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ASPARAGINE TRANSPORT IN *LACTOBACILLUS PLANTARUM* AND *STREPTOCOCCUS FAECALIS*

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SUMMARY

The properties of the asparagine transport systems in *Lactobacillus plantarum* and *Streptococcus faecalis* are described. In both organisms the uptake of isotopically labeled L-asparagine was markedly stimulated by glucose. Kinetic studies yielded curvilinear Lineweaver–Burk plots in both organisms. These data were most consistently accounted for in both organisms by assuming the operation of two catalytic uptake components in addition to a diffusion component. The occasional limitation of kinetic studies in distinguishing between single or multiple catalytic components is illustrated. A large selection of structurally related amino acids and other substances were tested as competitors in initial rate studies. In *L. plantarum* the most effective competitors were L-asparagine and L-glutamine. A small number of structurally related dicarboxylic acid amide derivatives were only moderately effective competitors. In contrast, the most effective competitors of L-asparagine uptake in *S. faecalis* were relatively small neutral amino acids such as L-alanine, L-serine, L-α-aminobutyric acid, L-cysteine and L-methionine, suggesting that asparagine enters this organism by reaction with a catalyst having relatively unspecific structural discrimination among neutral amino acids. Both organisms rapidly converted a large proportion of the transported asparagine to aspartic acid. In *S. faecalis*, the deamidation of L-asparagine was shown to be relatively insensitive to inhibition by those amino acids which were most effective in reducing the asparagine entry rate.

INTRODUCTION

The apparent effectiveness of L-asparaginase as a growth inhibitor of some neoplastic cells has focused attention on the relatively incomplete understanding of the metabolic reactions of asparagine. This lack of information extends also to the transport of this amino acid amide. Although several investigators have used asparagine as a competitor in surveys of various amino acid transport systems in mammalian cell types[1–4], there has not been a systematic description of the transport properties of this substance. Since the nutritional effectiveness of asparagine very likely is partially dependent on its interaction with transport systems, and an under-

Abbreviations: MES, 2-(*N*-morpholino)ethanesulfonic acid; HEPES, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid.

standing of these relationships might provide a means for controlling its utilization, it seemed additionally of interest to characterize these catalysts. Some properties of the asparagine transport systems in *Lactobacillus plantarum* and *Streptococcus faecalis* will be described here. Asparagine transport in mammalian ascites tumor cells also has been studied and will be described separately. During the preparation of this manuscript Willis and Woolfolk[5] described a relatively specific asparagine transport system in *Escherichia coli*. A preliminary report of this study has appeared[6].

METHODS AND MATERIALS

Organisms and culture conditions

Lactobacillus plantarum 17-5 (ATCC 8014) and *Streptococcus faecalis* R (ATCC 8043) were maintained in stab culture and grown in synthetic media for use in uptake experiments as described previously[7–9]. After collection by centrifugation the cells were washed by resuspending once in 1/4 to 1/3 the original volume of 10-fold diluted uptake buffer (0.012 M in phosphate for *L. plantarum*, 0.018 M in phosphate for *S. faecalis*).

Uptake experiments

Uptake of L-[^{14}C]asparagine was carried out as described previously for *S. faecalis*[9] in a phosphate-buffered salts solution containing glucose as energy source. *L. plantarum* was studied under identical conditions except the buffered salts solution contained 0.12 M phosphate[7]. Both organisms were incubated at 37 °C generally in a volume of 1 ml with a cell concentration of 1.6 mg per ml. In most experiments L-[^{14}C]asparagine was used at a final concentration of 2 mM with *L. plantarum* and 0.5 mM with *S. faecalis*. The specific activity was adjusted to a level (0.2–1.0 Ci/mole) which permitted rapid counting of cellular isotope to a high level of statistical significance. Incubations were terminated by removing 100-μl samples to 1 ml of ice-cold uptake buffer and filtering through chilled Millipore filters. The cells and filters were washed twice with 1-ml aliquots of cold buffer and then removed to scintillation counting vials containing 2.5 ml absolute ethanol when using a toluene-based counting fluid, or 1 ml of 15% ethanol when using a dioxane-based counting fluid. The asparagine molar equivalence of detected isotope was calculated using standards in which asparagine solutions of known specific activity were applied to Millipore filters coated with 0.16 mg cells in scintillation vials prior to the addition of ethanol and counting fluid.

The pH optimum was determined using the following buffers: 2-(*N*-morpholino)ethanesulfonic acid (MES) at pH values between 4.8 and 6.0; phosphate, pH values between 5.6 and 7.2; *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES), pH values between 6.8 and 7.6. In all cases these substances were present at a final concentration of 0.12 M for *L. plantarum* and 0.18 M for *S. faecalis*. The phosphate buffers used in the pH experiments were made by mixing equimolar solutions of the mono- and dibasic phosphates each containing equimolar concentrations of Na^+ and K^+. $MgCl_2$ was added at concentrations described previously. MES and HEPES were adjusted to the desired pH values using HCl or a solution containing equimolar amounts of NaOH and KOH as required.

Metabolism of asparagine during transport

L. plantarum and *S. faecalis* were incubated for 2 min at 1.6 mg/ml (final volume 3.8 ml) in uptake buffer containing glucose and 2 mM L-[^{14}C]asparagine. The tubes were chilled, the cells separated by centrifugation and cell extracts prepared using 5% trichloroacetic acid as described previously[9]. Aliquots of extracts equivalent to 3.2 mg of cells were chromatographed in a Technicon Amino Acid Autoanalyzer using norleucine as an internal standard. The columns were run for 4.5 h at 30 °C and 16.5 h at 60 °C. There was no detectable deamidation of glutamine or asparagine under these conditions. Two columns were operated in parallel. A control derived from cells incubated with nonradioactive asparagine was passed through one column to obtain a trace describing the separated ninhydrin-reactive components. A duplicate sample from cells incubated with radioactively labeled asparagine was passed through the other column and the eluate collected directly in tubes on a fraction collector without reaction with ninhydrin. Aliquots of these fractions were analyzed for isotope by scintillation counting. Identification of column fractions was confirmed by paper electrophoresis of hydrolyzed and unhydrolyzed samples from tubes showing significant isotope content.

