) and in rat kidney cortex (Segal and Smith, 1969). The interdependence of separate transport sites and their regulation are complicated (Benko *et al.*, 1967, 1968; Roess and DeBusk, 1968) and not yet well understood.

During an investigation of arginine metabolism in cell

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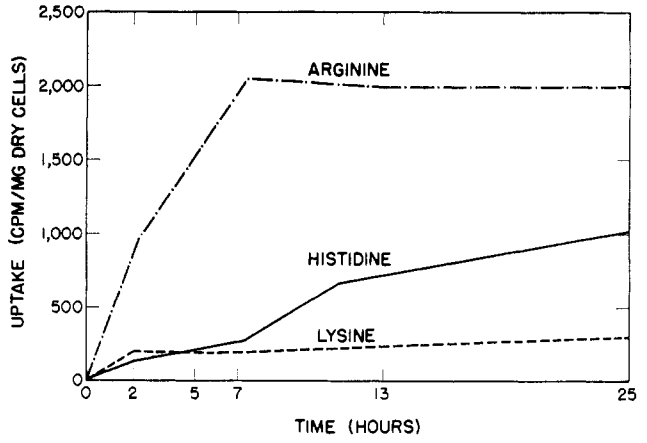

FIGURE 1: Comparison of the total ethanol-soluble radioactivity taken up by sugarcane cells during 25-hr incubations on [^{14}C]-arginine (262 mCi/mmole), [^{14}C]histidine (267 mCi/mmole), [^{14}C]-lysine (271 mCi/mmole). The incubation medium was synth II with two modifications: 3 μCi of [^{14}C]arginine, -histidine, or -lysine was added; and carrier concentrations of 17.2, 19.3, or 20.5 μM, respectively, replaced the normal concentration of the three amino acids. Cells were depleted of the amino acid investigated by incubation on synth II lacking this particular amino acid for 60 hr prior to addition of the radioisotope.

suspension cultures of sugarcane we noted the rapid rate of arginine uptake by the cells in comparison with several other amino acids (Maretzki *et al.*, 1969a). No obvious relationship between the uptake rate of arginine and its growth-stimulatory function in sugarcane cells was apparent, but arginine manifested its stimulatory role in the cells only when a relatively complex mixture of amino acids was used for the medium. This finding suggested a regulatory mechanism, possibly functioning by the inhibition of a detrimental factor in the presence of arginine.

Experimental Methods

Maintenance of Growth of Cell Cultures. Sugarcane variety H 50-7209 was used throughout this investigation. The cells originated from parenchyma explants and have been maintained as suspension cultures since 1963. Stocks are propagated by transfer at monthly intervals, using a White's medium containing 2,4-dichlorophenoxyacetic acid, a mixture of vitamins, sucrose, and yeast extract (Nickell and Maretzki, 1969). The cultures are incubated (69°F) on rotary shakers (New Brunswick Scientific Co., Inc.) at approximately 260 oscillations per minute.

Two types of media were used throughout this investigation. In one of these the yeast extract of the above-mentioned medium was replaced by a mixture of amino acids. This amino acid mixture has previously been described as synthetic mixture M-2 (Nickell and Maretzki, 1969) and the total medium will be referred to in this report as "synthetic II" (synth II). The other medium consisted of a White's mixture of inorganic constituents as well as the other constituents mentioned above but lacking a source of organic nitrogen; this medium will be called "basal."

Normally 6.0-ml inocula from 1-month-old stock cultures were pipetted into the experimental medium *via* a transfer to sterile distilled water. Incubations of cells for periods of more than 15 min were carried out on a rotary shaker.

All cell cultures used for uptake or transport studies were obtained by aseptically decanting the medium from 3-week-old stock cultures, replacing it with synth II without arginine and without lysine, or with basal, and incubating the cells on the shaker for 60 hr except where noted otherwise.

Incubations for Transport Rate Determinations. Short-period cell incubations of durations less than 15 min were conducted in flasks placed in a water bath over a magnetic stirrer. Aeration was provided by passing a stream of H_2SO_4-dried air into the flasks. The suspension was gently agitated with a slow-spinning magnetic stirrer. High-speed stirring caused disruption of the cells. Chemicals were added to the cell suspensions with a pipet, and cell aliquots were withdrawn with a wide-mouth pipet at 30-sec to 1-min intervals. The cell samples withdrawn from the suspension were immediately collected on a suction filter and washed with distilled water, and the damp pellet frozen.

