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PHYSIOLOGICAL BASIS FOR PREFERENTIAL UPTAKE OF D-α-AMINOADIPATE OVER THE L-ISOMER BY *ALCALIGENES DENITRIFICANS*

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SUMMARY

Alcaligenes denitrificans, pre-incubated with D-α-aminoadipate and assayed for L-isomer uptake without removal of extracellular D-isomer, exhibits a reduced rate of uptake and a reduced level at which steady state is achieved. During D- or L-isomer uptake, intracellular α-aminoadipate is exclusively the L-configuration. These data are consistent with an intracellular, mediated reduction in L-isomer uptake as the physiological basis for preferential D-α-aminoadipate uptake by *A. denitrificans* growing on racemic α-aminoadipate. Translocated D-α-aminoadipate is rapidly metabolized to form an L-isomer pool which subsequently reduces the rate of L-isomer uptake and the level at which steady state occurs resulting in a preferred D-isomer uptake. Competitive inhibition of L-α-aminoadipate uptake by the D-isomer or a difference in the maximum rates of uptake of each isomer does not contribute to preferential uptake. D-α-aminoadipate uptake is an inducible process expressed only in the presence of that compound and while uptake of L-α-aminoadipate is also inducible there is a low rate of constitutive uptake. While L-α-aminoadipate uptake occurs against a concentration gradient, uptake of the D-isomer is not against a gradient. D- and L-isomer uptake are active processes since both are inhibited by azide, cyanide and 2,4-dinitrophenol.

INTRODUCTION

Alcaligenes denitrificans (ATCC 15173) growing on DL-α-aminoadipate exhibits a diauxic growth in which the two phases are often separated by a diauxic lag [1]. The first growth phase represents a preferential uptake of the D-isomer while the second phase represents an increased uptake of α-aminoadipate after depletion of the D-isomer. Cells at either phase of growth oxidize D- or L-α-aminoadipate at

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immediate linear rates negating the possibility that the D-isomer is genetically repressing induction of either an L-α-aminoadipate transport system or synthesis of enzymes for its catabolism. One explanation for preferential D-isomer uptake is competitive inhibition of L-α-aminoadipate uptake by the D-isomer at the extracellular side of the membrane. Since inhibition of L-isomer uptake at the intracellular side of the membrane by translocated D-α-aminoadipate or its catabolites is an equally valid explanation, a study of transport by preventing subsequent metabolism via a metabolic inhibitor or metabolic mutant seemed undesirable. Accordingly, we studied the translocation of D- and L-α-aminoadipate in the presence of subsequent metabolism, which in the terminology of Ghei and Kay [2] is uptake rather than transport, to determine the physiological basis for preferential uptake by *A. denitrificans* of D-α-aminoadipate over the L-isomer.

MATERIALS AND METHODS

Chemicals

DL-α-Amino[6-^{14}C]adipate was obtained from Schwarz/Mann or synthesized by the method of Rothstein and Claus [3]. Isolation of D- and L-α-amino[6-^{14}C]-adipate which were free of radioactive impurities and at least 99.7 % pure with respect to the other enantiomer was by the method of Pekala and Hartline [4]. DL-α-Amino-adipate was synthesized and resolved according to Wood and Hartline [5]. All other chemicals were of reagent or analytical grade and were obtained from commercial sources. Chromatography of α-aminoadipate was with Whatman No. 4 filter paper in butanol/acetic acid/water (12 : 3 : 5, by vol.). Radioactivity on chromatograms was detected by autoradiography with Kodak nonscreen X-ray film or by direct scanning on a Vanguard 880 autoscanner.

Cell growth

Growth of *A. denitrificans* (ATCC 15173) was at 30 °C with shaking at 300 rev./min in mineral salt medium [6] with DL-α-aminoadipate at 12 mM as the sole source of carbon and nitrogen. Cultures were one-fifth the growth flask volume. Growth was followed turbidimetrically on a Bausch and Lomb Spectronic 20 at 660 nm.

