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Cysteine Transport into Cultured Tobacco Cells

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ABSTRACT

Cysteine transport by tobacco cells (*Nicotiana tabacum* L. var. Xanthi) cultured on liquid B-5 medium was examined.

Transport was linear with time or amount of tissue and had a pH optimum of 4.5. Cysteine transport over a wide concentration range was biphasic. The isotherm, for descriptive convenience, was divided into two segments both of which obeyed Michaelis-Menten kinetics. The Km for high affinity transport was in the range 1.7 × 10^{-5} M(±0.17) while the Km for low affinity transport was in the range 3.5 × 10^{-4} M(±0.13). Maximum velocities were 3 to 6 nmoles/g fresh weight/minute and 13 to 16 nmoles/g fresh weight/minute, respectively.

Azide and 2,4-dinitrophenol caused more than 90% inhibition of net transport by either system. N,N'-Dicyclohexylcarbodiimide was not inhibitory while the inhibition by carbonylcyanide *m*-chlorophenylhydrazone was dependent on the cysteine concentration. Only those compounds that were inhibitory to transport caused significant efflux of labeled material from preloaded cells.

Tobacco cells that had been preincubated in iodoacetamide or N-ethylmaleimide did not transport cysteine while similar treatments with dithiothreitol were only slightly inhibitory or had no effect on transport.

Transport by either system was, to some extent, inhibited by all other tested amino acids and analogs. Alanine, methionine, and S-methyl cysteine were most effective in inhibiting cysteine transport. Both alanine and methionine were competitive inhibitors of cysteine transport by either system with inhibition constants that were similar to the Km for the particular system.

The transport of amino acids has been studied in bacterial systems (23). Lombardi and Kaback (16) provided evidence for 19 separate amino acid transport systems in *Escherichia coli* plasma membrane vesicles. Several fungal systems have been characterized in detail. Both basic and acidic amino acid transport systems have been described in *Penicillium* (10), as have uptake systems in *Neurospora* (24). Several amino acid permease mutants have been isolated in *Saccharomyces cerevisiae* (7).

Reports of amino acid transport into higher plant material are limited (22). Transport has been examined in root tissue of carrot (1) and barley leaf tissue (25). Cell suspension cultures of soybean (13, 14) sugarcane (17), and tobacco (8) have been used to study amino acid transport.

A limited study of cysteine entry into tobacco cells was conducted by Hart and Filner (8). They demonstrated that cysteine was a potent inhibitor of sulfate uptake by tobacco cells. This inhibition was reduced by various amino acids including arginine, lysine, and leucine. These antagonists were subsequently shown to be inhibitors of cysteine entry into the cells. The present research was carried out to further characterize cysteine entry into tobacco cells.

MATERIALS AND METHODS

The tobacco cell culture, XD line, (*Nicotiana tabacum* L. var Xanthi) was originally obtained from P. Filner and cultured on B-5 medium (5). Amino acids and inhibitors were obtained from Sigma Chemical Co., L-[^{35}S]cysteine from Amersham/Searle Corp., Handifluor scintillation fluid and analytical grade chemicals were obtained from commercial suppliers.

Transport Experiments. Eight-day-old cells were harvested by vacuum filtration and washed with 50 ml of $^1/_{10}$ strength B-5 medium (0.1 B-5 medium). The cells (0.2–0.4 g fresh wt) were placed in a 50-ml Erlenmeyer flask containing 20 ml of transport medium composed of 10 mM sodium citrate (pH 4.5) and 0.5 mM L-[^{35}S]cysteine (0.2 μCi). The flasks were stoppered with cotton plugs and placed on a rotary shaker for 1 or 2 hr at 25 C. At termination of transport experiments, the cells were again harvested by vacuum filtration and washed with 50 ml of unlabeled cysteine (concentration 10 times that used in incubation) to remove any adsorbed radioactive cysteine. Transported cysteine was extracted by one of two methods; either cysteine was isolated by placing the cells in 20 ml of boiling water (27) and 5-ml aliquot of the extract added to 10 ml of Handifluor, or the cells were placed directly in 1 ml of water and 10 ml of Handifluor. All radioactivity was determined by liquid scintillation counting in a Packard 3310 Tri-Carb scintillation spectrometer with external standardization. There were no differences in radioactivity recovered by either of these two methods.