Asparagine metabolism in cell-free extracts of S. faecalis

Experiments to determine the relative effectiveness of various substances as competitors of asparagine deamidation were performed as follows. Cells grown and washed as described above were suspended in 0.01 M phosphate buffer (pH 7.0) to a density of 8–10 mg/ml. The suspension was sonicated with glass beads in a Branson Sonifier (Model W185C, Heat Systems Co., Melville, N.Y.) until the decline in absorbance deviated markedly from linearity. Generally this required from 30 to 50 min at which time the absorbance was 20 to 25% of its initial value. Sonication was carried out in a rosette cooling cell immersed in an ice-water bath. The unit was operated for 3 to 5 min periods between which a minute was allowed for the suspension to cool. After sonication the suspension was centrifuged at $8000 \times g$ for 10 min. The supernatant was decanted carefully and used in the metabolism experiments. In most experiments cell extract equivalent to 4–5 mg cells was incubated in a volume of 2 ml with 2 mM L-[^{14}C]asparagine in 0.05 M phosphate buffer (pH 7.5) at 37 °C in the absence and presence of various competitors at 10 mM. After 30 min the tubes were chilled in ice and 2 ml of cold 5% trichloroacetic acid was added. After 30 min the tubes were centrifuged at $4000 \times g$ for 5 min and the clear supernatant decanted. The residue was re-extracted with 2 ml of 5% trichloroacetic acid. The two supernatants were combined and extracted repeatedly with ether to remove trichloroacetic acid. The supernatant was adjusted to pH 6–7 and passed through a small column (0.8 cm × 4 cm) of Dowex AG-1 (X8, 200–400 mesh) in the acetate form. The sample was washed into and through the column using water. An initial 15 ml and five 2-ml fractions were collected which contained all the asparagine present in the sample. Aspartic acid was eluted by adding 2 M HCl to the column. A 6-ml and three 2-ml fractions were collected. Aliquots of all fractions were assayed for isotope by scintillation counting.

Chemicals

L-[U-^{14}C]Asparagine was obtained either from New England Nuclear, Boston, Mass., or Amersham/Searle, Arlington Heights, Ill. To minimize the presence of

trace amounts of labeled aspartic acid, the preparations used in these experiments were chromatographed on columns of Dowex 1 (AG-1 X8, 200–400 mesh, acetate form) and eluted with water (see above under Metabolism). The presence of such high specific activity contaminants can greatly distort the results of substrate competition studies. Most of the aspartic and glutamic acid derivatives tested were provided by Dr E. Roberts of this department who obtained them from Merck, Sharpe and Dohme, Rahway, N.J. All other substances were the highest grade commercially available.

RESULTS

Time-course of uptake and glucose dependence

The time-course of L-[^{14}C]asparagine uptake by *L. plantarum* and *S. faecalis* in the absence and presence of glucose is shown in Fig. 1. Apparent pool sizes based on isotope content are high, in the order of magnitude observed previously with the respective dicarboxylic acid. As will be demonstrated below, there is considerable metabolism of the transported amide, and the asparagine pool sizes, while substantial, are considerably lower than indicated in Fig. 1. Glucose stimulated uptake in both organisms, although there was significant uptake by *S. faecalis* in the absence of this exogenous energy source. A clearer indication of the dependence of the initial uptake rate on glucose over a broad concentration range is shown in Fig. 2 for *L. plantarum*.

As can be seen more clearly in Figs 3A and 3B, there is some uptake of isotope

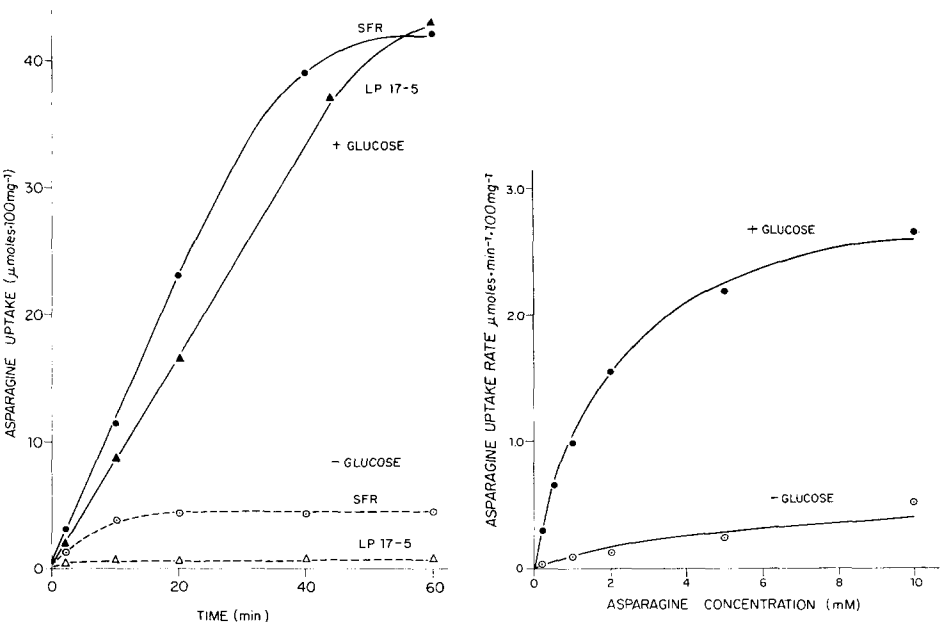

Fig. 1. Time course of L-[^{14}C]asparagine uptake by *L. plantarum* (LP) and *S. faecalis* (SF) in the absence and presence of glucose. Extracellular asparagine concentration, 2.0 mM.

