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α-METHYL-L-GLUTAMIC ACID UPTAKE BY HIGH AFFINITY DICARBOXYLIC AMINO ACID TRANSPORT SYSTEM IN *STREPTOCOCCUS FAECALIS*

JOSEPH T. HOLDEN, NEDRA M. UTECH and K. GARTH REID

Division of Neurosciences, City of Hope National Medical Center, Duarte, Calif. 91010 (U.S.A.)

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SUMMARY

The transport of α-methyl-L-glutamic acid was studied in *Streptococcus faecalis*. Energy-dependent uptake against substantial concentration gradients was observed. Kinetic experiments indicated that, in contrast to L-glutamic acid, only a single catalytic component (high affinity) and a diffusion controlled process participated in α-methyl-L-glutamic acid uptake. At concentrations up to 10 mM, α-methylglutamate transport was almost completely abolished in a mutant strain lacking a high affinity dicarboxylic amino acid transport system. In competition experiments, α-methylglutamic acid antagonized glutamate uptake via the high affinity system, and only slightly via the low affinity system. Column chromatography of cell extracts showed that very little (approx. 5 %) of the accumulated amino acid was converted to metabolites during short term incubations. These studies indicate that, at concentrations up to 3–5 mM, α-methyl-L-glutamic acid can be used as a specific, relatively metabolically inert substrate of the high affinity dicarboxylic amino acid transport system in *S. faecalis*.

INTRODUCTION

We have previously reported that *Streptococcus faecalis* contains two kinetically distinguishable catalytic systems for transporting dicarboxylic amino acids [1, 2]. During those studies, the possibility was considered that one of these transport components might be a metabolic system which contributed to the uptake of isotopically labeled substrate by converting it to glutamate metabolites. Comparative metabolic and transport studies using the parental strain and a transport defective mutant produced no evidence favoring this postulate. Although less than 20% of the accumulated amino acid was converted to metabolites in such experiments, we sought to improve their reliability by using a substrate analog having a greater resistance to metabolic alteration. In preliminary studies several substances were found to be reasonably effective inhibitors of glutamic acid uptake, indicating an ability to interact with the relevant transport catalysts. One of these, α-methyl-DL-glutamic acid was

shown to be relatively resistant to metabolic alteration including decarboxylation and transamination. Accordingly, the racemate was resolved and the L-isomer labeled with tritium to facilitate its use in transport studies. As will be shown below, α-methylglutamic acid reacts significantly with only one of the two dicarboxylic amino acid transport systems in *S. faecalis*, the so-called high affinity component. A preliminary account of some of these experiments has been presented [3].

METHODS AND MATERIALS

Most of these experiments were performed at the same time as previously described studies on the kinetics of glutamic acid transport in *S. faecalis* R (ATCC 8043), referred to below as strain ATCC, and in a dicarboxylic amino acid transport mutant of this organism designated R-4 [1, 2]. These reports should be consulted for detailed descriptions of all procedures including growth media, culture conditions, uptake experiment protocols and procedures for calculating kinetic constants.

The uptake studies utilized a rapid multisample millipore filtration procedure to terminate incubations. Radioactivity associated with filtered cells was determined by liquid scintillation spectrometry. Kinetic constants were derived from initial uptake rate data using a curve fitting procedure [1]. Successive approximations of the constants were made until the experimentally observed and calculated velocities agreed closely assuming the operation either of one or two catalytic systems with or without a diffusion component.