All manipulations prior to the addition of radioactive cysteine were performed in a sterile room using autoclaved media and equipment. Each point reported in this research represents the mean of at least two separate experiments with internal replicates.

RESULTS AND DISCUSSION

General Characteristics. Cysteine transport by tobacco cells was linear with time for at least 5 hr (Fig. 1) and with amount of tissue in the range 0.15 to 0.4 g. fresh wt. Approximately 10% of the total labeled material associated with the cells after rapid washing with unlabeled cysteine was released when cells were suspended in 0.1 B-5, 10 mM citrate (pH 4.5) for 1 hr (see Table II). This release of labeled material is probably not due to exchange either from exterior sites or internal pools since preloaded cells that were suspended in unlabeled cysteine or buffer alone did not lose significantly different amounts of labeled material.

Analysis of the labeled material within the cells indicated that when the incubation period was 2 hr at least 60% of the intracellular labeled material was identified as free cysteine. However, as the incubation period was increased progressively less of the labeled material was identifiable as free cysteine, indicating rapid metabolism and incorporation into protein. The metabolism of cysteine in these cells is currently being investigated.

Cysteine was transported against a concentration gradient, as shown in Figures 1 and 2. A transport rate of between 390 and 720 nmol/g fresh wt·hr. was observed in the presence of 0.5 mM cysteine (500 nmol/ml) and as indicated above, the majority of the labeled material was identified as free cysteine. The steepness of the gradient is unknown since it depends upon the volume of the cell occupied by the pool into which cysteine is transported and the initial cysteine content of this pool.

Transport was maximal in cells from logarithmically growing cultures (Fig. 2). Cells from 8-day-old cultures were routinely used in all experiments. A decline in transport was observed in cells taken from stationary cultures. However, the transport rate is relatively uniform when compared to the transport of sulfate into these cells where a 20 nmol/g fresh wt·hr to 100 nmol/g fresh wt·hr rate increase was observed during the logarithmic increase in fresh wt (26).

The pH optimum for cysteine uptake was 4.5 in 10 mM sodium citrate buffer and the transport rate increased with decreasing pH when bis-tris propane was used as a buffer (Fig. 3). The pH optimum for serine transport into tobacco cells was approximately 4 when the incubation period was 1 hr. However, if the incubation period was reduced to 15 min, optimal transport was observed at pH 3 (Smith, unpublished results). Serine was rapidly transported at pH 3 for 15 to 30 min, but the transported material was lost from the cells during the next 30 min so that the net accumulation in 1 hr was low. Since serine and cysteine are probably transported by the same system, the apparent decline in cysteine transport below pH 4.5 (Fig. 3) may be due to increased loss of transported material at low pH resulting in lower observed accumulation. The sharp decline in transport above pH 4.5 is not due to stimulated loss since previous investigations (27) showed that cells placed in pH 10 bicine buffer did not lose significant amounts of sulfur-containing metabolites in a 6-hr period.

The stimulation of amino acid uptake into tobacco cells by low pH is in agreement with a previous investigation of α-aminoisobutyric acid uptake by barley leaves (25) but contrasts with a pH optimum of 7 for sulfate transport by tobacco cells (27). Shtarkshall and Reinhold (25) believe that in barley leaves the transported species is the zwitterion because the increase in cation concentration between pH 6 and 4 would be insufficient to account for the marked increase in transport rate. In addition, they conclude that the primary effect of pH is upon the protonation/deprotonation of the transport site. Alternatively, the increased transport at low pH may be due to the direct utilization of protons in transport; in *E. coli* neutral amino acids are taken up by a symport mechanism with one proton/one amino acid stoichiometry (2).

Variation of B-5 concentration had marked effects on cysteine transport (Fig. 4). The major constituents of B-5 are mM $CaCl_2$, $MgSO_4$, NaH_2PO_4, $(NH_4)_2SO_4$, 25 mM KNO_3 and 20 g/l of sucrose (for complete medium see ref. 5). Maximum uptake rates were observed in 0.1 B-5 medium and in general transport decreased with increasing concentrations of B-5. Sulfate transport into tobacco cells also decreased with increasing salt concentration, but remained relatively constant over a wide range of sucrose concentrations (27).

