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Properties of an Inducible Uptake System for β-Ketoadipate in *Pseudomonas putida*

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Wild-type strains of *Pseudomonas putida* form an inducible uptake system that appears to act on β-ketoadipate under normal physiological conditions. The system is induced by β-ketoadipate and is repressed by catabolites derived from it. Adipate is metabolized very slowly by wild-type *P. putida* cultures; [^{14}C]adipate was used as an analogue of β-ketoadipate to measure the transport activity in wild-type cells and in cells that constitutively produced the uptake system. Constitutive cells that contained high levels of the uptake system concentrated adipate to a level up to 200-fold above the concentration in the external medium. The process was energy dependent. The activity of the system with radioactive adipate was inhibited by β-ketoadipate, by β-ketoadipate analogues, and by some compounds (e.g., acetate, glucose) that are structurally unrelated to β-ketoadipate; it is not known if the inhibitory effects are exerted directly by the compounds themselves or indirectly by catabolites derived from the compounds. The discovery of the β-ketoadipate uptake system is surprising in view of earlier studies that had indicated that β-ketoadipate does not permeate the membrane of wild-type *P. putida* cells. Contradictions between the former investigations and the present analysis are due primarily to the relatively high concentrations of substrate used in the earlier experiments. The existence of the β-ketoadipate uptake system indicates that β-ketoadipate may exist as a selective nutrient in the natural niche of *P. putida* and may play a determinative role in the evolution of induction mechanisms that are characteristic of fluorescent pseudomonads.

β-Ketoadipate (Fig. 1) occurs frequently as a catabolite in the dissimilation of aromatic compounds by microorganisms (23). Two lines of evidence have suggested that β-ketoadipate itself does not serve as a growth substrate for bacteria in the natural environment. First, β-ketoadipate does not induce the enzymes required for its utilization in some organisms. For example, the enzymes that act upon β-ketoadipate are induced by the metabolic precursors protocatechuate or *cis,cis*-muconate in *Acinetobacter calcoaceticus* (4; Fig. 2). Since the enzymatic reactions that convert these precursors to β-ketoadipate are essentially irreversible, β-ketoadipate cannot give rise to them. Consequently, growth with β-ketoadipate can be achieved only after a regulatory mutation leading to the constitutive production of the enzymes that act upon the compound in *Acinetobacter* (2, 5). Second, manometric experiments have indicated that β-ketoadipate does not readily permeate the cell membrane of organisms in which growth with β-ketoadipate is not precluded by induction mechanisms. For example, β-ketoadipate induces the enzymes that participate in its utilization in fluorescent pseudomonads (11, 16; Fig. 3), and yet Stanier's classical studies of simultaneous adaptation (22) revealed that the oxidation of β-ketoadipate by these bacteria was severely limited by permeability barriers.

The induction mechanisms characteristic of each of the foregoing bacterial groups are rigorously conserved: β-ketoadipate invariably acts as an inducer in *Pseudomonas* and appears never to induce enzymes in *Acinetobacter*. These observations had been interpreted as evidence that the β-ketoadipate pathway may have evolved independently in each bacterial genus (3, 23), but comparative examination of protein structures has made this view untenable: muconolactone isomerases from *A. calcoaceticus* and *Pseudomonas putida* share a number of physical characteristics, and the amino terminal amino acid sequences of the proteins bear only one difference in the first 14 residues (20).

Another hypothesis to account for the evolu-

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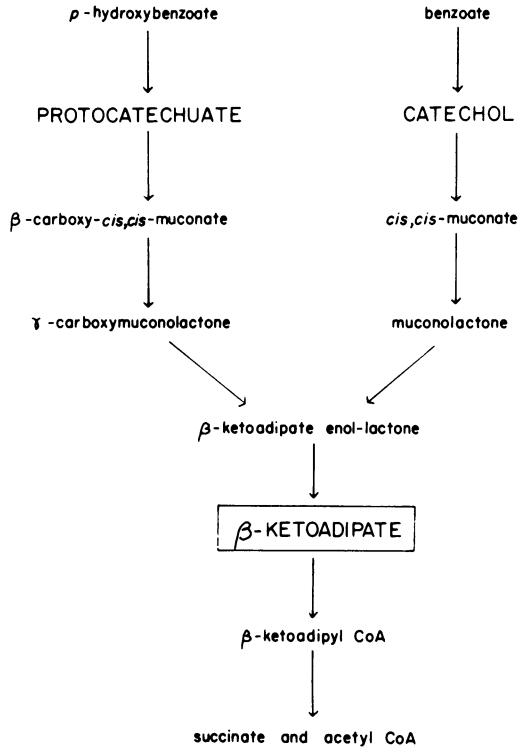