Preparation of α-methyl-L-glutamine

The procedure of Kagan et al. [4] based on a stereospecific amidation reaction was used to prepare α-methyl-L-glutamine from commercially available α-methyl-DL-glutamic acid. Glutamine synthetase from sheep brain was prepared according to Pamiljans et al. [5]. The purification was carried through Step 6. The precipitated enzyme was dissolved in 0.005 M 2-mercaptoethanol as described by these authors and used without dialysis or further purification. At this stage, the preparation had a specific activity of 9.5 units/mg protein as determined by the hydroxamate assay. Several large scale incubations of α-methyl-DL-glutamic acid and the glutamine synthetase preparation were carried out as described by Kagan et al. [4]. In a typical preparation 14 mmol α-methyl-DL-glutamic acid, 1 mmol ATP, 2 mmol $MgCl_2$, 7.7 mmol phosphoenolpyruvate, 10 mmol NH_4Cl, 7 mg (917 units) pyruvate kinase, 0.15 mmol 2-mercaptoethanol and 6.2 ml (1080 units) of glutamate synthetase in a final volume of 31 ml were incubated at 37 °C for 8–10 h. The reaction mixture was processed as described by Kagan et al. [4] to yield a purified preparation of α-methyl-L-glutamine. A portion of this material was hydrolyzed with acid to yield α-methyl-L-glutamic acid.

Preparation of α-[^3H]methyl-L-glutamic acid

500 mg of α-methyl-L-glutamine were tritiated by the Amersham-Searle Corp. (Arlington Heights, Ill.). A water solution of the treated material was applied to the top of a column of Dowex 50W (200–400 mesh, 8X, 2.8 × 5 cm, H^+ form). The column was washed with 140 ml H_2O and eluted with 120 ml 2 M NH_4OH. Approximately 85 % of the radioactivity applied to the column appeared in the water fraction,

suggesting that the amide had cyclized to the pyrollidone form during the titration procedure. Therefore, the material in the water fraction was purified. Easily exchanged counts were removed by repeated concentration in a flash evaporator. The residual material was dissolved in 3 M HCl and hydrolyzed at 120 °C for 30 min. HCl was removed by repeated concentration in a flash evaporator. The remaining material was dissolved in water and applied to a Dowex 50 column as described above. Approximately 60 % of the radioactivity applied to the column appeared in the NH_4OH fraction. Two-dimensional chromatography on paper (phenol/ammonia/H_2O) followed by butanol/acetic acid/H_2O (4 : 1 : 2) followed by radioautography revealed that most of the radioactivity migrated with carrier α-methyl-L-glutamic acid. A labeled contaminant which migrated faster than α-methylglutamic acid in both solvents was removed by chromatographing the NH_4OH eluate one dimensionally on Whatman No. 1 paper (46 × 57 cm) which had previously been washed for several days each with 2 M acetic acid and 95 % ethanol. Butanol/acetic acid/H_2O was the solvent. The band of α-methylglutamic acid was located by ninhydrin treatment of both edges of the chromatogram, and the material eluted with water. The material was repurified on Dowex 50 as previously described. The fraction eluted with 2 M NH_4OH contained essentially all the radioactivity which had been applied to the column. The material was freed of ammonia by flash evaporation and redissolved in water. Two-dimensional chromatography established the presence of single coincident ninhydrin reactive and radioactive spots whose migration corresponded to that of carrier α-methyl-L-glutamic acid. The final product had an estimated specific activity of 4.2 Ci/mol. When chromatographed on an amino acid analyzer as described below, only a single major peak was observed containing 99 % of the recovered radioactivity in the position expected for α-methylglutamic acid. The labeled compound generally was diluted to 0.1–1.0 Ci/mol with nonradioactive α-methyl-L-glutamic acid for use in uptake experiments.

Chemicals

All other chemicals used in this study were the highest grade commercially available. L-[U-^{14}C]Glutamic acid was obtained from Schwarz BioResearch and Amersham Searle and was purified before use by passage through columns of Dowex 50 and Dowex 1 as described previously [1].