Kinetics. The cysteine absorption isotherm over a wide concentration range (10^{-6} to 10^{-3} M) is shown in Figure 5. Absorption isotherms which fit rectangular hyperbolas have traditionally been interpreted as evidence for a carrier system and a direct analogy has been drawn with the Michaelis-Menten kinetic treatment of enzyme-catalyzed reactions using one substrate (4). The observation of biphasic or multiphasic absorption isotherms has resulted in attempts to dissect the curves into two or more Michaelis-Menten curves and assign appropriate con-

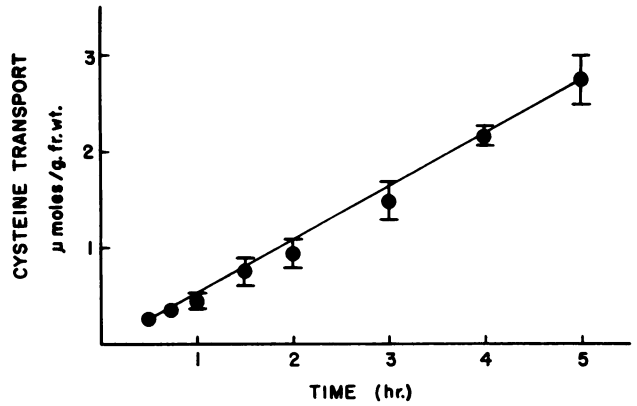

FIG. 1. Linearity of cysteine transport with time. Transport measured as described in text. Cysteine concentration was 5×10^{-4} M.

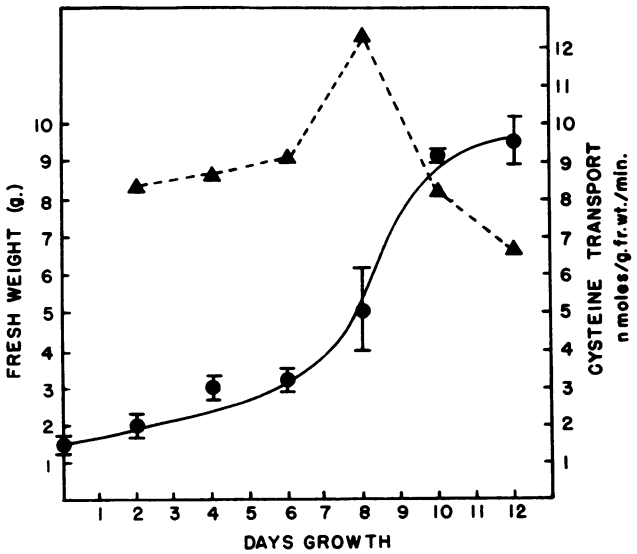

FIG. 2. Effect of culture age on cysteine transport. Transport was measured in 5×10^{-4} M cysteine in transport medium (pH 4.5). ●——●: growth curve; ▲- - -▲: cysteine transport.

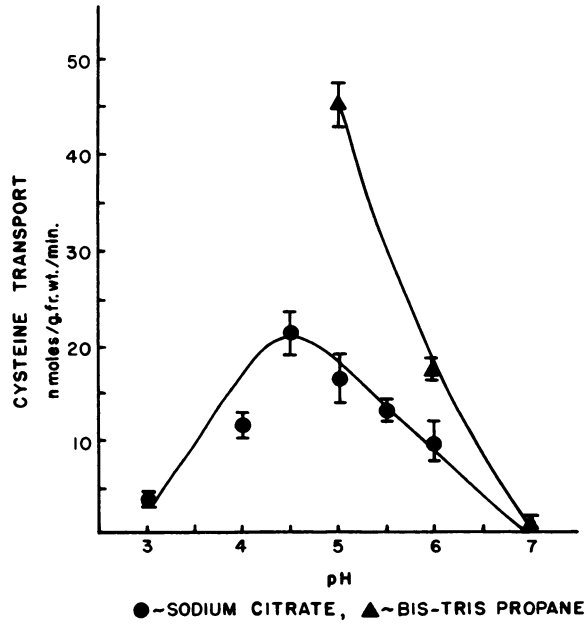

FIG. 3. Effect of pH on cysteine transport. Transport was measured in 5×10^{-4} M cysteine in 0.1 B-5, 0.01 M buffer. ●——●: sodium citrate; ▲——▲: bis-tris propane.