FIG. 1. *β-Ketoadipate (in box) as a catabolite formed in the dissimilation of aromatic compounds by bacteria. Many aromatic precursors (including p-hydroxybenzoate and benzoate, which were used as growth substrates in this study) are converted to one of two diphenols, protocatechuate or catechol. The diphenols are converted to succinate and acetyl CoA by a series of convergent metabolic steps known as the central reactions of the β-ketoadipate pathway.*

tionary conservation of the induction mechanisms is that they were selected by nutritional opportunities available to the bacteria in their microniches. Thus the *Pseudomonas* induction mechanisms would have been favored if bacteria in this genus encountered β-ketoadipate in their evolutionary history more frequently than did representatives of *Acinetobacter*. This proposal appears to be contradicted by the relative impermeability of fluorescent *Pseudomonas* species to β-ketoadipate in the manometric analysis of simultaneous adaptation (22). But the concentrations (1 to 10 mM) of β-ketoadipate used in such studies are high when compared with those that might exist in the natural environment, and transport mechanisms that are effective with low concentrations of β-ketoadipate would not have been revealed by the manometric observations. Therefore, we undertook an investigation of the uptake of β-ketoadipate in *P. putida* by using relatively sensitive radiochemical techniques. Adipate does not serve as a growth substrate for natural isolates of the species (24); radioactive adipate was found to be a substrate for a β-ketoadipate uptake system elaborated by *P. putida* and therefore was used to measure its activity. In this report we describe the properties of the uptake system that is induced by β-ketoadipate and concentrates adipate intracellularly.

MATERIALS AND METHODS

Bacterial strains. All of the bacterial strains used in this study were derived from Stanier's strain 90 (ATCC 12633) of *P. putida* biotype A (24). Strain PRS2000 was selected from strain 90 as a mutant organism that grew well at the expense of *cis,cis*-muconate (25). This growth property, an apparent consequence of an increased permeability to *cis,cis*-muconate, was lost during storage of strain PRS2000 so that now the organism is phenotypically indistinguishable from its parental strain (15). Strain PRS2015 is a spontaneous mutant strain that differs from strain PRS2000 in that it contains a deletion in the *cat B* (*cis,cis*-muconate lactonizing enzyme) structural gene (25). Strain PRS2178 produces the β-ketoadipate uptake system constitutively. It was selected from strain PRS2015 by a procedure analogous to the method used by Cohen-Bazire and Jolit (6) to select *lac* constutitive strains of *Escherichia coli*, except that aliquots of cultures were serially transferred between noninducing growth medium containing succinate as the sole growth substrate and growth medium in which the sole growth substrate was β-ketoadipate. The physiological properties of strain PRS2178 and like mutant strains are complex, and details concerning their isolation and characterization will be published separately. The mutant organisms differ from their parental strains in that they produce constitutively the β-ketoadipate uptake system, β-carboxy-*cis,cis*-muconate lactonizing enzyme, γ-carboxymuconolactone decarboxylase, and β-ketoadipate enol-lactone hydrolase. The mutant strains resemble the wild-type organisms in that they are inducible for β-ketoadipate-succinyl coenzyme A (CoA) transferase. Strain PRS2241 is a spontaneous mutant strain that cannot form the transferase. Orginally referred to as strain PRS2105 (19), its designation has been changed to PRS2241 to avoid confusing it with strain PRS2015.

Growth conditions. Cultures for uptake experiments were grown overnight at 30 C in defined mineral medium (17) from 1% inocula that had been grown in the same medium. β-Ketoadipate is chemically unstable. Therefore it was filter sterilized as a 0.5 M solution and stored at −20 C until immediately before use. Other carbon sources were sterilized as 1.0 M solutions by autoclaving. All carbon sources were transferred aseptically to growth flasks containing sterile mineral media immediately before the addition of cells. Unless otherwise stated, carbon sources were present in growth media at a concentration of 10 mM.