Measurement of uptake

Cells were harvested at late log phase (20 h) by centrifugation for 10 min at 10 000 × *g* at 5 °C, washed with 10 ml cold mineral salt medium, centrifuged and suspended in mineral salt medium to 75 or 5.4 μg (dry wt) per ml for long term and short term assays, respectively. These suspensions, maintained in the cold, retained uptake ability for 4 h.

For long term assays (to 1 min), 1 ml of cell suspension in a 10-ml flask containing chloramphenicol at 120 μg/ml was incubated for 10 min at room temperature. Uptake was initiated by addition of D- or L-α-amino[6-^{14}C]adipate (spec. act. 30, 47 or 57 Ci/mol) to the flask containing 75 μg (dry wt) of cells in a final volume of 1.5 ml (radioactive α-aminoadipate at 2 μM). During uptake assays at room temperature, the cell suspensions were aerated by means of a magnetic stirrer. At intervals between 5 s and 1.0 min, aliquots of 0.2 ml were transferred to 0.22 μM type GSWP Millipore filters and vacuum filtered. The filters were immediately washed with 10 ml

mineral salt medium at room temperature and after 84 s placed in scintillation vials containing 5 ml of scintillation fluor [7].

For short term assays (to 8 s), 6 ml of cell suspension containing chloramphenicol at 120 μg/ml was incubated at room temperature for 10 min. 5 ml of this suspension containing 27 μg (dry wt) of cells were transferred to a 0.22 μM type GSWP Millipore filter and vacuum filtered. The top of the filter holder was removed and uptake initiated by placing a volume of D-α-amino[6-^{14}C]adipate solution at the desired concentration directly onto the filter containing the cells. After the radioactive solution passed through, the filter was washed with 10 ml mineral salt medium at room temperature and immediately placed in a scintillation vial containing 5 ml of scintillation fluor [7]. The time required for the radioactive solution to pass through the filter, which was proportional to the volume of solution, was considered the duration of uptake. Using volumes of 0.25–1.5 ml we were able to assay from 1.2 to 8 s for concentrations of 0.05–5 μM obtaining linear uptake rates that could be extrapolated through the origin. Appropriate subtractions were made for radioactivity not washed free of the filters when D-α-amino[6-^{14}C]adipate solutions were filtered in the absence of cells. However, residual radioactivity was insignificant except for the larger volumes (above 1 ml) of high concentrations (above 1 μM) which were about twice the background.

After 4 h, the time required for complete solubilization of filter contents, racioactivity was measured with a Packard Tri-Carb Model 3320 liquid scintillation spectrometer. Transport rates are expressed as nmol of α-aminoadipate transported/min per mg dry cell wt. All rates were calculated by least square plots.

Recovery of intracellular α-aminoadipate

To study intracellular accumulation of translocated α-aminoadipate, twenty 2-ml aliquots from 10-s, 30-s, and 1-min uptake incubations were filtered as in a long term uptake assay, except that the filters were 0.5 μM type EHWP Millipore filters. The filters were washed with three 10-ml portions of mineral salt medium at room temperature and immediately placed in 100 ml of water at 95 °C for 1 h. The water was decanted and the filters boiled for 30 min in an additional 50 ml of water. Cell residue was removed from the pooled water fractions by filtration on a Millipore filter. The filtrate was evaporated in vacuo at 40 °C to 5 ml and applied to a 1 × 5 cm Dowex 1-X8 (acetate) ion-exchange column. Salts were removed by an initial 100 ml wash and the α-aminoadipate isolated during an acetic acid nonlinear gradient elution [8]. Radiopurity of isolated α-amino[^{14}C]adipate was determined on chromatograms by direct scanning. Intracellular water, determined with [^{3}H]inulin as described by Kotyk and Janacek [9], is 66 % of the wet wt (2.3 μl of cell water per mg of dry cell wt).