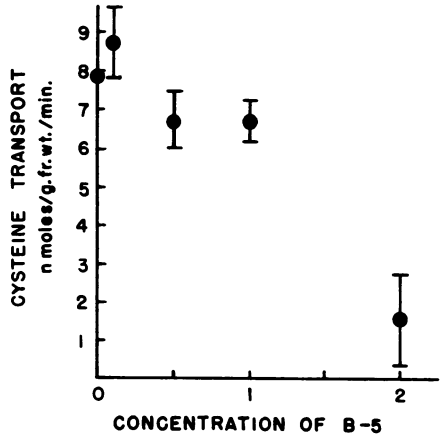

FIG. 4. Effect of B-5 concentration on cysteine transport. Transport measured in 0.01 M sodium citrate (pH 4.5).

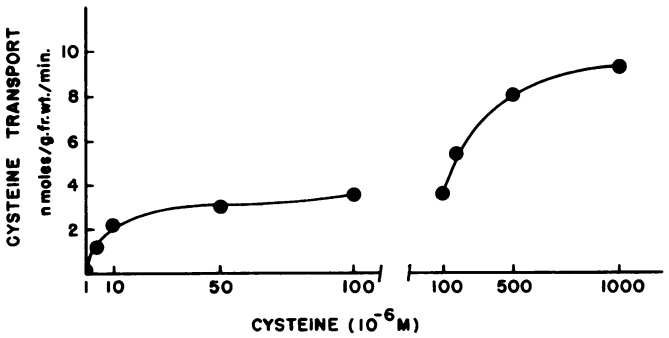

FIG. 5. Effect of wide substrate concentration range on cysteine transport. Transport measured as described in text.

stants (20). Alternatively, Cram (3) states that Michaelis-Menten kinetic data are not applicable to absorption isotherms in plants and these isotherms do not provide mechanistic information.

The transport of a metabolizable amino acid into a plant cell probably involves the initial binding to a membrane-bound carrier system which could be analyzed using Michaelis-Menten kinetics. However, the translocation of the amino acid across the membrane may be accompanied by co-transport with H^+, in which case transport should be treated minimally as a bisubstrate reaction. Alternatively, amino acid transport may require the operation of the γ-glutamyl cycle (18, 28) and the kinetics of γ-glutamyl transfer to the transported amino acid might be more significant than the original binding to the external carrier system. In addition, in bacteria amino acids are transported into an expandable pool (15) which can provide resistance to further transport as the pool approaches maximal expansion and may markedly affect prior steps in the transporting process.

We agree with Thellier (29) that the use of Michaelis-Menten parameters is a convenient method of summarizing experimental results but does not characterize the mechanism of transport. Accordingly, we use the term "high affinity system" to describe cysteine transport at low cysteine concentrations (10^{-6} to 10^{-4} M) and "low affinity system" to describe transport at higher cysteine concentrations (10^{-4} to 10^{-3} M) for descriptive convenience, not as a statement of mechanism.

The high affinity system has a *Km* of 1.7×10^{-5} M (±0.17) and a V_{max} in the range 3 to 6 nmol/g fresh wt min (Figs. 5 and 6). The low affinity system has a *Km* of 3.5×10^{-4} M (±0.13) and a V_{max} in the range 13 to 16 nmol/g fresh wt min. (Figs. 5 and 7). The possibility that the characteristics of the absorption isotherm were due to changes in the oxidized/reduced state of the substrate or membrane sulfhydryl groups was examined