Separation of labeled intracellular pool components

The method used to separate and quantitatively estimate metabolites of α-methyl-L-glutamic acid formed during brief uptake incubations is closely similar to that used in previously described experiments with L-glutamic acid [1]. Following incubation with α-[^{3}H]methyl-L-glutamic acid for 2 min, the cells were collected by centrifugation and cell extracts were prepared using 5 % trichloroacetic acid. An aliquot of the extract equivalent to 3.2 mg of cells was chromatographed in a Technicon amino acid autoanalyzer using norleucine as an internal standard. The columns were run at 25 °C to prevent deamidation of glutamine and any other labile amides. As described previously, two columns were run in parallel. A control sample derived from cells which had been incubated with nonradioactive α-methyl-L-glutamic acid was passed through one column in the normal manner to obtain a trace describing the separated ninhydrin reactive pool components. The comparable labeled sample

was passed through the other column and the eluate passed directly to tubes on a fraction collector without reaction with ninhydrin. Aliquots of all these fractions were assayed for isotope by scintillation spectrometry. These experiments were preceded by control studies which established the elution volume and purity of the α-[^{3}H]-methyl-L-glutamic acid.

RESULTS

Time-course of uptake and glucose dependence

The time-course of α-methyl-L-glutamate uptake in the parental strain of *S. faecalis* (ATCC) is shown in Fig. 1. Uptake was dependent on an external energy source, in this case glucose. At the relatively high external concentration used in this experiment (3 mM), a calculated concentration gradient (cells/buffer) of only 15 was attained. However, using lower external concentrations (0.03–0.1 mM), concentration gradients on the order of 200–300-fold were observed. Mutant strain R-4, which lacks the high affinity dicarboxylic amino acid transport system, was incapable of taking up α-methylglutamate appreciably at concentrations up to 3 mM (less than 4 % of the uptake of the parental strain). Although this strain transports L-glutamic and L-aspartic acids relatively poorly at concentrations below 0.1 mM, at concentrations above 1.0 mM the undamaged low affinity transport component enables it to

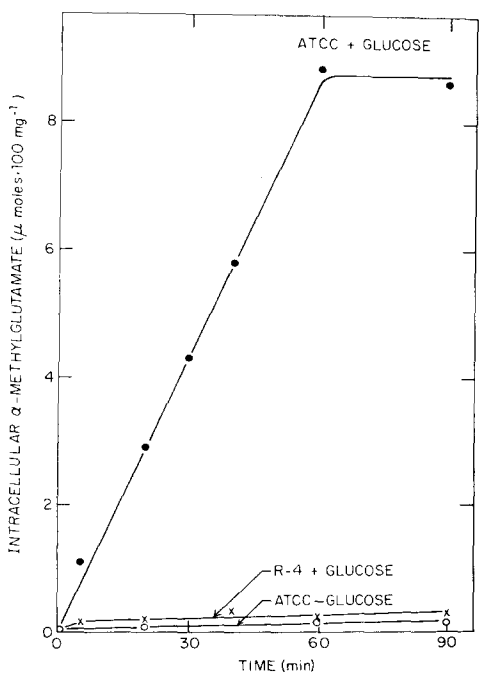

Fig. 1. Time-course and glucose dependence of α-methyl-L-glutamic acid uptake in *S. faecalis* ATCC and the dicarboxylic amino acid transport mutant, R-4. Cells were incubated with 3.0 mM α-[^{3}H]-methyl-L-glutamic acid as follows: ●, parental strain ATCC with glucose; ○, ATCC without glucose; ×, mutant R-4 with glucose.

accumulate glutamate at 50–70 % of the parental strain rate. (In the experiment shown, control suspensions accumulated the following amounts of glutamate from a 3 mM solution in 60 min: strain ATCC, 23.3 μmol · 100 mg^{-1}; strain R-4, 17.2 μmol · 100 mg^{-1}.) These findings suggested that α-methylglutamic acid reacted poorly with the low affinity dicarboxylic amino acid transport system, and that uptake of this glutamate analog by the parental strain was probably due predominantly to the activity of the high affinity system.