Determination of the configuration of intracellular α-aminoadipate

Configuration determination, based on the ability of *Pseudomonas putida* P2 (ATCC 25571) to utilize L-α-aminoadipate but not the D-isomer, was according to the method of Pekala and Hartline [4]. α-Aminoadipate that disappeared from the medium when an aliquot of recovered intracellular radioactive α-aminoadipate was incubated with *P. putida* P2 would be of the L-configuration; α-aminoadipate not taken up from the medium would be the D-isomer. Incubations with isolated α-aminoadipate were run simultaneously with a control consisting of *P. putida* P2

incubated with equivalent amounts of L-α-amino[6-^{14}C]adipate to ascertain the ability of the organism to utilize completely the potential amount of L-isomer present.

Pre-incubation of cells the in presence of the D- *or* L-*isomer*

Cell suspensions (1 mg dry wt/ml) were incubated for 5 min at 30 °C in medium containing D- or L-aminoadipate at 200 μM, diluted 30-fold by addition of ice-cold medium and centrifuged. The pellet was suspended in cold medium and centrifuged. The supernatant fluid was decanted and the pellet suspended in 1.3 ml of ice-cold medium. An identically treated cell suspension incubated without addition of α-aminoadipate served as a control. Assay for L-isomer uptake was initiated by addition of 0.1 ml of cell suspension to 1.4 ml of medium at room temperature containing L-α-amino[6-^{14}C]adipate at 2μM.

During the first phase of growth on DL-α-aminoadipate when cells exhibit limited L-isomer uptake D-α-aminoadipate is present extracellularly [1]. To approximate these conditions 1.4 ml cell suspensions (50 μg dry wt/ml) containing the desired amount of D-isomer were incubated for 5 min at 30 °C and, allowing any extracellular D-isomer to remain in the medium, immediately assayed for uptake by addition of L-α-amino[6-^{14}C]adipate.

Using cells incubated with radioactive D- or L-α-aminoadipate for 5 min at 30 °C, efflux of intracellular α-aminoadipate was determined according to Winkler and Wilson [10] except that efflux was assayed at room temperature.

RESULTS

Time-course of uptake

Uptake of L-α-aminoadipate is linear for 45 s before reaching steady state while uptake of the D-isomer is biphasic; a rapid uptake followed by a reduced rate of uptake (Fig. 1). It is conceivable that the first phase of D-isomer uptake is an initial

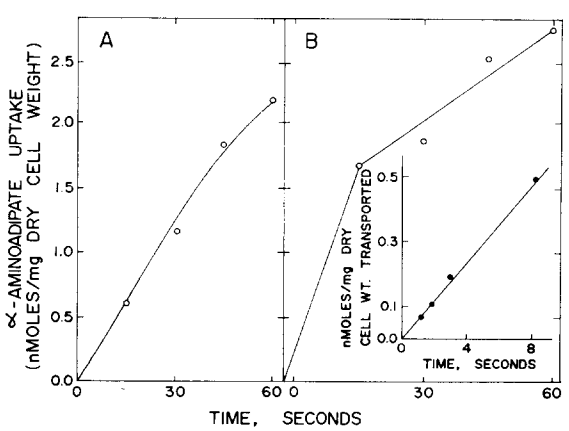

Fig. 1. Time-course for uptake of L- and D-α-aminoadipate (A and B, respectively); insert in 1B is short term assay for D-uptake.

rapid translocation process while the slower second phase results from intracellular metabolism. Assuming uptake during the initial seconds of an assay represents mostly translocation we examined uptake of D-α-aminoadipate for the first 8 s. Uptake of D-α-aminoadipate during the initial 8 s is linear (Fig. 1B insert) and concentration dependent (see Fig. 4).

Uptake of D- *or* L-α-*aminoadipate by cells grown on various carbon sources*

Cells grown on succinate, D- or L-glutamate or D- or L-α-aminoadipate take up L-α-aminoadipate while uptake of the D-isomer occurs only in cells grown on DL-α-aminoadipate or the D-isomer (Table I).