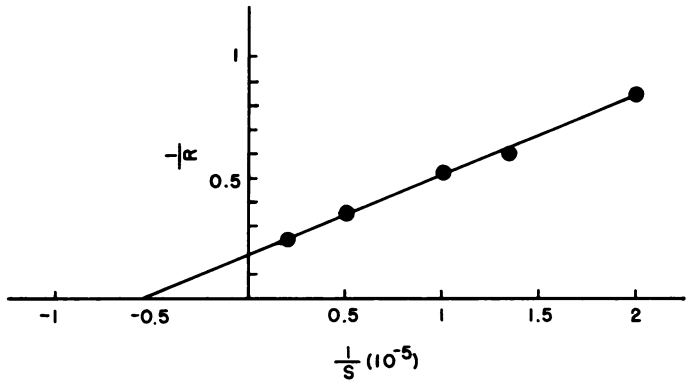

FIG. 6. High affinity *Km*. Double reciprocal plot. Transport measured as described in text; cysteine concentration range was 5×10^{-5} M to 5×10^{-6} M.

using dithiothreitol. Cells were either incubated with DTT (2×10^{-4} M for 1 or 2 hr) prior to measurement of transport or DTT (2×10^{-4} M) was included in the transport medium. Variable results were obtained in separate experiments, but the variation from the control curve did not exceed 10% throughout the entire cysteine concentration range 10^{-6} to 10^{-3} M. Therefore, the low and high affinity systems are not due to differential velocities of cysteine *versus* cystine transport or to the differential velocity of cysteine transport by an oxidized *versus* a reduced carrier system. These results are contrary to the bacterial transport model proposed by Kaback and Barnes (11) where the oxidized carrier has a much higher affinity for the substrate than the reduced carrier. The majority of biphasic or multiphasic transport systems are found in plants and are responsible for the transport of a variety of solutes (4, 6, 19, 21, 22). The biphasic uptake represented in Figure 5 is a characteristic of the transport system and is in agreement with previous observations of multiphasic uptake of amino acids in plants (13, 25).

Respiratory Inhibitors. At a cysteine concentration of 5×10^{-4} M net absorption was inhibited more than 90% by 10^{-5} M azide or 10^{-4} M DNP[1] (Table I), which is in general agreement with the effects of these compounds on sulfate transport into tobacco cells (27). Since CCCP can react with −SH groups (12) the effect of this compound was tested either when present in the transport medium or in preincubation experiments. In the latter, cells were preincubated for 1 hr in 0.1 B-5 containing CCCP and washed with 100 ml of inhibitor-free 0.1 B-5 before addition to the transport medium. There were no differences in rate determined by either of these methods. The effectiveness of CCCP (10^{-5} to 10^{-4} M) as an inhibitor depended on the cysteine concentration. At high cysteine concentration (5×10^{-4} M) inhibition was approximately 50% while at low concentration (2×10^{-5} M) inhibition was approximately 80% (Table I). DCCD was not inhibitory at the concentrations of CCCP (10^{-6} M) and DCCD (10^{-4} M) which completely inhibited sulfate transport into tobacco cells (27).

The effect of the above inhibitors on the efflux of labeled material from cells was also examined (Table II). Cells were allowed to transport L-[^{35}S]cysteine (10^{-4} M) for 4 hr; washed with 50 ml of 0.1 B-5 containing 1 mM unlabeled cysteine, and then placed in 40 ml of 0.1 B-5 0.01 M citrate (pH 4.5) containing 10^{-5} M inhibitor. In the absence of inhibitor, approximately 12% of the label was released into the medium in 1 hr, but subsequent loss was not significant (Table II). The rate of label efflux was greatest in the presence of azide with 90% of the label released in 2 hr. Ethanol induced a slight increase in

[1] Abbreviations: DNP: 2,4-dinitrophenol; CCCP: carbonylcyanide *m*-chlorophenylhydrazone; DCCD: N,N'-dicyclohexylcarbodiimide; NEM: N-ethylmaleimide.