Kinetics of α-methylglutamate transport

Initial rate studies (Fig. 2) also showed that the transport mutant strain R-4 was markedly handicapped in transporting α-methylglutamate over a broad concentration range. The failure of the mutant to substantially increase its uptake relative to the parental strain at higher substrate concentrations (> 1.0 mM) (in contrast to its behavior with glutamic acid) suggested again that the low affinity dicarboxylic amino acid system, which is fully active in the mutant strain, may be incapable of transporting α-methylglutamate at a measurable rate. The increased amounts of α-methylglutamate associated with R-4 cells at 5 and 10 mM (Fig. 2) were not larger than the amount that might have been expected to enter the cells by diffusion operating at a rate observed in subsequent experiments (theoretical points marked X). Unfortunately, the rate of α-methylglutamate uptake by the R-4 strain, which is

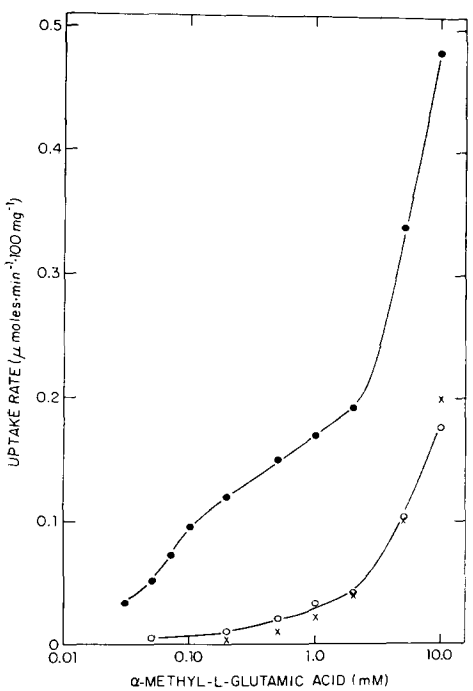

Fig. 2. Effect of α-methyl-L-glutamic acid concentration on the initial rate of uptake by the *S. faecalis* parental (ATCC) and transport mutant (R-4) strains. ●, ATCC; ○, R-4; × shows the uptake rates predicted by a diffusion component having a rate constant k_D = 0.02 ml · min^{-1} · 100 mg^{-1}.

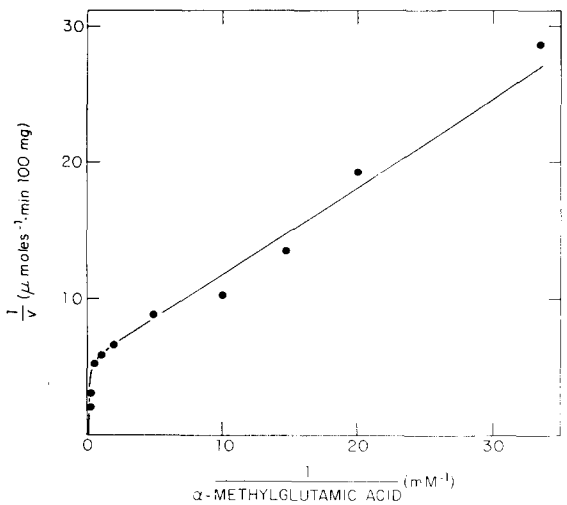

Fig. 3. Reciprocal plots of initial uptake rates and extracellular α-methyl-L-glutamic acid concentrations. The points represent experimental values. The line was drawn assuming a single catalytic component (K_m, 0.10 mM; V, 0.16 μmol · min^{-1} · 100 mg^{-1}) a diffusion component (k_D, 0.03 ml · min^{-1} · 100 mg^{-1}) and deriving values for v_t by substituting in the equation $v_t = VS/(K+S) + k_DS$.

very low even in the presence of glucose, became extremely erratic in the absence of an energy source, preventing a more definitive examination of this question. Additional support for the conclusion that the low affinity dicarboxylic amino acid system was incapable of transporting α-methylglutamic acid, at least in the concentration range studied, was obtained by further analysis of such kinetic experiments.