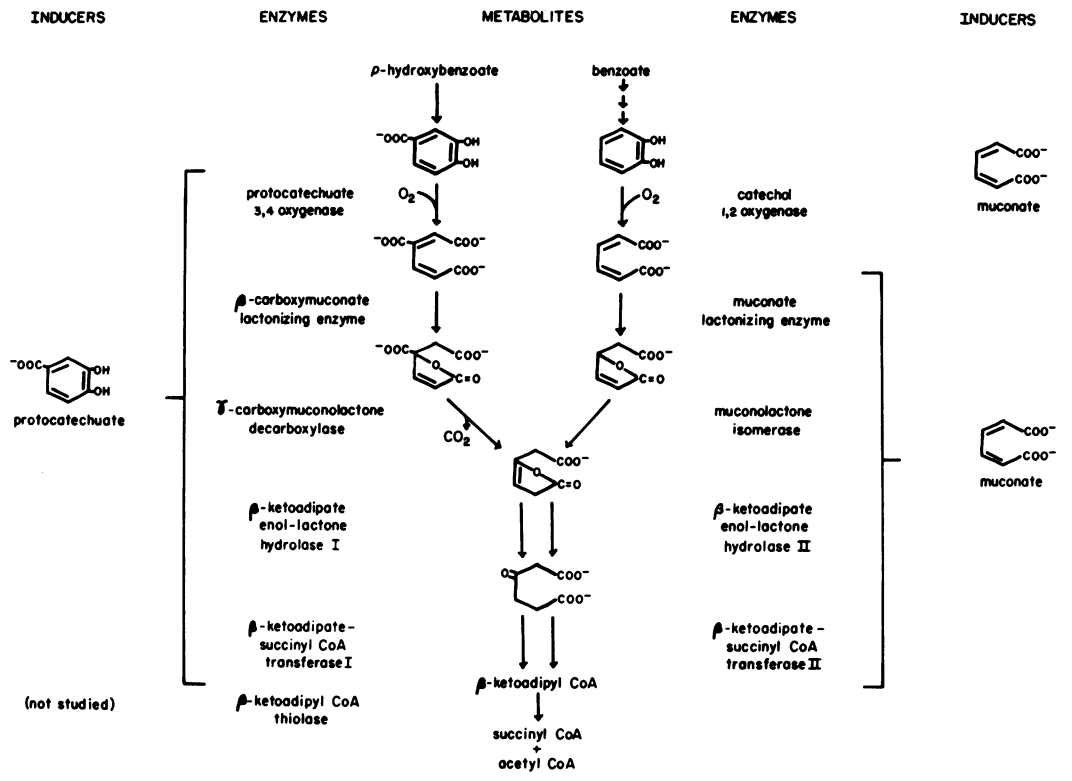

FIG. 2. *Regulation of the β-ketoadipate pathway in Acinetobacter. Enzymes subject to coordinate induction are enclosed by brackets. Note that the enzymes that act upon β-ketoadipate (β-ketoadipate succinyl CoA transferases I and II) are induced by metabolic precursors of β-ketoadipate (protocatechuate and cis,cis-muconate, respectively).*

Constant aeration was provided by a New Brunswick gyratory environmental shaker as the liquid cultures of 150 ml were grown in 500-ml baffled Erlenmeyer flasks. The cultures were in late exponential or stationary phase when harvested.

Preparation of cells for uptake experiments. All operations, including the uptake experiments, were conducted at ambient temperature (22 C). Cells were harvested by centrifugation at 3,000 rpm for 10 min in the GSA rotor of a Sorvall RC2-B centrifuge. The cells were resuspended in 10 ml of the basal mineral medium (17) from which the ammonium sulfate had been omitted. This buffered medium was used throughout the uptake experiments. The cells were centrifuged, washed again with 10 ml of the buffered medium, and resuspended in the medium to a final cell concentration corresponding to 12.5 mg (dry weight) per ml. Cells that were stored for up to 12 h in the medium had no detectable loss of uptake activity.