Cell Extractions. Lyophilized cells were extracted either with 70% aqueous alcohol or with 0.01 M phosphate buffer (pH 7.0). The buffer-extractable radioactivity was found to give an acceptable measure of amino acid taken up by the cells in the short-period experiments. The specific activities of the radioisotopes, as noted by the manufacturer, were used for calculation of the amounts of amino acid transported.

The initial sample (withdrawn either 30 or 60 sec after addition of the radioisotope) gave a spurious high count for lysine and frequently for arginine, and did not fit the linear relationship of the subsequent samples measured over a 10-min period. The problem is assumed to arise from non-active absorption of label on the cell surface (Benko *et al.*, 1967). Therefore, in calculating transport rates the plots were not extrapolated to zero time but to the value of the first sample withdrawn and subsequent samples to 6 min.

Radioactivity Measurements. Radioactive counts were determined in a scintillation counter (Beckman Instruments, Inc., Model SL-150), using aliquots of the extracts (usually 200 μl) in a 2,5-diphenyloxazole, 1,4-bis[2-(5-phenyloxazolyl)]benzene, toluene cocktail and employing "Bio-Solv BBS-3" (Beckman Instruments, Inc.) to ensure miscibility. Radioisotopes used in this investigation were uniformly labeled [^{14}C]arginine and [^{14}C]lysine (specific activities as indicated under results) obtained from New England Nuclear Corp.

Results

Relative Rate of Uptake of the Three Major Basic Amino Acids. Previously we were able to demonstrate that sugarcane cells take up arginine from the surrounding medium much more rapidly than they take up either valine or threonine (Maretzki *et al.*, 1969a). A similar striking difference in uptake rates has also been noted when leucine or tyrosine was compared with arginine (unpublished results). In the present study we measured comparative uptake rates of the three major basic amino acids–lysine, histidine, and arginine (Figure 1). The three uniformly labeled amino acids used in this experiment had similar specific activities, and in each instance the cells were deprived for 60 hr of an extracellular supply of the amino acid which was later added as the radio-

TABLE I: Effect of Metabolic Inhibitors on Uptake of [^{14}C]-Arginine and [^{14}C]Lysine by Sugarcane Cells.[a]

	Per Cent of Control without Inhibitor		
	0.5[b]	3.5[b]	7.0[b]
[^{14}C]Arginine			
p-Chloromercuribenzoate (10^{-3} M)	100.0	31.5	18.6
Arsenate (10^{-2} M)	33.7	9.3	13.1
Cyanide (10^{-3} M)	18.6	29.4	0.2
N-Ethylmaleimide (10^{-3} M)	58.1	76.0	52.3
Azide (10^{-3} M)	0	9.2	12.6
Cysteine (10^{-3} M)	14.0	1.7	0.5
2,4-Dinitrophenol (10^{-4} M)	0	3.4	6.9
Fluoride (10^{-2} M)	100.0	65.5	37.9
EDTA (10^{-3} M)	0	15.9	8.3
Iodoacetate (10^{-3} M)	85.0	24.1	14.3
[^{14}C]Lysine			
Fluoride (10^{-2} M)	100.0	78.7	38.9
2,4-Dinitrophenol (10^{-4} M)	60.0	31.9	20.0

[a] Cells were depleted of both lysine and arginine prior to radiosotope addition; arginine or lysine uptake was measured for a 10-min period, as described under Methods. [b] Minutes after addition of inhibitor.

isotope in the presence of carrier. Before equilibrium became established arginine was more rapidly assimilated by the cells than either histidine or lysine.