Fig. 2. Effect of glucose on the initial rate of L-asparagine uptake by *L. plantarum* in the concentration range 0.2–10.0 mM.

in the absence of glucose and one of the more notable features of this process is that in both organisms the uptake reaches a plateau level at approximately the same time regardless of the external asparagine concentration. The height of the plateau, on the other hand, is determined by the external concentration. The quantitative

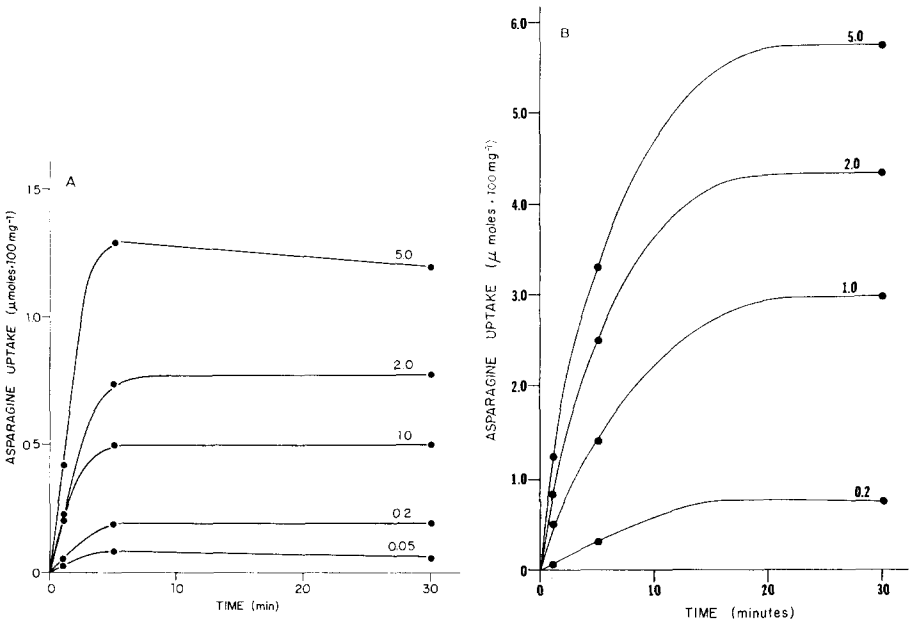

Fig. 3. Time course of asparagine uptake by (A) *L. plantarum*, (B) *S. faecalis* in the absence of glucose. The figures on the curves indicate the initial extracellular asparagine concentrations (mM).

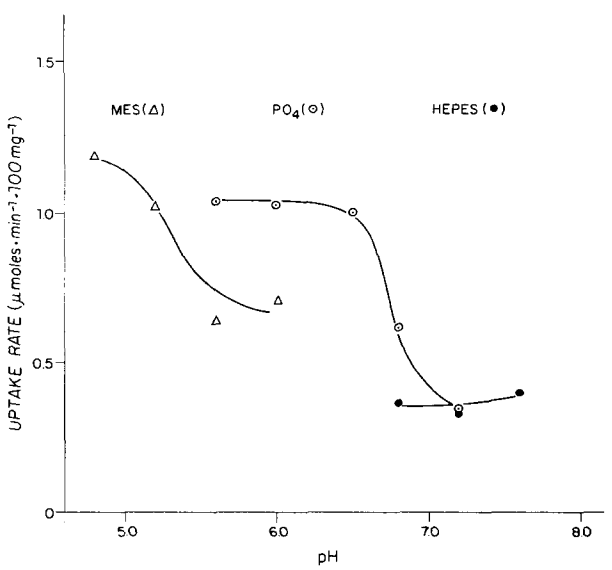

Fig. 4. Effect of buffer pH and composition on the initial rate of L-[^{14}C]asparagine uptake by *L. plantarum*. The indicated buffer was used at a final concentration of 0.12 M.

relationship shown in Figs 3A and 3B could not be accounted for by assuming the presence of intracellular asparagine and aspartic acid pools of various sizes with which the external molecules exchange. The abrupt termination of isotope uptake after 5 and 15 min in *L. plantarum* and *S. faecalis* regardless of external concentration suggests instead that the phenomenon may be related to the non-stoichiometric depletion of an intracellular component whose metabolism or exit from the cell can promote the entry of extracellular labeled molecules.

Effect of pH on initial rate

The effect of buffer composition and pH on the asparagine uptake rate in *L. plantarum* is shown in Fig. 4. In phosphate there was a broad maximum between pH 5.6 and 6.5. The activity at lower pH values (to 4.8) very likely is at least as high but was difficult to establish with certainty because the buffer used in this range (2-(*N*-morpholino)ethanesulfonic acid) appeared to inhibit uptake somewhat. Similar results were obtained with *S. faecalis*. In view of the necessity to avoid as much as possible the degradation of asparagine to aspartic acid, most experiments have been carried out in a phosphate buffer at pH 6.5. These conditions also eliminated a pronounced lag in the uptake rate which was consistently observed at lower pH values.