As shown in Fig. 3, a nonlinear reciprocal rate curve was obtained using the parental strain. Employing a previously described curve fitting procedure to obtain kinetic constants, the experimental data were most satisfactorily accounted for by assuming the operation of a single relatively high affinity catalytic component and a non-saturable (diffusion-controlled) process. No evidence was obtained in such studies for the operation of a second catalytic component of lower affinity, as was the case when glutamic or aspartic acids were used as substrates. It was extremely difficult to obtain reliable kinetic data with strain R-4 because the rates of α-methylglutamate uptake were very low and the amounts taken up in one or two min were only slightly higher than a variable adsorption of this compound to the millipore filters. However, in the most reliable experiments, the R-4 strain also yielded curvilinear Lineweaver-Burk plots which could again be accounted for by a non-saturable and a single catalytic system. The V of the catalytic system, however, was only 5 % of the one observed in the parental strain. These data suggest that the high affinity system in the R-4 strain may not be completely inactivated by the genetic modification. Its activity, however, is so low that its presence is masked by the low affinity component when superior substrates such as glutamate or aspartate are used to measure uptake.

The possibility exists, of course, that transport of α-methylglutamate on the low affinity system is possible at much higher concentrations. Unfortunately, rates at concentrations above 10 mM could not be reliably determined using strain R-4 due to the extreme variability of the data described above.

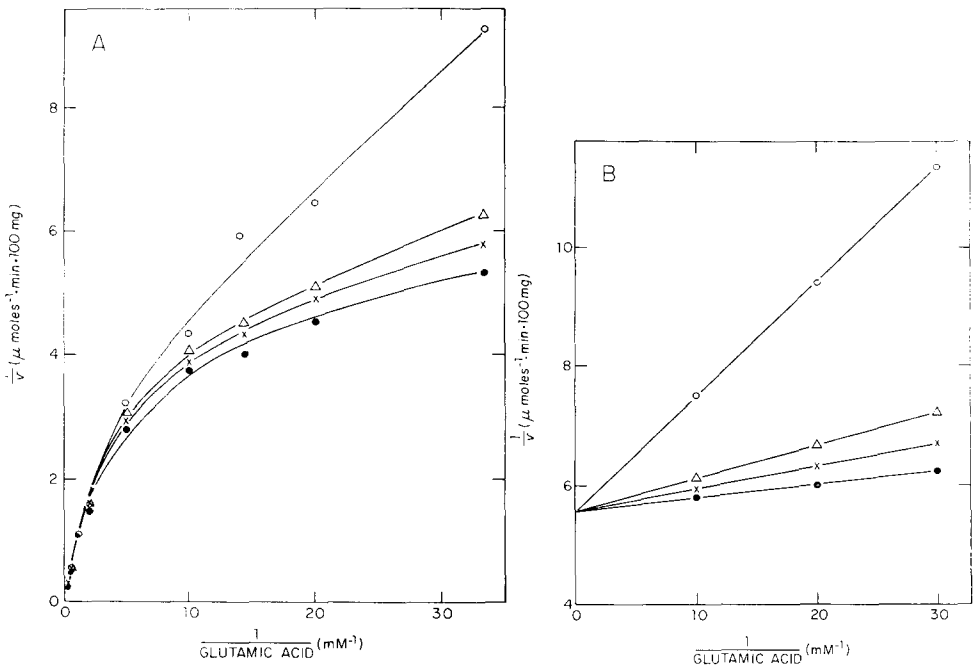

Fig. 4. Effect of α-methyl-L-glutamic acid on L-glutamic acid uptake rate. (A) Reciprocal rate plot of L-[^{14}C]glutamic acid transport measured in the absence (●) and in the presence of the following concentrations of α-methyl-L-glutamic acid; 0.05 mM (×); 0.2 mM (△); 1.0 mM (○). The curve fitting procedure yielded the following kinetic constants for the high and low affinity systems. In all cases V of the high affinity system was 0.18 μmol · min^{-1} · 100 mg^{-1}. The K_1 values were: control, 0.0042 mM; with 0.05 mM α-methylglutamate, 0.0070 mM; with 0.20 mM α-methylglutamate, 0.010 mM; with 1.0 mM α-methylglutamate, 0.035 mM. For the low affinity system, in all cases V was 10 μmol · min^{-1} · 100 mg^{-1}. The K_2 values were: control, 10 mM; with 0.05 mM α-methylglutamate, 11 mM; with 0.20 mM α-methylglutamate, 12 mM; with 1.0 mM α-methylglutamate, 12.5 mM (B) The kinetic constants derived above were used to calculate the theoretical rates of glutamic acid uptake via the high-affinity transport system in the absence and presence of the indicated concentrations of α-methyl-L-glutamic acid using the equation $1/v = (K_1/V_1 \cdot 1/S)+1/V_1$. Symbols are as above.