Transport Rates of Arginine and Lysine. The transport rates of arginine and lysine across the cellular barrier were compared in cells which were incubated in basal (Figure 2a) or in synth II (Figure 2b). Synth II was the normal growth medium employed in prior studies and the high rate of arginine uptake was first observed in this medium. The relative transport rates of arginine and lysine were similar in each of these two media, although the overall rates were higher in basal than in synth II. The presence of other amino acids, therefore, influences the total but not significantly the relative transport rates of arginine and lysine. The transport rate of arginine was high at concentrations below 10 μM and approached—but did not reach—saturation at higher concentrations of added arginine. On the other hand, endogenous lysine accumulation proceeded at an appreciably slower rate at low concentrations, but exceeded arginine transport above initial exogenous lysine concentrations of about 40 μM, and remained linear until extracellular lysine concentration exceeded 100 μM. Previous chromatographic evidence had shown that [U-^{14}C]arginine was taken up but not appreciably metabolized by the sugarcane cells during a 10-min incubation period (Maretzki *et al.*, 1969b). Chromatography has shown also that metabolism of [U-^{14}C]lysine in sugarcane cells was negligible during the first several hours of incubation (unpublished results). Uptake measurements for 6 min were, therefore, assumed not to be complicated by metabolic modifications of these amino acids.

Effect of Metabolic Inhibitors on Arginine and Lysine

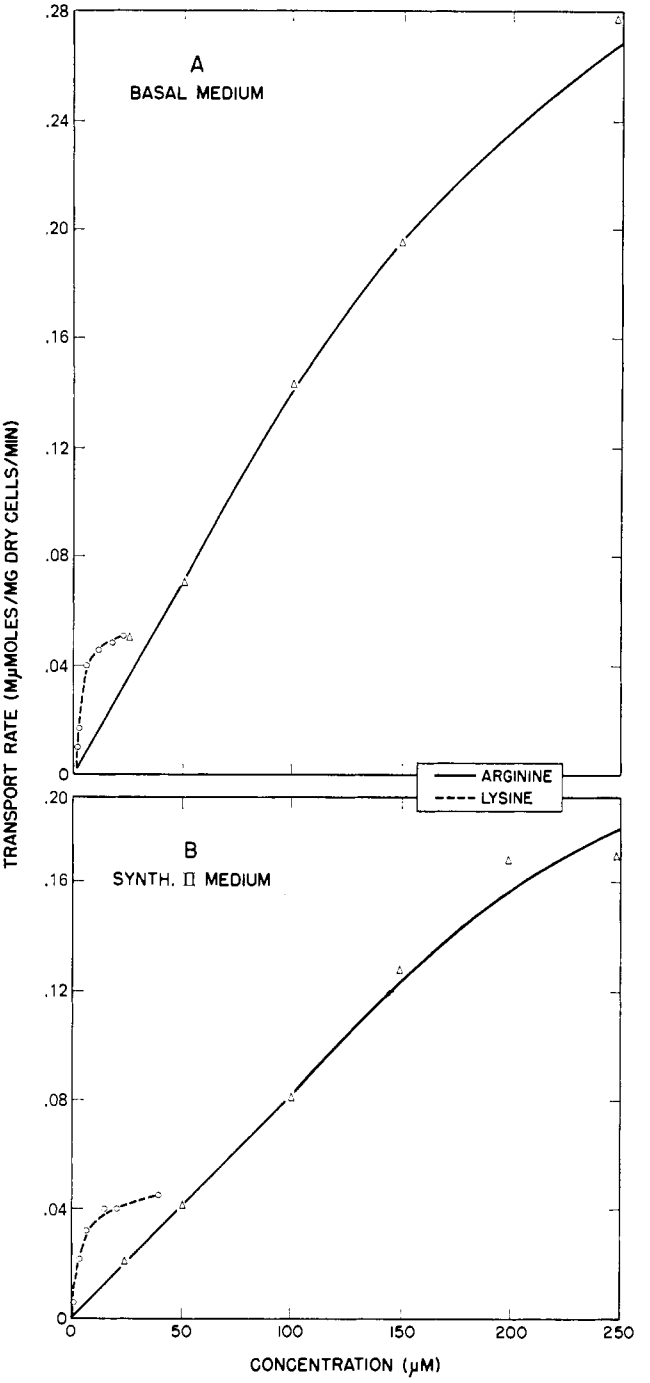

FIGURE 2: The transport rates of arginine and lysine in sugarcane cells on basal (A) or synth II (B) as a function of increasing concentration in these media. Prior to addition of the radioisotope, cells were pretreated for 60 hr either on basal medium or on synth II lacking lysine and arginine. Transport rates were calculated from the uptake of either arginine or lysine as described under Methods.