In *L. plantarum* uptake was not affected by the absence of Na^+ or K^+ at low or high extracellular asparagine concentrations. The omission of Mg^{2+} from the buffer reduced the uptake rate 35%. Substitution of Sr^{2+} for Mg^{2+} prevented most of this decline in rate.

Metabolism of asparagine

The distribution of isotope among intracellular pool components is summarized in Table I. Both organisms rapidly converted most of the asparagine taken up to

TABLE I

METABOLISM OF L-[^{14}C]ASPARAGINE IN *L. PLANTARUM* AND *S. FAECALIS*

Pool component	*% of pool radioactivity**		
	L. plantarum	*S. faecalis*	*Unincubated control***
Asparagine	12.6	22.5	97.7
Aspartic acid	76.9	61.4	—
Unidentified:			
Acidic No. 1	4.9	3.0	0.9
Acidic No. 2	4.4	7.7	1.0
Neutral	—	0.5	—

* Cells were incubated with L-[^{14}C]asparagine for 2 min as described under Methods and Materials. With *S. faecalis*, 96.2% and with *L. plantarum* 99.3% of the isotope applied to the column was recovered in individual fractions. The figures shown represent the percentage of recovered isotope present in the indicated fraction. The balance of the recovered isotope was distributed in small amounts among several additional minor fractions.

** An aliquot of L-[^{14}C]asparagine was added to a trichloroacetic acid cell extract which was then chromatographed as described for experimental samples.

other substances most notably aspartic acid. The unincubated control shows that the asparagine preparation used was essentially free of labeled aspartic acid, but contained detectable amounts of two unidentified acidic impurities. The rapid conversion of asparagine to aspartic acid raised the possibility that this reaction might have occurred in the outer region of the cell membrane and that most of the isotope entered the cell as aspartic acid. Two observations are in conflict with this interpretation. As shown in Fig. 5 an *S. faecalis* mutant (R-4) which lacks a high affinity dicarboxylic amino acid transport system, and, consequently, is markedly deficient in aspartic acid transport[10] was not at all handicapped in accumulating asparagine-derived isotope over a broad concentration range. This observation indicates also that the inactivity of this dicarboxylic amino acid transport component does not impair the retention of intracellular aspartic acid formed from asparagine despite an exceptionally large concentration gradient. In addition, aspartic acid was found to be a very poor competitor of L-asparagine uptake (see below). Both observations suggest that the conversion of asparagine to aspartic acid occurs after or at the earliest, during the transport event.

In view of this extensive metabolism, it is not possible to speak with any assurance about the size of the asparagine pool which these organisms might be capable of accumulating. In *S. faecalis* where 8% of the intracellular isotope after 40 min incubation was still present in asparagine, a minimal value would be approximately 3.2 μmoles/100 mg cells (8% of 40 μm/100 mg, Fig. 1). This is equivalent to an intracellular concentration of approximately 16 mM. Since the initial outside concentration was 2.0 mM, at least an 8-fold concentration gradient can be formed, indicating the operation of an active transport process. It would be necessary to

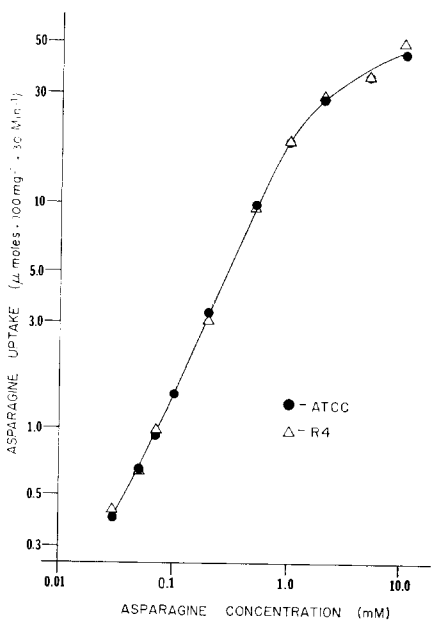

Fig. 5. Effect of concentration on the uptake of L-[^{14}C]asparagine by the parental (●, ATCC) and dicarboxylic amino acid transport mutant (△, R-4) strains of *S. faecalis*.

inhibit metabolism to estimate with confidence how large a gradient these systems could establish.

Kinetics of asparagine transport

Lineweaver–Burk plots of initial rate uptake data obtained with *S. faecalis* and *L. plantarum* are presented in Fig. 6. In both organisms curvilinear plots generally were obtained, although the deviation from linearity occasionally was not marked. Using a curve-fitting procedure involving successive approximation of the kinetic constants as described previously[9], and assuming the operation of two catalytic transport components and a diffusion term, the constants shown in Table II were derived. The close correspondence between experimentally observed rates and those predicted by calculated constants in an experiment with *L. plantarum* is illustrated in Fig. 7.

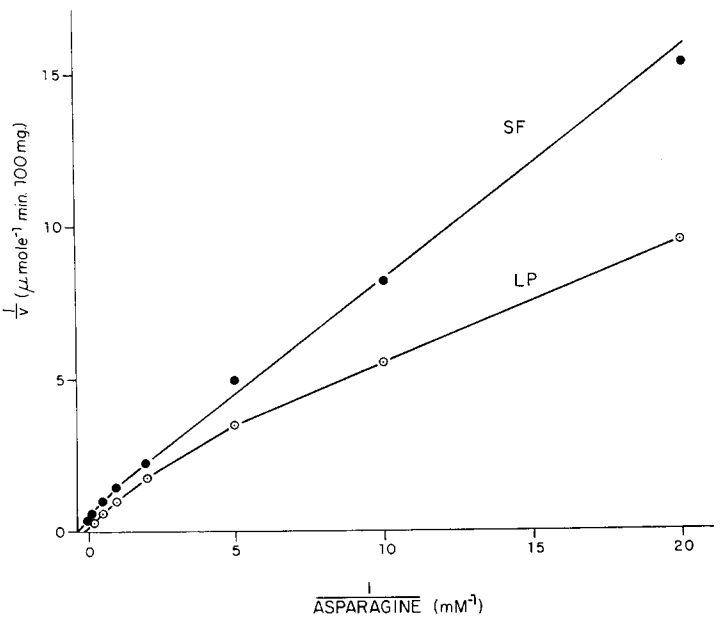

Fig. 6. Reciprocal plots of initial rates of asparagine uptake and extracellular asparagine concentration for *S. faecalis* (SF, ●) and *L. plantarum* (LP, ⊙).