Further evidence that α-methyl-L-glutamic acid was taken up via the high affinity dicarboxylic amino acid system was provided by competition studies in which the initial rate of L-[^{14}C]glutamic acid uptake at various concentrations was measured in the absence and presence of several concentrations of α-methyl-L-glutamic acid. Lineweaver-Burk plots of the resultant rate observations are shown in Fig. 4A. Using the previously described curve fitting procedure, kinetic constants for the high and low affinity systems were derived which showed clearly that α-methylglutamate increased the apparent K_m value of the high affinity system for glutamate. At the concentrations used, there was no significant effect on the kinetic constants of the low affinity system. Using these derived constants, the initial velocities of L-glutamic acid uptake via the high affinity system could be calculated showing, as illustrated in Fig. 4B, that α-methylglutamate and glutamate interacted competitively in this system. The calculated K_i value of 0.12 mM for α-methyl-L-glutamate agreed reasonably well with its K_m value (0.10 mM) for transport by this system.

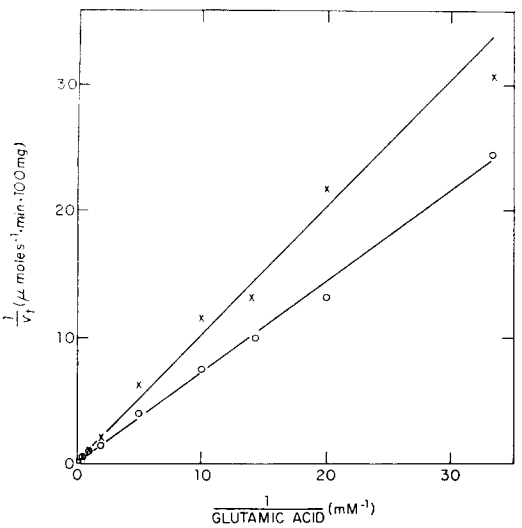

Fig. 5. Effect of α-methyl-L-glutamic acid on the initial rate of L-[^{14}C]glutamic acid transport in mutant strain R-4. Glutamate uptake was measured in the absence (○) and presence (×) of 10 mM α-methyl-L-glutamic acid. The following kinetic constants were calculated: control K_m = 22 mM, V = 30 μmol · min^{-1} · 100 mg^{-1}; with 10 mM α-methylglutamate, K_m = 30 mM, V = 30 μmol · min^{-1} · 100 mg^{-1}.

Comparable experiments were also carried out in the mutant strain R-4 to determine with greater certainty whether or not α-methylglutamic acid had any effect on the operation of the low affinity dicarboxylic amino acid system. As shown in Fig. 5, there was a small but significant competitive effect, suggesting that while α-methylglutamic acid probably is not translocated across the membrane by this catalyst, it can interfere in its reaction with L-glutamic acid.

TABLE I

METABOLISM OF α-[^{3}H]METHYL L-GLUTAMIC ACID BY *S. FAECALIS*

Cells were incubated with the indicated concentrations of α-[^{3}H]methyl L-glutamic acid for 2 min as described under Methods and Materials. With 0.1 mM α-methyl-L-glutamate 99.0 % and with 5 mM, 98.8 % of the isotope applied to the column was recovered in discrete peaks. The figures shown represent the percentage of recovered isotope present in the indicated fractions.