Transport. Arginine and lysine entry into sugarcane cells is a metabolically active process (Table I). For arginine, uncouplers of oxidative phosphorylation and inhibitors of the cytochrome system were immediate and total in their interference with the uptake mechanism. Iodoacetate and *p*-chloromercuribenzoate were slower in their effectiveness

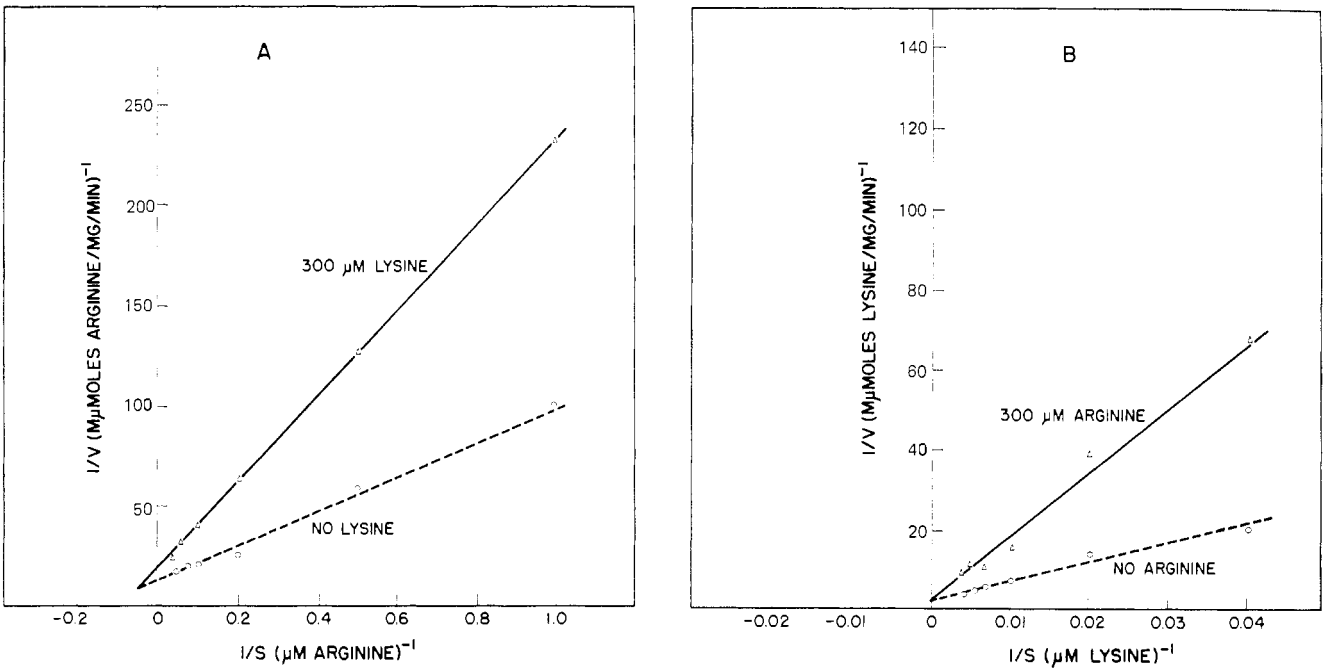

FIGURE 3: Lineweaver–Burk plots of arginine or lysine transport in a basal medium. (A) Arginine transport was plotted in the absence of lysine and in the presence of 300 μM lysine. Arginine uptake was measured at concentrations ranging from 1 to 20 μM. (B) Lysine transport was plotted in the absence of arginine and in the presence of 300 μM arginine. Lysine uptake was measured at concentrations ranging from 25 to 250 μM. In both A and B, cells were preincubated on a basal medium for 60 hr prior to measurement of transport rates and all other experimental conditions were similar to those described under Methods.

as inhibitors, and *N*-ethylmaleimide was only partially effective, possibly indicating a relatively protected positioning of an essential SH group. Cysteine was found to be highly inhibitory. Although fluoride was only partially inhibitory, EDTA inhibition was immediate and complete. Only fluoride and 2,4-dinitrophenol were tested for their effectiveness as metabolic inhibitors of lysine uptake. Fluoride had a delayed effect on the lysine system, similar to that noted for arginine uptake. Uncoupling of oxidative phosphorylation by 2,4-dinitrophenol was reflected more slowly in lysine uptake than it was in arginine uptake.