TABLE II

KINETIC CONSTANTS FOR ASPARAGINE TRANSPORT IN *S. FAECALIS* AND *L. PLANTARUM*

Organism	*No. of expts*	K_1 (*mM*)	V_1 (*μmoles·min⁻¹· 100 mg⁻¹*)	K_2 (*mM*)	V_2 (*μmoles·min⁻¹· 100 mg⁻¹*)	k_D (*ml·min⁻¹· 100 mg⁻¹*)
S. faecalis	4	0.16±0.01	0.10 ±0.02	2.9±0.15	2.1±0.10	0.15 ±0.11
L. plantarum	6	0.17±0.07	0.060±0.051	1.7±0.18	2.0±0.21	0.037±0.015

With both organisms it was found in some experiments that the observed rates could be accounted for equally well by assuming a diffusion term and either one or two catalytic components. An example of such an experiment with *S. faecalis* is illustrated in Fig. 8. In most experiments however, a successful fit to the experimental data could be obtained only by assuming two saturable uptake systems, and it is on this basis that we concluded that there is more than one system catalyzing the entry of asparagine in these organisms.

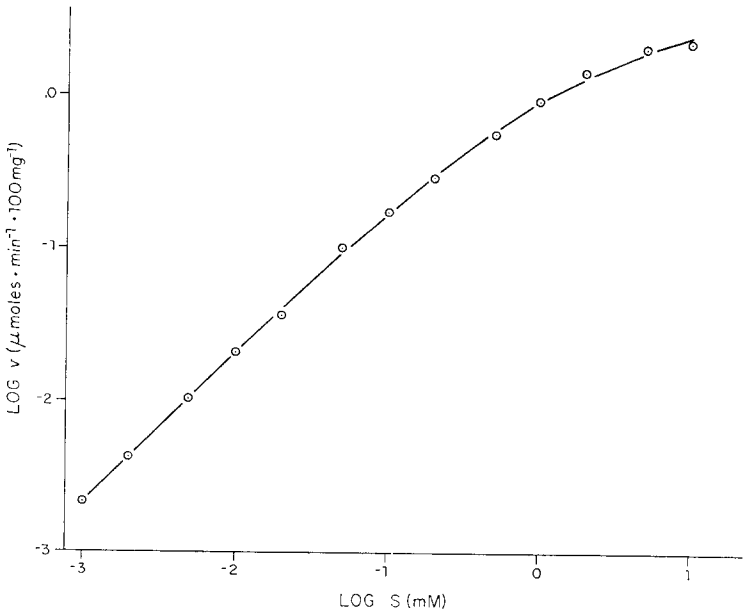

Fig. 7. Variation in the initial rate of L-asparagine uptake by *L. plantarum* over the concentration range 0.001 to 10.0 mM. The points are experimentally derived values. The line was drawn assuming the following constants: $K_1 = 0.151$ mM, $V_1 = 0.148$ μmoles·min^{-1}·100 mg^{-1}, $K_2 = 1.92$ mM, $V_2 = 2.36$ μmoles·min^{-1}·100 mg^{-1} and a diffusion component with $k_D = 0.03$ ml min^{-1}·100 mg^{-1}.

Structure specificity of asparagine transport in L. plantarum

The structural requirements for interaction with the transport catalysts were investigated by measuring the initial rates of L-[^{14}C]asparagine uptake in the absence and presence of a large number and variety of potential competitors. Competitors were tested at two concentrations and the percentages of control uptake rates were calculated. In some cases the average of these two values was used for comparative purposes. Table III lists the most active competitors encountered and Table IV shows most of the compounds tested using *L. plantarum* arranged according to structure type. It is apparent that the so-called low affinity system which predominates at the asparagine concentrations used in these experiments (2 mM) is relatively specific since L-[^{12}C]asparagine and glutamine were by far the most effective competitors of L-[^{14}C]asparagine uptake. The next most active compound was L-2-amino-3-ureidopropionic acid (albizzin) a substance reported to be an antagonist of glutamine in purine biosynthesis[11]. The hydrazide and hydroxamate derivatives had significant activity, although the hydrazide of glutamic acid was only marginally effective. The

α-methyl derivative of glutamine had significant competitive activity, but the corresponding asparagine derivative was ineffective. L-Tryptophan had significant competitive activity as it did also in *S. faecalis*. The D-isomers of asparagine and glutamine