Effect of metabolic and sulfhydryl group inhibitors

Table II shows the affect of these inhibitors on uptake of D- and L-α-aminoadipate. Inhibition by iodoacetamide, *N*-ethylmaleimide and *p*-hydroxymercuri-

TABLE I

UPTAKE OF D- AND L-α-AMINOADIPATE BY *A. DENITRIFICANS* GROWN ON DIFFERENT COMPOUNDS AS THE SOLE SOURCE OF CARBON

Carbon source (12 mM)	Rate of uptake in mmol/min per mg dry cell wt	
	D-Isomer	L-Isomer
DL-α-Aminoadipate	3.6	2.40
D-α-Aminoadipate	11.02	8.96
L-α-Aminoadipate	0	4.52
D-Glutamate	0	6.38
L-Glutamate	0	11.42
Succinate + NH_4Cl (20 mM)	0	1.34

TABLE II

EFFECT OF METABOLIC AND SULFHYDRYL GROUP INHIBITORS ON UPTAKE OF D- AND L-α-AMINO [6-^{14}C]ADIPATE BY *A. DENITRIFICANS*

Transport was measured as usual except that the preliminary 10-min incubation contained the indicated inhibitor at 10 mM unless otherwise stated. Rates are expressed relative to the inhibitor free control set at 100.

Addition	Relative rate of transport	
	D-Isomer	L-Isomer
None	100	100
N-Ethylmaleimide	0	0
p-Hydroxymercuribenzoate (1 mM)*	0	0
Iodoacetamide	35	18
Cyanide	0	0
Azide	0	0
2,4-Dinitrophenol	0	0

* The effect of *p*-hydroxymercuribenzoate was measured by suspending the cells in glycylglycine buffer at pH 8.5 and uptake compared to cells in glycylglycine buffer without inhibitor.

TABLE III

EFFECT OF STRUCTURAL ANALOGS ON UPTAKE OF THE ISOMERS OF α-AMINOADIPATE

Incubations contained 2 μM of radioactive D- or L-α-aminoadipate and the indicated additions at 200 μM (100-fold). Rates are expressed relative to the no-addition control set at 100. Rates for D-isomer uptake were at 5 s while rates for the L-isomer were at 15 s.

Addition	Relative rate of uptake of	
	D-Isomer	L-Isomer
None	100	100
L-α-Aminoadipate	33, 100*	0
D-α-Aminoadipate	0	65, 100*
L-Glutamate	63	6
D-Glutamate	20	51
L-Aspartate	44	77
D-Aspartate	60	51

* Rate when the potential inhibitor was at a concentration 10 times (20 μM) that of the isomer being taken up.

benzoate could be due to alkylation of a transport protein while inhibition by cyanide, azide and 2,4-dinitrophenol presumably reflects interference with energy production.

Inhibition by structural analogs

Uptake of D-α-aminoadipate is strongly inhibited by L-α-aminoadipate and D-glutamate and moderately inhibited by L-glutamate and both isomers of aspartate all at 100 times the concentration of D-α-aminoadipate (Table III). L-α-Aminoadipate uptake is strongly inhibited by L-glutamate and moderately inhibited by D-α-aminoadipate, D-glutamate and both isomers of asparatate at 100 times the concentration of L-α-aminoadipate. At a 10-fold concentration neither isomer of α-aminoadipate inhibits uptake of the opposite enantiomer.

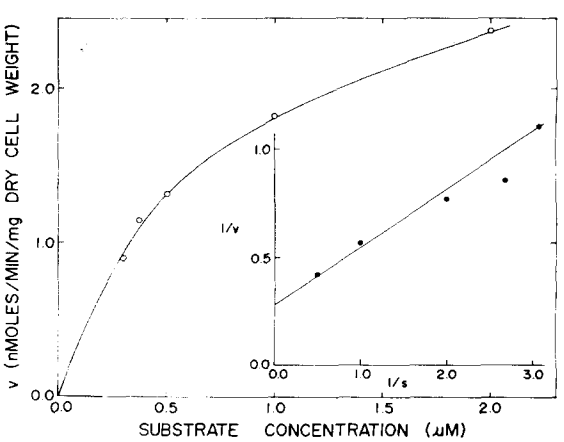

Fig. 2. Double reciprocal plot of L-amino[6-^{14}C]adipate uptake. Uptake was by long term assay. The insert shows the data plotted according to Lineweaver and Burk [11] to obtain K_m and V.