Measurement of the uptake of adipate. Radioactive adipate (1,6-^{14}C; 7.6 μCi/μmol) was purchased from ICN Isotope and Nuclear Division. The specific activity of the compound was not altered as it was diluted in buffered medium for the uptake experiments. Unless noted otherwise, preparations for uptake experiments contained 1.25 mg (dry weight) of cells per ml and 10^{-6} M adipate in buffered medium. Reaction volumes varied from 1 to 3 ml in test tubes (1 by 10 cm) that were incubated at 22 C. Experiments routinely were initiated by the addition of adipate; initiation of uptake experiments by the addition of cells had no detectable influence on the kinetics of uptake. When the inhibition of adipate uptake was being examined, the experiments were started by the addition of a solution containing the amounts of both adipate and inhibitor necessary to achieve the desired concentrations. Samples of 0.2 ml containing 0.25 mg (dry weight) of cells were removed after timed intervals and placed on membrane filters (Millipore Corp., Bedford, Mass.; type PH filters, 0.45-μm pore size). The medium was drawn off by suction, and the cells were washed with 2.0 ml of buffered medium three times. The washed membrane filters were placed in 5 ml of Bray solution (1), and the radioactivity was determined in a Tri-Carb liquid scintillation counter (Packard Instrument Co., Inc., Downers Grove, Ill.). In the figures we usually represent the intracellular adipate concentration as picomoles per 0.25 mg (dry weight). This is the value that was determined experimentally. The intracellular volume of bacteria frequently is assumed to be 4 μl per mg (dry weight) (12). If this assumption is made, the concentrations shown on the ordinates of the graphs correspond roughly to the ratio of the intracellular

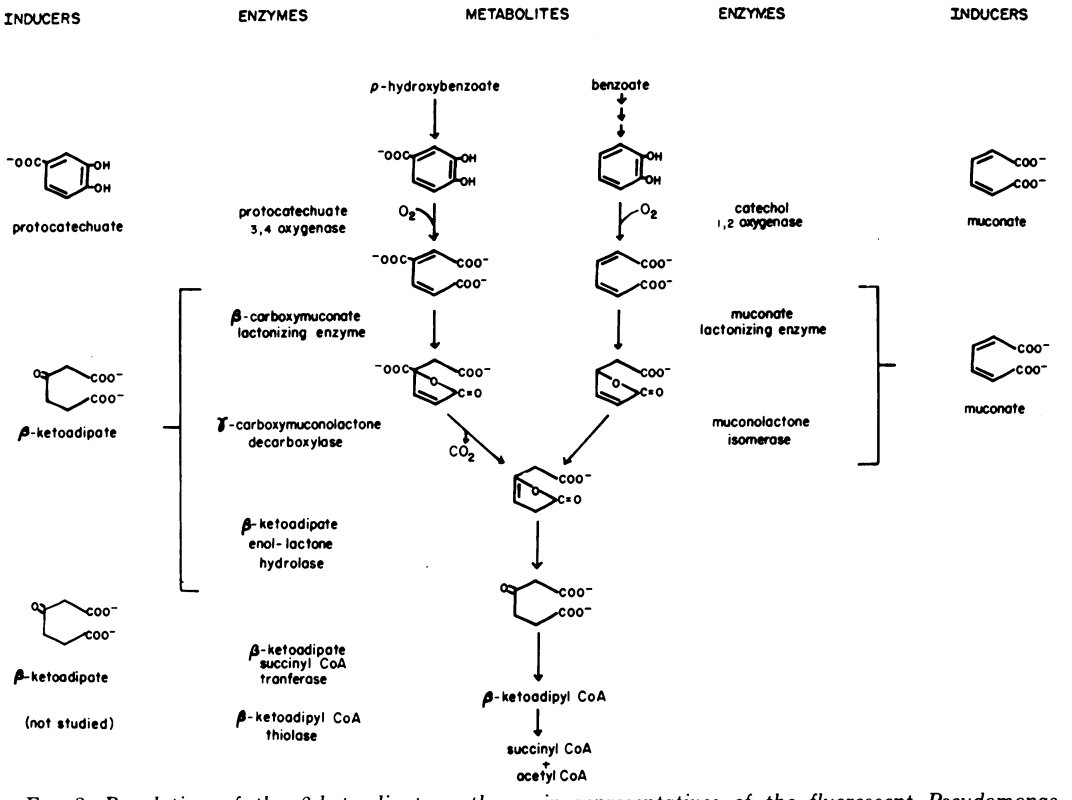

FIG. 3. *Regulation of the β-ketoadipate pathway in representatives of the fluorescent Pseudomonas ribonucleic acid homology group (18). Enzymes subject to coordinate induction are enclosed by brackets. Note that β-ketoadipate induces the enzyme that acts upon it (β-ketoadipate succinyl CoA transferase) as well as the three enzymes that give rise to it from β-carboxy-cis,cis-muconate.*

adipate concentration to the initial extracellular concentration (when the latter value was 10^{-6} M). Recent estimates of the intracellular volume of *Pseudomonas* cultures suggest that this estimate of the ratio may be from 16% (9) to 40% (8) too high.