TABLE II: The Specificity of Inhibition by Arginine of [^{14}C]-Lysine Uptake in Sugarcane Cells.[a]

Inhibitor	Radioactivity of Extract (cpm/mg of dry cells) × 10^{-3}
None (control)	5.42
(+) L-Arginine (57 μM)	1.88
(+) D-Arginine (57 μM)	5.44
(+) Canavanine (57 μM)	4.02

[a] Cells were depleted of extracellular arginine and lysine for 60 hr prior to addition of [U-^{14}C]lysine (264 mCi/mmole), using synthetic II with 1.4 M (0.2 ppm) lysine carrier and the inhibitor in place of L-arginine as shown. Cells were incubated in the presence of the labeled lysine for a period of 5 hr prior to extraction.

Mutual Inhibition of Arginine and Lysine Transport. Preliminary experiments using synth II had shown a much higher inhibition of lysine uptake in the presence of excess arginine that was found with the reverse condition. To clarify this point, the velocity of linear uptake of arginine and lysine by the cells from basal was measured over wide concentration ranges. A double reciprocal plot of the data according to Lineweaver and Burk (1934) showed that inhibition of arginine by lysine most nearly fitted "uncompetitive inhibition" (Figure 3a). The maximum velocity of arginine transport, 0.11 mμmole/mg per minute, was reduced to 0.06 mμmole/mg per minute in the presence of 300 μM lysine. The affinity of arginine for its primary transport site was relatively unaffected by the simultaneous addition of a 50-fold greater lysine concentration (K_m = 1.03 × 10^{-4} M *vs.* 1.27 × 10^{-4} M).

Similar treatment of lysine transport gave a maximum velocity value of 0.5 μmole/mg per minute in both the presence and absence of arginine (Figure 3b) but a threefold decrease in the affinity of lysine for its transport site in the presence of 300 μM arginine (K_m = 2.45 × 10^{-3} M *vs.* 7.90 × 10^{-3} M). A typical competitive situation was suggested by the Lineweaver–Burk plot in this case. This was further substantiated by treating the inhibition of lysine transport according to the method of Dixon (1953) (Figure 4b). On the other hand, arginine transport inhibition by lysine appeared to be more akin to a noncompetitive relationship when plotted by the Dixon method (Figure 4a).