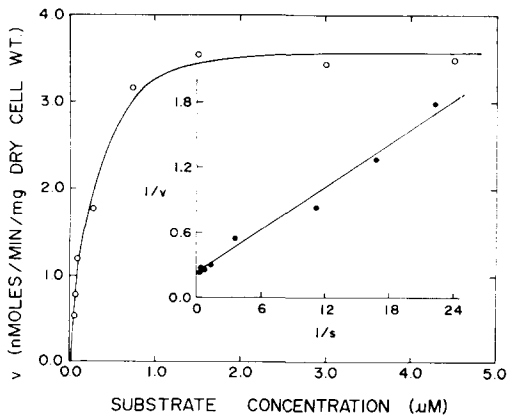

Fig. 3. Double reciprocal plot of D-α-amino[6-^{14}C]adipate uptake. Uptake was by short term assay. The insert shows the data plotted according to Lineweaver and Burk [11] to obtain K_m and V.

TABLE IV

CONCENTRATION OF INTRACELLULAR RADIOACTIVE L-α-AMINOADIPATE DURING TRANSPORT OF L- OR D-α-AMINO[6-^{14}C]ADIPATE BY *A. DENITRIFICANS*

The initial extracellular (ex) concentration of each isomer was 1.5 μM; in, intracellular concentration.

Duration of uptake (s)	Uptake of L-α-aminoadipate		Uptake of D-α-aminoadipate	
	Intracellular concn (μM) of L-isomer	Ratio L-isomer$_{in}$/ L-isomer$_{ex}$	Intracellular concn (μM) of L-isomer	Ratio L-isomer$_{in}$/ D-isomer$_{ex}$
10	365	243	100	67
30	485	320	150	100
60	508	338	206	138

Kinetics of D- *and* L-α-*aminoadipate uptake*

The K_m and V for uptake of L-α-aminoadipate is 1.13 μM and 3.57 nmol/min per mg dry cell wt, respectively; the K_m and V for D-α-aminoadipate uptake are 0.27 μM and 4.12 nmol/min per mg dry cell wt, respectively (Figs 2 and 3).

Intracellular isomer concentration following uptake of D- *and* L-α-*aminoadipate*

Intracellular α-aminoadipate from uptake of either isomer after 10, 30 and 60 s is exclusively in the L-configuration (Table IV).

Uptake of L-α-*aminoadipate by cells pre-incubated with* α-*aminoadipate*

Cells pre-incubated with D- or L-α-aminoadipate and assayed for L-isomer uptake after removing extracellular α-aminoadipate exhibit rates of uptake of 12 and 18 %, respectively, of cells pre-incubated without addition of α-aminoadipate (data not shown). While L-isomer uptake by the control cells reaches steady state after 30 s, L-isomer uptake by cells pre-incubated with either isomer does not come to steady state during the 1-min assay period. Cells preincubated with radioactive D- or L-α-aminoadipate show a negligible efflux of accumulated α-aminodipate.

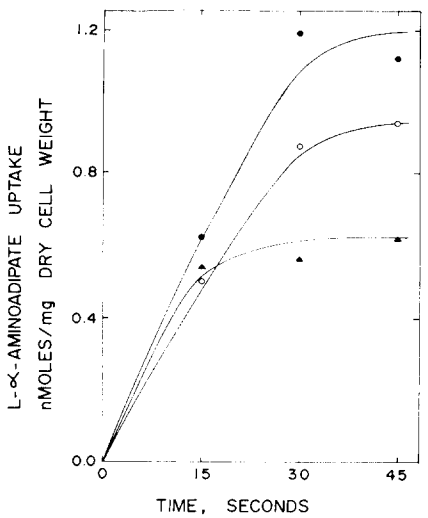

Fig. 4. Time-course for uptake of L-α-amino[6-^{14}C]adipate at 2 μM by cells preincubated for 5 min with and without D-α-aminoadipate; ●, no D-α-aminoadipate; ○, 0.5 μM D-α-aminoadipate; ▲, 2.5 μM D-α-aminoadipate. Extracellular D-isomer remaining from pre-incubation was not removed prior to assay for L-isomer uptake.