Identification of radioactive material accumulated by cells. Glucose-grown cultures of the constitutive strain PRS2178 were incubated at a concentration of 1 mg/ml in buffered medium containing 10^{-5} M [^{14}C]adipate. Samples of 0.5 ml were collected on membrane filters and rinsed three times with 2.0 ml of buffered medium. The filters were transferred to a tube containing 4.0 ml of water, and the cells were resuspended with a Vortex mixer. The cell suspension was removed and treated with a drop of toluene to disrupt the cell membranes. After cellular debris had been removed by centrifugation, 100% of the radioactivity was recovered in the supernatant liquid. The extract was dried in vacuo at 55 C overnight; 0.2 ml of 6 N HCl was added, and the material was dried once more. In an analogous series of experiments, the supernatant liquid of toluenized cells was acidified with 0.1 ml of 1.0 M H_2SO_4 and extracted three times with 5.0 ml of ethyl acetate. The organic phase that contained the radioactivity was dried in vacuo at 23 C. Qualitatively similar results were obtained with the two methods.

The dried preparation was dissolved in 20 μl of ethanol, and a sample of 2 μl was subjected to ascending chromatography on a sheet (20 by 20 cm) of Whatman no. 1 paper. The solvent system contained 160 parts ethanol, 10 parts concentrated ammonium hydroxide, and 30 parts water. Radioactivity was detected by cutting the sample lanes of 2.0 cm into 0.5-cm strips and counting the radioactivity in 5 ml of Bray solution (1).

RESULTS

Uptake of radioactive adipate by induced and uninduced wild-type cells. *p*-Hydroxybenzoate is metabolized via β-ketoadipate in *P. putida* (Fig. 3). After growth with *p*-hydroxybenzoate, wild-type cells took up radioactive adipate at a significant rate. The initially rapid rate of uptake departed from linearity within 5 min of the addition of adipate to the cells (Fig. 4). The uptake system approached a steady state 30 min after the start of the experiment. At this point the intracellular concentration of adipate exceeded the external concentration of the compound by about 25-fold. In contrast to

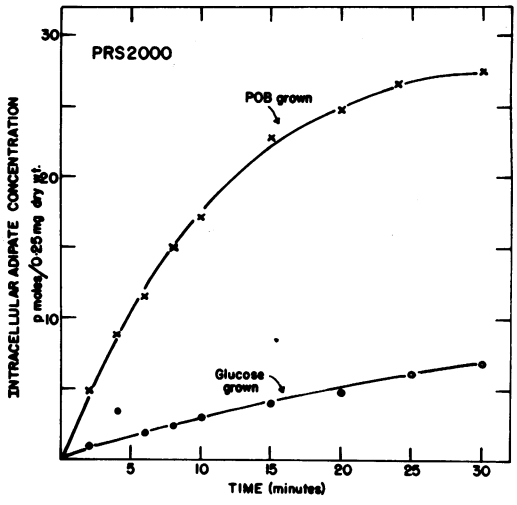

FIG. 4. *Uptake of adipate by wild-type cultures of P. putida strain PRS2000 after growth with 5 mM p-hydroxybenzoate (POB, ×) or with 10 mM glucose (○). The uptake experiments were started by adding [1,6-^{14}C]adipate to bring its concentration to 10^{-6} M. Samples of 0.2 ml containing 0.25 mg (dry weight) of cells were removed after the indicated intervals for the determination of intracellular radioactivity. Details of the experimental procedures are given in the text.*

p-hydroxybenzoate-grown cells, glucose-grown cells did not take up adipate rapidly (Fig. 4). Therefore, the uptake system appears to be induced by either *p*-hydroxybenzoate or a metabolite derived therefrom.