Canavanine can be used as an analog of arginine but its

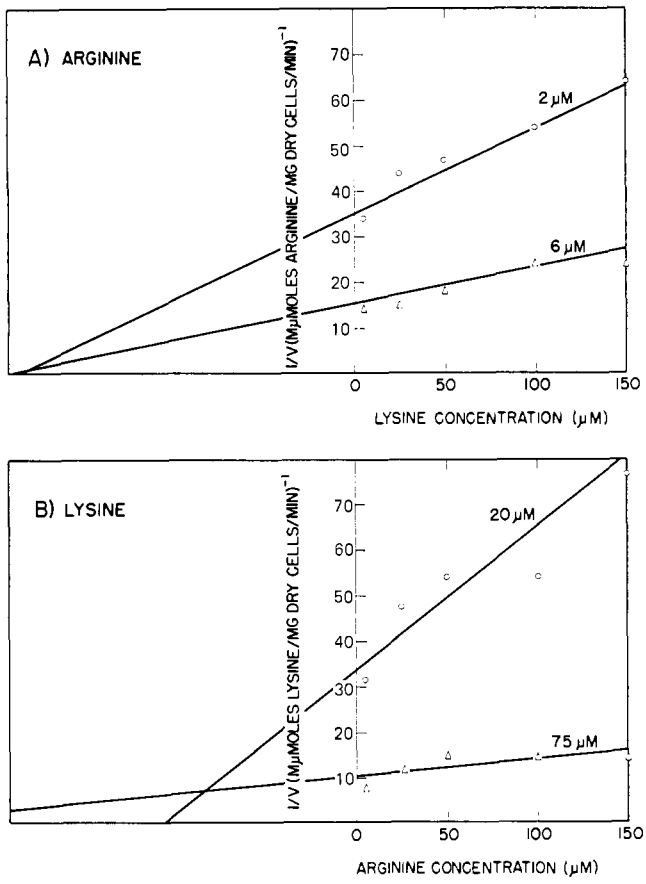

FIGURE 4: Inhibition of transport in sugarcane cells using a basal medium. (A) Inhibition of arginine transport by lysine. Data treated according to the method of Dixon (1953) at two concentrations of arginine (2 and 6 μM) in the presence of 5–150 μM lysine. (B) Inhibition of lysine transport by arginine. Data treated according to the method of Dixon (1953) at two concentrations of lysine (20 and 75 μM) in the presence of 5–150 μM arginine. In both A and B, cells were pretreated on a basal medium for 60 hr prior to measurement of transport rates and all other experimental conditions were similar to those described under Methods.

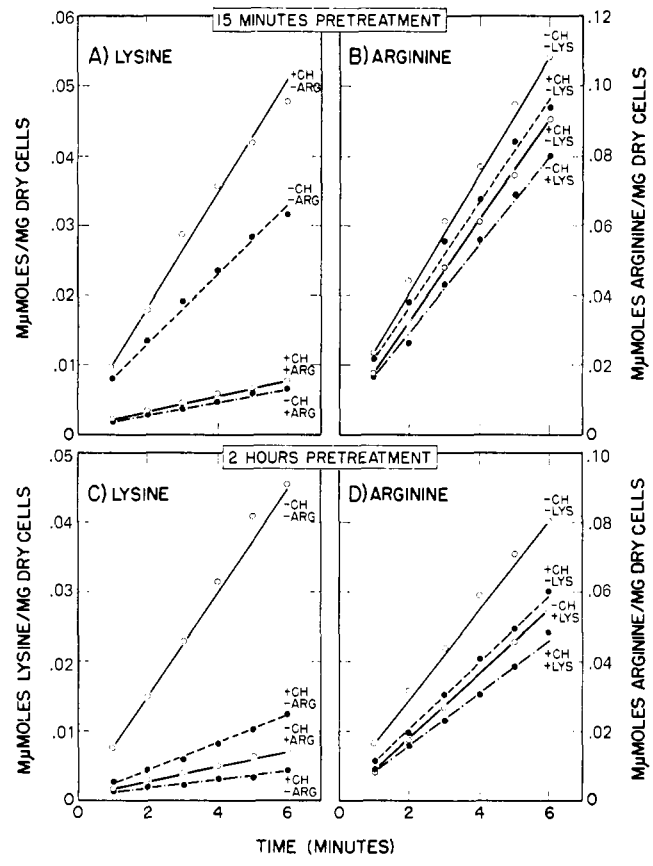

FIGURE 5: Effect of pretreatment of sugarcane cells with cycloheximide (10^{-4} M) on the uptake of [^{14}C]lysine (1.0 μCi, 270 mCi/mmole) or [^{14}C]arginine (0.5 μCi, 260 μCi/mmole) in a basal medium. Cells were treated with cycloheximide for either 15 min (A and B) or 2 hr (C and D) prior to uptake determinations and all cells were incubated for 60 hr on the basal medium before cycloheximide addition. The uptake determinations were made in a basal medium containing: for lysine uptake, 1 μM lysine carrier and either 25 μM arginine or no arginine (A and C), or for arginine uptake, 1μM arginine carrier and either 50 μM lysine or no lysine (B and D).

effect on arginine transport in the sugarcane cell system is no greater than that shown by lysine. Neither D-arginine nor canavanine replaced L-arginine as inhibitors of lysine uptake (Table II), although canavanine repressed lysine uptake by 25%.

Effect of Cycloheximide on Arginine and Lysine Transport. Comparisons of substances known to interfere with normal protein formation in other biological systems showed cycloheximide to be the most effective protein inhibitor in sugarcane cell cultures. Including 10^{-4} M of cycloheximide in the incubation medium reduced amino acid incorporation by more than 95%.