Cells pre-incubated with D-α-aminoadipate and assayed for L-isomer uptake without removing extracellular D-isomer show a modest decrease in the rate of uptake and come to steady state after taking up less L-α-aminoadipate than cells pre-incubated without addition of D-isomer (Fig. 4). If cells are grown on L-α-aminoadipate rather than racemic α-aminoadipate, L-isomer uptake is not affected by pre-incubation with D-α-aminoadipate.

DISCUSSION

D-α-Aminoadipate uptake by *A. denitrificans* is an inducible process promoted exclusively by D-α-aminoadipate. Metabolism of translocated D-α-aminoadipate to the L-isomer could account for the ability of D-α-aminoadipate to induce for L-isomer uptake. Conversely, the apparent inability of cells to convert L-α-aminoadipate to the D-isomer accounts for the failure of L-α-aminoadipate to induce for D-isomer uptake. Since metabolism of D-α-aminoadipate proceeds through the L-isomer and since L-α-aminoadipate does not induce for D-isomer uptake, we assume that D-α-aminoadipate induces its own transport system. Why cells grown on racemic α-aminoadipate take up each isomer at a lower rate than cells grown on the individual isomers is not known.

The extremely effective induction of L-α-aminoadipate uptake by L-glutamate could reflect simultaneous uptake by an induced L-α-aminoadipate system, an induced L-glutamate system, and a constitutive L-glutamate system. L-Glutamate couldinduce the L-α-aminoadipate system by virtue of structural similarity. Evidence for possible participation by the induced and constitutive L-glutamate systems is the observed competitive inhibition by L-α-aminoadipate of induced and constitutive L-glutamate

uptake in *A. denitrificans* (Bon, C. and Hartline, R. A., unpublished data). D-Glutamate could induce L-α-aminoadipate uptake directly or via L-glutamate arising from intracellular metabolism, or both. Uptake of L-α-aminoadipate in cells grown on succinate presumably occurs through the constitutive L-glutamate system.

The concentration of intracellular L-α-aminoadipate during L-isomer uptake being larger than the initial extracellular concentration is evidence for uptake against a concentration gradient. D-α-Aminoadipate is not taken up against a gradient since an intracellular D-isomer pool is not maintained. Inhibition of D- and L-isomer uptake by cyanide, azide and 2,4-dinitrophenol is evidence that both processes are active.

Lack of inhibition of L-α-amino[6-^{14}C]adipate uptake by D-α-aminoadipate at a 10-fold concentration negates involvement of competitive inhibition in preferential D-isomer uptake from a racemic mixture. The lower K_m for D-α-aminoadipate uptake indicates that less D-isomer in needed to achieve V for D-α-aminoadipate uptake than the amount of L-isomer required to reach V for uptake of L-α-aminoadipate. However, the similar V values for uptake of D- and L-α-aminoadipate indicates that when each isomer is present at a concentration that saturates its respective uptake system the rates of D- and L-isomer uptake would be essentially equal. *A. denitrificans* growing on 3 mM DL-α-aminoadipate preferentially takes up the D-isomer in the first phase of growth [1]. Since during most of this phase both isomers are present at saturating concentrations, we conclude that a difference in the rates of isomer translocation could not be the basis for preferential uptake.

The exclusive L-configuration of intracellular α-aminoadipate during uptake of D-isomer is evidence that the limited L-isomer uptake by cells in the first phase of growth on racemic α-aminoadipate results from an L-isomer pool maintained by metabolism of D-α-aminoadipate. The reduced rates of L-isomer uptake by cells pre-incubated with D- or L-α-aminoadipate and assayed for uptake after removal of extracellular α-aminoadipate are consistent with inhibition of L-isomer uptake resulting from an intracellular L-isomer pool.