Growth with β-ketoadipate also elicited the formation of the uptake system. Two strains (PRS2000 and PRS2015) that are wild type with respect to the β-ketoadipate uptake system took up adipate more rapidly after growth with β-ketoadipate than after growth with glucose (Fig. 5). The uptake of adipate in *p*-hydroxybenzoate-grown cells consistently was more rapid than adipate uptake in β-ketoadipate-grown cells (Fig. 5). The differences in rates of uptake may be due to either a relatively high concentration of the inducer or a relatively low concentration of metabolites that repress synthesis of the uptake system in the *p*-hydroxybenzoate-grown cells. The metabolism of β-ketoadipate could be blocked by a mutation deleting the synthesis of β-ketoadipate-succinyl CoA transferase. Higher levels of the uptake system were formed in a transferaseless mutant strain (PRS2241) grown in the presence of β-ketoadipate than in wild-type cells (PRS2000) grown at the expense of β-ketoadipate (Fig. 6). Thus it appears that β-ketoadipate itself is the inducer of the uptake system and that the synthesis of the uptake system may be repressed by catabolites derived from β-ketoadipate.

Adipate did not induce the synthesis of the uptake system: the rate of adipate uptake was identical in cultures of wild-type cells grown with glucose alone or with glucose supplemented with adipate at concentrations ranging from 10^{-5} to 10^{-2} M.

Since the uptake system is induced by β-ketoadipate but not by adipate, it appears that the biological function of the uptake system is to concentrate β-ketoadipate and that adipate acts as an analogue of the substrate β-ketoadipate. The K_m of the uptake system with adi-

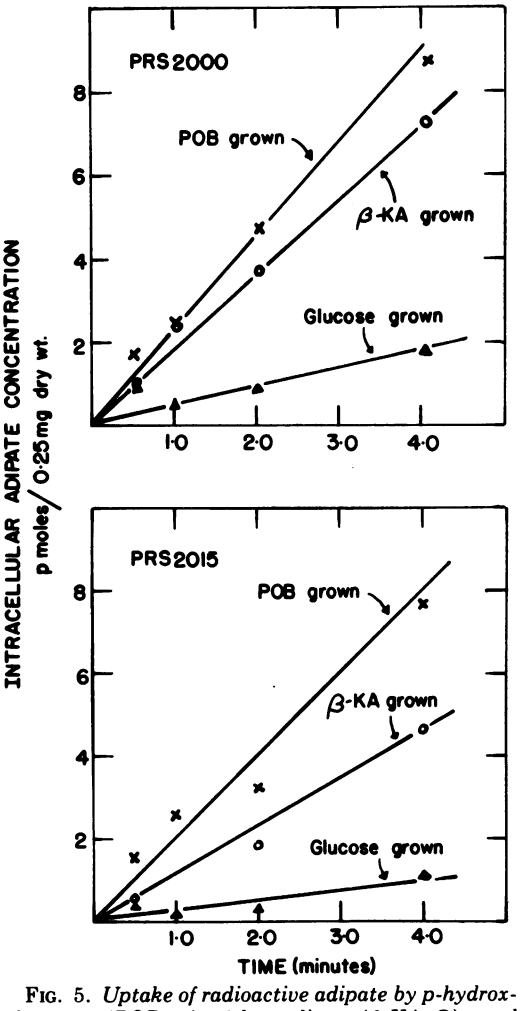

FIG. 5. *Uptake of radioactive adipate by p-hydroxybenzoate (POB, ×)-, β-ketoadipate (β-KA, ○)-, and glucose (Δ)-grown cultures of P. putida. Strains PRS2000 and PRS2015 are wild type with respect to the uptake system. Experimental procedures for the uptake experiments are summarized in the legend to Fig. 4.*

pate was 2.3×10^{-4} M (Fig. 7). The V_{max} with adipate (Fig. 7) was 2.6 μmol/min per g (dry weight) of *p*-hydroxybenzoate-grown cells or 0.65 mmol/min if the intracellular volume (in microliters) is assumed to be four times the dry weight (in milligrams) of the cells (12).