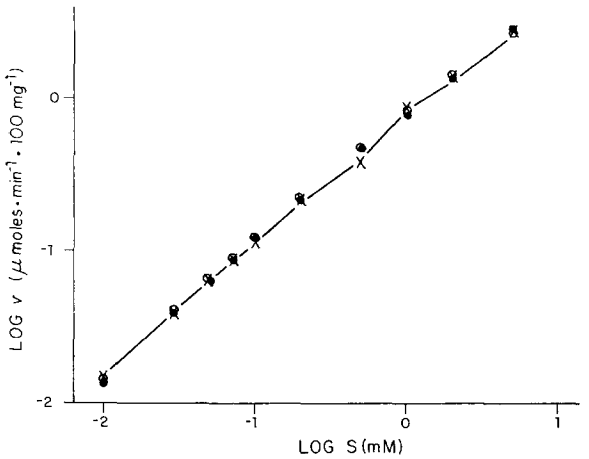

Fig. 8. Variation in the initial rate of L-asparagine uptake by *S. faecalis* over the concentration range 0.01 to 5.0 mM. The line connects the experimentally derived values (×). Values indicated by (●) refer to the rates predicted by the following assumed constants; $K_m = 0.472$ mM, $V = 0.418$ μmoles·min^{-1}·100 mg^{-1} and a diffusion component with $k_D = 0.50$ ml·min^{-1}·100 mg^{-1}; (⊙) refers to the rates predicted by the following constants; $K_1 = 0.165$ mM, $V_1 = 0.085$ μmoles·min^{-1}·100 mg^{-1}, $K_2 = 3.05$ mM, $V_2 = 2.00$ μmoles·min^{-1}·100 mg^{-1}, $k_D = 0.26$ ml·min^{-1}·100 mg^{-1}.

TABLE III

RELATIVE EFFECT OF STRUCTURAL ANALOGS AS INHIBITORS OF L-ASPARAGINE TRANSPORT RATE IN *L. PLANTARUM*

Uptake rates were measured by millipore filtration as described under Methods and Materials using L-[^{14}C]asparagine at 2 mM. Rates measured in the presence of the indicated nonradioactive competitors were divided by the rate measured in the absence of competitor to derive the % control uptake rate values.

Rank No.	*Compound*	*% Control uptake rate*		
		5 mM	*25 mM*	*Av. of 5 and 25 mM*
1	L-Asparagine	30	16	23
2	L-Glutamine	28	26	27
3	L-2-Amino-ureidopropionic acid	63	30	47
4	DL-Aspartic acid-β-hydrazide	83	53	68
4	L-Glutamic acid-γ-hydroxamate	74	62	68
6	DL-Aspartic acid-β-hydroxamate	77	66	72
6	L-Tryptophan	81	62	72
8	L-Valine	91	63	77
9	α-Methyl-DL-glutamine	88	67	78
10	L-2,4-Diaminobutyric acid	80	80	80

TABLE IV

RELATION BETWEEN COMPETITOR STRUCTURE AND INHIBITION OF L-ASPARAGINE TRANSPORT RATE IN *L. PLANTARUM*

See Table III for incubation conditions. The percentage of control uptake rates using competitors at 5 and 25 mM were determined and the averages of these values were calculated. The following additional compounds were tested. The value given after each substance is the average of the % control uptake rates. D-Aspartic acid, 93; D-glutamic acid, 94; DL-α-amino-β-phosphonopropionic acid, 88; α-methyl-DL-aspartic acid, 93; α-methyl-DL-glutamic acid, 93; β-methyl-DL-aspartic acid, 86; β-methyl-β-hydroxy-DL-aspartic acid, 91; L-glutamic acid γ-ethyl ester, 100; *N*-carbobenzoxy-L-asparagine, 85; *N*-carbamyl-L-aspartic acid, 99; glycylglycine, 117; α-aminoisobutyric acid, 102; L-isoleucine, 87; L-leucine, 98; cycloleucine, 84; DL-*C*-allylglycine, 90; L-serine, 87; DL-*O*-methylserine, 92; L-methionine sulfoxide, 93; L-methionine sulfone, 102; L-ethionine, 101; *S*-methyl-L-cysteine, 90; DL-penicillamine, 107; L-proline, 108; L-hydroxyproline, 96; 6-aminopenicillanic acid, 95; pyrrole-2-carboxylic acid, 108; L-ornithine, 98; L-arginine, 94; L-histidine, 103; L-phenylalanine, 94; DL-*p*-fluorophenylalanine, 87; *N*-acetylglycine amide, 105; β-alanine, 95; DL-β-aminobutyric acid, 90; L-citrulline, 88; aminooxyacetic acid, 113; DL-α-hydroxy-ε-aminocaproic acid, 95; glutaric acid, 113; α-ketoglutaric acid, 115; succinic acid, 120; 2-amino-4-carboxy-5-chloropyrimidine, 90; D-glucosamine, 106; D-glucosaminic acid, 103; D-glucosamine 6-phosphate, 93; D-glucuronic acid, 108.

Structure type	*Compound*	*Av. % control uptake rate*
L-Isomer	L-Asparagine	23
	L-Glutamine	27
D-Isomer	D-Asparagine	82
	D-Glutamine	93
Dianionic	L-Aspartic acid	97
	L-Glutamic acid	91
	L-Cysteic acid	85
α-Hydrogen substitution	α-Methyl-DL-asparagine	101
	α-Methyl-DL-glutamine	78
β- or γ-carboxyl derivative	DL-Aspartic acid β-hydroxamate	72
	DL-Aspartic acid β-hydrazide	68
	L-Glutamic acid γ-hydroxamate	68
	L-Glutamic acid γ-hydrazide	88
	L-Aspartic acid β-methyl ester	90
	DL-Glutamic acid γ-ethylamide	105
α-Amino derivative	*N*-Carbamyl-L-asparagine	93
	Glycyl-L-asparagine	105
Neutral, aliphatic amino acids	Glycine	84
	L-Alanine	91
	L-α-Aminobutyric acid	103
	L-Valine	77
Hydroxy amino acids	L-Threonine	87
Sulfur amino acids	L-Methionine	87
	S-Carbamyl-L-cysteine	81
	S-Carboxymethyl-L-cysteine	89