Pool component	Fraction number	% of pool radioactivity	
		α-[^{3}H]Methyl-L-glutamate	
		0.1 mM	5.0 mM
α-Methylglutamic acid	41–45	94.2	96.5
α-Methylglutamine	27–29	0	0
Unidentified			
Acidic	3–5	0	0
No. 1	7–9	3.0	2.0
No. 2	47–48	2.6	1.3

Metabolism of α-[^3H]methyl-L-glutamic acid

Table I summarizes the distribution of isotope in column fractions of cell extracts following a short incubation (2 min) with α-[^{3}H]methyl-L-glutamic acid. This is the maximum incubation time used in kinetic studies. Approximately 5 % of the isotope was found in fractions other than α-methylglutamic acid. The identity of these substances is unknown. α-Methylglutamine was not formed in measurable amounts. In contrast to glutamic acid, no acidic metabolites were formed from α-methylglutamate.

DISCUSSION

These studies have shown that α-methyl-L-glutamic acid can serve as a useful analog for studying dicarboxylic amino acid transport. As expected, this substance was metabolized much less extensively than L-glutamic acid by *S. faecalis*. The lack of acidic metabolites is consistent with the expected resistance of this compound to transamination and deamination. Another potentially useful property of this substance is that in the concentration range used to study the dicarboxylic amino acid carriers in *S. faecalis*, α-methylglutamate was apparently transported exclusively by the high affinity transport system. Although it appeared to compete weakly against L-glutamic acid in the low-affinity system, there was no indication in kinetic studies that it was translocated by this catalyst. Therefore, in the concentration range up to 3–5 mM, α-methyl-L-glutamate could be used as a selective substrate for studying the kinetic properties of the high-affinity transport system.

Consequently, methods are now available to study individually both dicarboxylic amino acid transport systems in *S. faecalis*; i.e. the low affinity system can be studied in mutants such as R-4 which lack significant high affinity activity, and, as indicated above, the high-affinity system can be studied in the parental strain using α-methylglutamate as substrate. As reported previously [6], the aspartic acid analog phosphonoalanine (2-amino-3-phosphonopropionic acid) can also be used as a relatively specific substrate for the high-affinity system in this bacterium.

In concordance with our earlier findings, we have encountered no positive evidence indicating that either the low or the high affinity transport components in *S. faecalis* are metabolic systems which mimic a transport system by diverting some of the glutamate intracellulary to metabolic products (cf. ref. 1 for a detailed discussion of this question). Still, it is of some interest that the two poorly metabolized glutamic and aspartic analogs that we have studied (α-methylglutamate and phosphonoalanine, respectively), were not measurably transported by the low-affinity system. While this may be a coincidental finding, the available data do not exclude the possibility that transport via this system involves a cyclic metabolic process in which the substrate is derivatized during transport and regenerated on the other side of the membrane.

Another possible explanation for the failure of the low-affinity system to transport α-methylglutamate is that this system is derived from the high affinity catalyst by a substrate-induced allosteric modification, and while α-methylglutamate can react with the translocation site of the high affinity form, it is incapable of reacting at the modifier site to produce the low affinity configuration. Halpern and his coworkers have suggested that the *Escherichia coli* glutamate transport system contains

an allosterically reactive component [7]. We have attempted unsuccessfully to detect α-methylglutamate transport via the low affinity system using various concentrations of L-glutamate to effect such a hypothetical allosteric transformation.

Several other investigations have indicated that α-methylglutamate interacts with dicarboxylic amino acid specific membrane components. For example, Halpern and Even-Shoshan [7] observed a competitive effect of this substance in studies on the glutamate transport system of *E. coli*. Haldeman et al. have observed an inhibition by α-methylglutamate of glutamate stimulation of thalamic neurones [8].

Various investigators have described the interaction of several dicarboxylic amino acid specific enzymes with α-methylglutamate [4, 9–13]. Although some of these workers [12, 13] have reported that α-methylglutamate can undergo a decarboxylation dependent transamination reaction, the relatively high pH maintained in our experiments precluded extensive activity of the glutamic acid decarboxylase.

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