In cells pre-incubated with D-isomer at 0.5, 2.5 and 200 μM (Fig. 4; data not shown for 200 μM) and assayed for uptake without removal of extracellular D-isomer, the amount of L-α-aminoadipate taken up before steady state is 77, 50 and 50 %, respectively, of that taken up by cells pre-incubated without addition of D-α-aminoadipate. The greater amount of L-α-aminoadipate taken up by cells pre-incubated with 0.5 μM D-isomer compared with cells pre-incubated with 2.5 and 200 μM D-isomer probably reflects a depletion in extracellular D-α-aminoadipate due to catabolism during the pre-incubation period which, in turn, results in a reduced intracellular concentration of L-isomer. Cells preincubated with 2.5 and 200 μM D-α-aminoadipate taking up the same amount of L-isomer at essentially the same rate is consistent with saturation of an intracellular L-α-aminoadipate pool, formed by metabolism of the D-isomer, which promotes a reduction in both the rate of uptake and the amount of L-isomer taken up before steady state is achieved. The modest inhibition in the rate of L-α-aminoadipate uptake is consistent with the observation that cells pre-incubated with radioactive D-isomer exhibit a negligible efflux of intracellular α-aminoadipate. A significant efflux of L-α-aminoadipate would greatly decrease the specific activity of L-α-amino[6-^{14}C]adipate used in the uptake assay and cause an apparent pronounced reduction in the rate of uptake.

At present we cannot reasonably explain why different profiles of L-isomer uptake are observed depending on whether cells pre-incubated with D-isomer are assayed for uptake up the presence or absence of extracellular D-isomer. Data from both pre-incubation studies indicate that uptake of D-α-aminoadipate inhibits uptake of L-isomer. However, a common conclusion on the mechanism of inhibition is not applicable to data from both studies. The study with assay after removal of extracellular D-isomer indicates that uptake of D-α-aminoadipate inhibits L-isomer uptake by a severe reduction in the rate of uptake; an L-isomer uptake that should not reach steady state as long as extracellular D-isomer is available. Pre-incubation studies with assay in the presence of extracellular D-isomer indicate that uptake of D-α-aminoadipate inhibits L-isomer uptake by a modest reduction in the rate of uptake and by a reduction in the amount of L-isomer taken up prior to reaching steady state. Since assay for L-isomer uptake in the presence of D-α-aminoadipate approximates conditions during the first phase of cell growth on racemic α-aminoadipate, we presently consider inhibition of L-α-aminoadipate uptake, resulting from uptake of the D-isomer, to occur by reductions in the rate of uptake and the level at which steady state is attained.

The intracellular concentration of L-α-aminoadipate being lower during D-isomer uptake than during L-isomer uptake (Table IV) could indicate that cells taking up D-α-aminoadipate are not totally inhibited for L-isomer uptake but take up less than cells not exposed to D-α-aminoadipate. This is the case as evidenced by cells pre-incubated with D-α-aminoadipate reaching a steady state after taking up less L-isomer than cells pre-incubated without addition of D-isomer. A limited uptake of L-isomer in the presence of D-α-aminoadipate is also observed in cultures of *A. denitrificans* growing on racemic α-aminoadipate [1].

Data presented here are consistent with preferential uptake of D-α-aminoadipate over the L-isomer by *A. denitrificans* growing on DL-α-aminoadipate as a consequence of an intracellular L-α-aminoadipate pool maintained by metabolism of translocated D-isomer. The L-isomer pool subsequently reduces the rate of L-isomer uptake and the level at which steady state occurs resulting in a preferred D-isomer uptake. While it is possible that group translocation (conversion of D-α-aminoadipate to the L-isomer by "vectorial epimerization") could account for the exclusive presence of intracellular L-isomer following uptake of D-α-aminoadipate, it is difficult to understand D-α-aminoadipate inducing for its own uptake of it were not present, if only momentarily, inside the cell. Since racemase reactions are reversible, the lack of intracellular formation of D-α-aminoadipate from the L-isomer makes racemization as the means for conversion of D-α-aminoadipate to the L-enantiomer unattractive. Attempts to elucidate the metabolic steps for conversion of D-α-aminoadipate to the L-isomer in cell extracts were unsuccessful and will require more detailed study.

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