TABLE IV (*continued*)

Structure type	*Compound*	*Av. % control uptake rate*
Basic amino acids	L-2,4-Diaminobutyric acid	80
	L-Lysine	92
Aromatic amino acids	L-Tryptophan	72
Amino acid amides	L-Serine amide	91
	L-Valine amide	100
Miscellaneous amino acids	L-2-Amino-3-ureidopropionic acid	47
	γ-Aminobutyric acid	104

TABLE V

RELATIVE EFFECTS OF STRUCTURAL ANALOGS AS INHIBITORS OF L-ASPARAGINE TRANSPORT RATE IN *STREPTOCOCCUS FAECALIS*

Uptake rates were measured by Millipore filtration as described under Methods using L-[^{14}C]-asparagine at 0.5 mM. Competitive activities were calculated as described in Table III. The following additional compounds were tested. The value given after each substance is the average of the % control uptake rates observed with this compound at 1 and 5 mM. L-Glutamic acid, 81; DL-1-aminoethylphosphonic acid, 83; L-ornithine, 84; D-threonine, 84; D-asparagine, 85; α-methyl-DL-glutamine, 85; L-methionine sulfoxide, 86; L-2,4-diaminobutyric acid, 86; L-serine amide, 89; L-aspartic acid, 90; DL-aspartic acid β-hydroxamate, 92; L-glutamic acid γ-hydrazide, 92; L-lysine, 93; L-methionine sulfone, 93; α-amino-*n*-propylphosphonic acid, 94; 1-amino-1,3-propylene diphosphonic acid, 94; hydroxyproline, 95; DL-β-aminobutyric acid, 95; γ-aminobutyric acid, 96; taurine, 96; DL-aspartic acid β-hydrazide, 100; α-methyl-DL-aspartic acid, 101; α-amino-γ-phosphonobutyric acid, 100; β-alanine, 100; α-methyl-DL-glutamic acid, 101; L-cysteic acid, 105; L-valine amide, 105; α-methyl-DL-asparagine, 107; D-glutamine, 108; DL-α-amino-β-phosphonopropionic acid, 111; L-proline, 119.

Rank No.	*Compound*	*% control uptake rate*		
		1 mM	*5 mM*	*Av. of 1+5 mM*
1	L-α-Aminobutyric acid	30	23	27
2	L-Alanine	40	19	30
3	L-Serine	52	38	45
3	L-Cysteine	63	26	45
5	L-Methionine	61	32	47
6	*S*-Methyl-L-cysteine	57	47	52
7	Glycine	62	43	53
7	L-Tryptophan	61	44	53
9	L-Ethionine	70	38	54
10	L-Threonine	63	48	56
11	DL-*C*-Allylglycine	65	51	58
12	L-Asparagine	81	38	60
13	D-Tryptophan	75	52	64
13	L-Valine	73	55	64
15	L-Histidine	81	48	65
16	L-Glutamine	82	50	66
17	D-Methionine	71	62	67
18	D-Alanine	78	57	68
19	Aminomethylphosphonic acid	75	67	71
19	L-Arginine	75	67	71
21	α-Aminoisobutyric acid	81	62	72
22	2-Amino-3-ureidopropionic acid	85	60	73

were much less active than the L-isomers. Of considerable interest in comparison to the behavior of *S. faecalis* was the virtual inactivity of the small neutral amino acids. In this respect, the systems in *L. plantarum* and *S. faecalis* are markedly different. With freshly prepared solutions of L-[^{14}C]asparagine treated by ion-exchange chromatography to remove trace amounts of aspartic acid contamination, the dicarboxylic amino acids were found to have little or no competitive activity. The acidic character of the terminal carboxyl group apparently prevents interaction with the transport catalyst. However, modification of the charge as in L-aspartic acid β-methyl ester does not produce an effective competitor.

Structure specificity of asparagine transport in S. faecalis

The relative activities of various amino acids as competitors of the initial rate of L-[^{14}C]asparagine uptake in *S. faecalis* are shown in Table V. In contrast to the findings with *L. plantarum* summarized above, the small neutral amino acids were by far the most active competitors in *S. faecalis*. Asparagine and glutamine were much less effective. Substances such as L-tryptophan, L-valine, L-histidine and even the D-isomers of tryptophan, alanine and methionine were essentially as effective competitors as L-asparagine. It would seem, therefore, that the predominant system operating in this concentration range (0.5 mM) is a small neutral amino acid-preferring system comparable in some respects to the alanine-preferring, A or ASC systems studied by Christensen and his co-workers[4,12] in Ehrlich ascites tumor cells. The relative competitive activities described above were not significantly altered when

TABLE VI

COMPARATIVE EFFECT OF AMINO ACIDS AS COMPETITORS OF ASPARAGINE METABOLISM AND UPTAKE IN *S. FAECALIS*

Competitor	*% Reduction of asparagine*	
	*Metabolism**	*Uptake***
L-Asparagine	78	40
L-Glutamine	65	36
L-Tryptophan	43	47
L-Serine	32	55
L-Alanine	15	70
Glycine	15	47
L-Methionine	11	53
L-α-Aminobutyric acid	2	73
L-Histidine	0	35

* Metabolism refers to the conversion of L-[^{14}C]asparagine to [^{14}C]aspartic acid by broken cell preparations as described under Methods and Materials. L-[^{14}C]Asparagine was used at 2 mM and competitors were used at 10 mM. The values were obtained by the following calculation: 100 − (amount aspartic acid formed in the presence of competition/amount formed in the control × 100).

** Uptake was measured as described in Table V. The values shown were obtained by subtracting from 100 the average % of control uptake rate values shown in Table V for the respective amino acids.