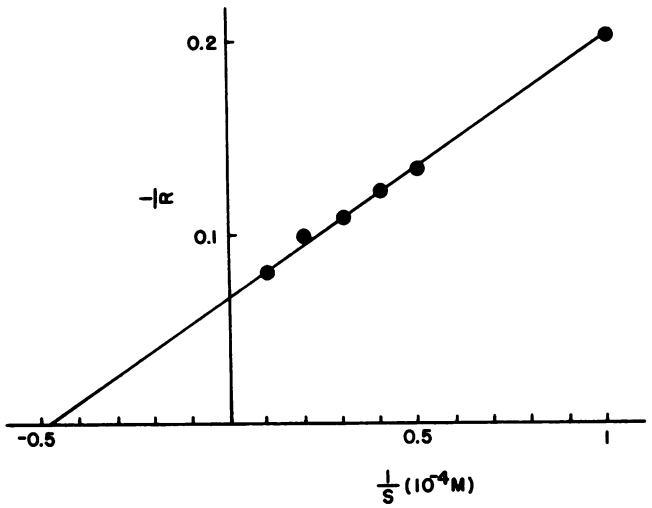

FIG. 7. Low affinity Km. Double reciprocal plot. Transport measured as described in text; cysteine concentration range was 10^{-3} M to 2 × 10^{-4} M.

Table I. Effect of metabolic inhibitors on cysteine transport. Transport was measured as described in text. Cells were incubated in CCCP and DCCD solutions (0.5% ethanol) for 1 hr. and washed before the beginning of the experiment. Control rates were: high affinity transport - 2.1 nmoles/g. fr. wt./min.; low affinity transport - 11.2 nmoles/g. fr. wt./min.

	Percent Inhibition of Net Absorption	
	Concentration Cysteine (M)	
Inhibitor	$2x10^{-5}$	$5x10^{-4}$
Azide 10^{-4}	-	97
10^{-5}	-	95
10^{-6}	-	4
CCP 10^{-4}	75	56
10^{-5}	83	50
10^{-6}	3	13
DCCD 10^{-4}	0	0
10^{-5}	0	0
10^{-6}	0	0
DNP 10^{-4}	-	93
10^{-5}	-	80
10^{-6}	-	13
Ethanol (0.5%)	11	11

efflux compared to the control and the ethanol figure is used as the standard for CCCP, DCCD, and DNP which were dissolved in ethanol. CCCP and DNP induced the loss of more than 80% of the label in 3 hr while DCCD was without effect. The above results indicate that material accumulated in 4 hr was almost totally lost when placed in either 10^{-5} M azide, CCCP, or DNP for 4 hr (Table II). The inhibition of net adsorption by these compounds (Table I) may be due to stimulated efflux (Table II) and not to an effect of these compounds on cysteine influx.

Effect of Sulfhydryl Reagents on Transport. As reported in the kinetics section, DTT had no significant effect on transport when present in the transport medium. Sulfate transport was not affected by preincubating cells with mercaptoethanol but thiol-reducing agents were potent inhibitors when included in the transport medium (*e.g.* 50 and 100% inhibition at 10^{-5} and 10^{-4} M, respectively) (27).

The sulfhydryl reagents N-ethylmaleimide and iodoacetamide were potent inhibitors of transport (Table III) which is in agreement with the observations of several investigators using a variety of organisms (9, 20). However, as reported previously (27), at inhibitory concentrations these compounds induce the total efflux of the sulfur-containing metabolites from tobacco cells and consequently are not useful for characterizing a specific transport system.

Effect of Amino Acids on Cysteine Transport. The effect of several amino acids on cysteine uptake by the high and low affinity systems was examined. At both cysteine concentrations, transport was inhibited by a wide range of amino acids (Table IV). In general, neutral aliphatic amino acids were most effective, which is in agreement with previous studies of cysteine transport in tobacco cells (8) and alanine transport into cultured soybean cells (14). These observations support the presence of

Table II. Effect of inhibitors on label efflux. Efflux measured as described in text. Cells were incubated in inhibitors for time periods indicated. Percentages are based on total labelled cell contents at time 0.

Inhibitor (M)	Time (hr)	Percent of total label to efflux
None	1	12
	2	13
	3	14
	4	12
Azide 10^{-5}	1	67
	2	90
	3	90
	4	88
Ethanol (0.5%)	1	15
	2	18
	3	21
	4	19
CCCP 10^{-5}	1	31
	2	62
	3	81
	4	79
DCCD 10^{-5}	1	14
	2	18
	3	21
	4	21
DNP 10^{-5}	1	23
	2	50
	3	90
	4	84

Table III. Effect of sulfhydryl reagents and dithiothreitol on cysteine transport. Transport was measured as described in text. Cells were preincubated in test compounds for 1 hr. and washed before the beginning of the experiment. Cysteine concentration was 5 X 10^{-4}M. Control rate was 13.3 nmoles/g. fr. wt./min.