Events at the cell barrier must occur during initial contact of the cell with the amino acid. The influence of cycloheximide on the transport mechanisms of arginine and lysine was measured over a 6-min time span following the addition of labeled amino acid to the basal medium. The cells were pretreated with the antibiotic for either 15 min (Figure 5a,b) or for 2 hr (Figure 5c,d). The presence of arginine at 25 times the concentration of lysine severely reduced the rate of lysine uptake, both with a 15-min cycloheximide pretreatment and in the absence of cycloheximide. In the absence of arginine, lysine uptake appeared to be initially stimulated by cycloheximide but with longer cycloheximide pretreatment (Figure 5c) was depressed almost to the low rate characteristic of cells in contact with excess exogenous arginine. No additive effect of cycloheximide and arginine was noted. In parallel experiments, arginine uptake showed the comparative insensitivity of this system to cycloheximide, even with a 2-hr pretreatment (Figure 5b,d).

Discussion

Our experimental evidence suggests the existence of at least two sites of transport for arginine and lysine in sugarcane cells. One arginine transport system has a high affinity for arginine at low extracellular concentrations. The diphasic nature of the arginine transport curve indicates that a second carrier system may function at higher arginine concentrations, similar to one suggested by Benko *et al.* (1967) for methionine transport in *Penicillium chrysogenum*. If so, a system less spe-

cific for arginine may be involved which may, in fact, be identical with the lysine transport system.

The rapid response of arginine and lysine transport to a number of metabolic inhibitors shows that the uptake mechanism requires energy. Uncouplers of phosphorylation are particularly rapid in their action, suggesting that ATP storage is limited or that stored ATP is unavailable and its continued formation must proceed for uptake to function normally.

In sugarcane cells lysine is a poor inhibitor of arginine transport. In this respect these cells resemble *E. coli* (Wilson and Holden, 1969) and *S. cerevisiae* (Grenson *et al.*, 1966), but differ from *N. crassa* where arginine transport was reduced by about 71% when a 10:1 ratio of lysine:arginine was used in the medium rather than arginine alone (Roess and DeBusk, 1968). Canavanine proved to be an equally poor inhibitor of arginine transport in sugarcane cells, contrary to its effect in *S. cerevisiae* where it substantially reduced arginine transport, apparently by competing for the uptake site (Grenson *et al.*, 1966).

In contrast to the specific lysine-transporting system reported in a wild-type strain of *S. cerevisiae*, which was unaffected by arginine (Grenson, 1966), the inhibition of lysine uptake by arginine in sugarcane cells was high.

The effects of preincubation with cycloheximide on transport of the two amino acids, inhibitory for lysine and much less so for arginine transport, provide further evidence for separate transport sites of these amino acids in sugarcane cells. Cycloheximide undoubtedly interfered with the normal ribosomal organization necessary for the formation of proteins essential for transport (Wiley and Matchett, 1968) but the protein associated with the arginine-transport site may be much more stable than the protein linked with the lysine-transport site. Other cycloheximide effects, such as those proposed by Grenson *et al.* (1968) and MacDonald and Ellis (1969), must also be considered.

Competition of arginine with lysine for the lysine transport site suggests that high concentrations of exogenous arginine may require the use of the lysine transport site preferentially for arginine accumulation in the sugarcane cell. Under these conditions exogenous lysine would enter the cell pools at a greatly reduced rate, a factor which might be of selective advantage to the survival and mitosis of the sugarcane cell. Many other factors undoubtedly influence the relative transport of exogenous basic amino acids into the cells of an intact plant. A recent report by Hart and Filner (1969) implicates both arginine and lysine as regulators for sulfate uptake by tobacco cells, and the plasmalemma is suggested as the possible transport barrier. Difficulties of distinguishing between plasmalemma and tonoplast transport have been pointed out by Cram (1969). One or the other, or both, of these membranes could account for the transport observations which we report.

It is apparent that plant cell cultures can provide information about transport-linked reactions which were heretofore not amenable to experimental manipulation in higher plants.

Summary

Evidence is presented for the existence of two distinct sites permitting the transport of extracellular arginine and lysine in sugarcane cells (variety H 50-7209). The rate of arginine transport is high compared with that of lysine but approaches saturation at a concentration at least ten times lower than does lysine transport. The rate of lysine transport is regulated by the extracellular concentration of arginine. The reverse dependence of arginine transport on lysine is very low.

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