L-[^{14}C]asparagine uptake was studied using 0.1 mM or 2.0 mM solutions. The kinetic experiments used to distinguish the number of operative transport catalysts are incapable of revealing the presence of multiple catalytic systems which have closely similar kinetic constants. Therefore, the operation of an additional system which is relatively specific for asparagine (and glutamine) comparable to that detected in *L. plantarum* is not excluded by these observations.

Despite the fact that these were short-term initial rate studies, since there is extensive deamidation of asparagine to aspartic acid, the possibility must be considered that the relative effectiveness of the substances listed in Table V might reflect a combined action on asparagine uptake and metabolism. This possibility is greatly diminished by the finding (Table VI) that the formation of aspartic acid from asparagine in broken cell preparations of *S. faecalis* was virtually insensitive to inhibition by small neutral amino acids like L-alanine and L-α-aminobutyric acid. In contrast, as expected, unlabeled L-[^{12}C]asparagine markedly reduced the conversion of L-[^{14}C]asparagine to aspartic acid.

DISCUSSION

It appears likely that several types of transport systems have been evolved which promote L-asparagine uptake. In *E. coli* a very specific system has been encountered which appears even to exclude L-glutamine[5]. In *L. plantarum* there is a relatively specific dicarboxylic amino acid amide system with which asparagine, glutamine and various structurally related compounds preferentially interact. Finally in *S. faecalis* and Ehrlich ascites tumor cells[1,4] (Holden, J. T. and Utech, N. M., unpublished) asparagine appears to be taken up primarily by a system for which small to moderate sized neutral amino acids have a relatively high affinity. It is not possible, however, solely on the basis of kinetic evidence to exclude the operation in *S. faecalis* of an additional catalyst with substrate specificity characteristics comparable to those observed in *L. plantarum*. Such a system might be masked if its kinetic constants were not significantly different from those of the general neutral amino acid system. We hope to resolve this question using mutants with defective transport systems for neutral amino acids. Preliminary experiments with a group of cycloserine-resistant strains of *S. faecalis* indicate that both alanine and asparagine transport are comparably reduced (Holden, J. T., Utech, N. M. and Bunch, J. M., unpublished). These observations are not compatible with the occurrence of a separate dicarboxylic amino acid amide-specific transport system.

Our conclusion concerning the number of catalytic systems promoting asparagine uptake in *S. faecalis* and *L. plantarum* is subject to a small reservation arising from limitations of the methods available to analyze kinetic data. All of our experiments demonstrated the existence of more than a single mode of entry, and most of them clearly indicated the operation of two catalytic systems in both organisms. However, as was suggested in a discussion of earlier experiments of this type[9], one could foresee that the curve-fitting procedure used to derive kinetic constants might yield several sets of constants that would account for the experimentally observed rates. In fact, two experiments yielded data that could be accounted for equally well by alternative sets of constants, one assuming a single catalyst (in addition to a diffusion component) and the other assuming two independent catalysts (Fig. 8). The

decision in favor of two catalytic systems was dictated by the great preponderance of experiments in which assumption of a single catalytic system could not predict the observed rates. This finding serves as a reminder of the potential limitation of kinetic evidence in establishing the operation of multiple transport catalysts, and emphasizes the importance of supporting such analyses with other types of observations such as studies with mutants lacking one of the suspected transport entities. It should also be noted that the apparent demonstration of two kinetically distinct asparagine transport components does not necessarily imply the occurrence of two independent, separately coded catalysts. As indicated previously[9,10], these findings could be accounted for by a single allosterically modified catalyst, as proposed for glutamate transport in *E. coli* by Halpern and Even-Shoshan[13].

Despite the apparent similarity of the kinetic properties of the asparagine transport systems in *L. plantarum* and *S. faecalis*, the substrate specificity properties of these systems differ markedly. This suggests that these catalysts differ significantly, and that the close correspondence of the kinetic constants may be a coincidental property.

Although the rapid metabolism of asparagine in these bacteria complicated studies with competitive substrates, control experiments with *S. faecalis*, indicated that the unusually high effectiveness of some substances as competitors (*e.g.* alanine, α-aminobutyric acid) was not based on an inhibition of metabolism. Nevertheless, caution must be exercised in interpreting the effects of substances like L- (or D-)-tryptophan, which on structural grounds would not appear likely to interact especially effectively with a receptor molecule that reacts preferentially with small neutral amino acids. The presence of small amounts of high specific activity contaminants in the isotopically labeled substrates used in competition studies could produce misleading observations. Many commercially available, reputedly high-purity grade amino acids contain microbiologically detectable amounts of contaminating amino acids[14]. For example, some samples of asparagine were found to contain histidine, lysine and tryptophan, and as many as 6 other amino acids have been detected in aspartic acid samples. (The two commercial sources from which we have purchased L-[^{14}C]-asparagine prepare this substance from L-[^{14}C]aspartic acid). The presence of such trace contaminants must be considered possible if not likely in isotopically-labeled amino acids isolated from natural sources. Such isotopically-labeled contaminants would not be diluted by non-radioactive carrier as is usually the case with the amino acid under investigation. The uptake even of small amounts of such high specific activity contaminants can contribute surprisingly large amounts of radioactivity to the intracellular pool. When the non-labeled form of such a contaminating amino acid is tested as a potential competitor of the intended substrate it will significantly reduce the uptake of isotope by diluting the radioactive contaminant and appear, therefore, to be acting as a competitor of the substrate amino acid. Any effort to establish relatively weak apparent competitive interactions of one amino acid with a carrier predominantly specific for another amino acid must take into account the exceptional sensitivity of the experimental model to errors of this type.

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