	Percent Inhibition of Transport		
	Inhibitor Concentration (M)		
Inhibitor	10^{-3}	10^{-4}	10^{-5}
DTT	10	0	0
Iodoacetamide	-	84	31
NEM		89	81

a neutral amino acid transport system in cultured plant cells, and is in agreement with observations of general amino acid transport in bacteria (23) and plants (14).

L-Cysteine transport was slightly inhibited by D-cysteine. The latter is able to support growth of these cells as a sole sulfur source (8). S-methyl cysteine was an effective inhibitor; however, cysteine methyl ester was relatively ineffective (Table IV). These results support the earlier conclusion that the zwitterion is the recognized and transported species. Methionine was more inhibitory than would be expected which is similar to its effects on arginine, glutamic acid, and alanine transport in soybean cells (14). Preincubation experiments indicated that the effect of methionine was not due to depletion of the endogenous ATP pool. Cells which had been preincubated with methionine for up to 2 hr had normal transport when the methionine was removed prior to the measurement of transport. The acidic amino acids glutamic acid and aspartic acid were, in general, the least effective inhibitors of the amino acids tested. The discrepancies in their behavior have been observed in soybeans, where aspartic acid was not inhibitory to glutamic acid uptake (14). There is a very close correlation between the ability of the tested amino acids to inhibit cysteine transport (Table IV) and their ability to accept the γ-glutamyl moiety in model studies of γ-glutamyl transpeptidase (28).

The effect of alanine and methionine on the kinetics of cysteine uptake was examined. Alanine was a competitive inhibitor of both the high and low affinity systems (Table V) with inhibition constants 3.8×10^{-5} M and 4.8×10^{-4} M, respectively. These values are within the range of the Km for cysteine transport for the two systems. Similarly, methionine was a competitive inhibitor of transport with Ki of 1.8×10^{-5} M and 2.3×10^{-4} M for the high and low affinity systems (Table V). As previously mentioned, Hart and Filner (8) reported inhibition of cysteine transport by several amino acids. This inhibition, however, occurred only after a 1- or 2-hr lag and not immediately as would be expected in the case of a competitive inhibition. There were no such lags observed in any of the experiments reported in this research. We feel that the mechanistic significance of the above observations must await a more detailed knowledge of the steps involved in cysteine transport and must include a consideration of the role of transinhibition by endogenous amino acid pools in cysteine uptake.

Table IV. Effect of amino acids and analogues on cysteine transport. Transport was measured as described in text. Control rates were: high affinity transport - 1.9 nmoles/g. fr. wt./min.; low affinity transport - 8.9 nmoles/g. fr. wt./min. Amino acid concentration was five-fold higher than cysteine concentration.

	Percent Inhibition of Transport	
	Concentration Cysteine (M)	
Amino Acid	$2x10^{-5}$	$5x10^{-4}$
D-Cys	35	15
S-methyl Cys	72	57
Cys methyl ester	27	10
Met	86	73
Gly	21	54
Ala	74	65
Ser	56	38
Thr	47	38
Glu	38	29
Val	28	47
Leu	44	56
Ileu	35	45
Asp	16	6

Table V. Inhibition constants for cysteine transport. Transport was measured as described in text. Antagonistic amino acid concentrations were $5x10^{-4}$M and 10^{-4}M (high affinity transport) and 10^{-3}M and $5x10^{-4}$M (low affinity transport). Control rates were: high affinity transport - 2.1 nmoles/g. fr. wt./min.; low affinity transport - 8.8 nmoles/g. fr. wt./min.

	Concentration Cysteine (M)	
Antagonist	$2x10^{-5}$	$5x10^{-4}$
Alanine	$3.8x10^{-5}$M	$4.8x10^{-4}$M
Methionine	$1.8x10^{-5}$M	$2.3x10^{-4}$M

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