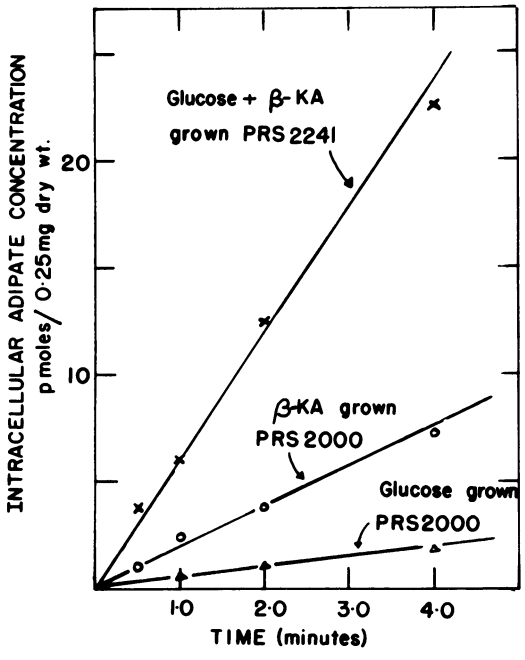

FIG. 6. *Uptake of adipate by glucose (Δ)- and β-ketoadipate (β-KA, O)-grown wild-type cells (PRS2000) compared with the uptake of adipate by a mutant strain (PRS2241, ×) that cannot metabolize β-ketoadipate. The mutant strain lacks β-ketoadipate succinyl CoA transferase; it was grown with 10 mM glucose in the presence of 10^{-10} M β-ketoadipate. Experimental procedures for the uptake experiments are summarized in the legend to Fig. 4.*

Uptake of radioactive adipate by a mutant strain that is constitutive for the uptake system. Further characterization of the uptake system was conducted with mutant strain PRS2178. The uptake of adipate by glucose-grown cultures of mutant strain PRS2178 and its parental strain PRS2015 is shown in Fig. 8. It can be seen that uninduced cells of the mutant strain PRS2178 took up adipate much more rapidly than uninduced cells of the parental strain. In fact, the rate of adipate uptake was considerably greater in the uninduced mutant strain PRS2178 than in *p*-hydroxybenzoate-grown wild-type cells. The V_{max} of strain PRS2178 grown on glucose was 4.3 μmol/min per g (dry weight) of cells (Fig. 9), which is 1.6 times that of the wild-type strain grown with *p*-hydroxybenzoate (Fig. 7). In the mutant strain the K_m with adipate was 2.0×10^{-4} M (Fig. 9), approximately equal to the value of 2.3×10^{-4} M found with the wild-type strain (Fig. 7). Therefore, it is evident that the mutant strain produces relatively high levels of an adipate uptake system that is physically similar to that found in the wild-type strain.

Low concentrations of β-ketoadipate effectively inhibited the transport of radioactive adipate by the uptake system (Fig. 10). This evidence further supports the inference drawn above that β-ketoadipate is the natural substrate of the uptake system.

Studies with the wild-type strain and a transferaseless mutant strain suggested that the synthesis of the uptake system might be repressed by catabolites derived from β-ketoadipate. Further evidence in support of this conclu-

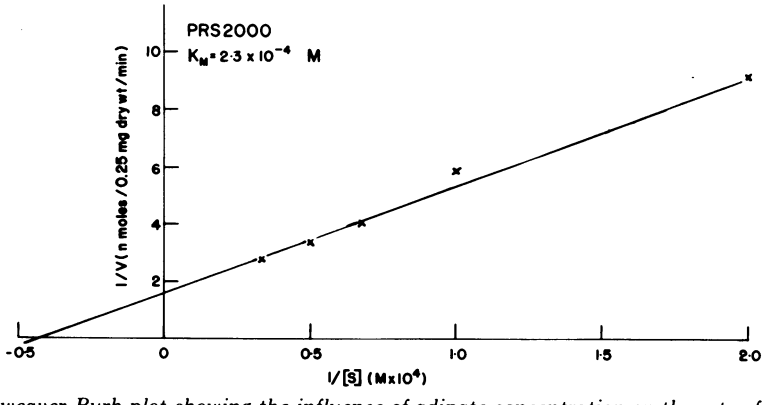

FIG. 7. *Lineweaver-Burk plot showing the influence of adipate concentration on the rate of adipate uptake by p-hydroxybenzoate-grown cultures of wild-type cells (strain PRS2000). The initial rates of uptake were measured under conditions summarized in the legend to Fig. 4, except that the substrate concentration was varied.*

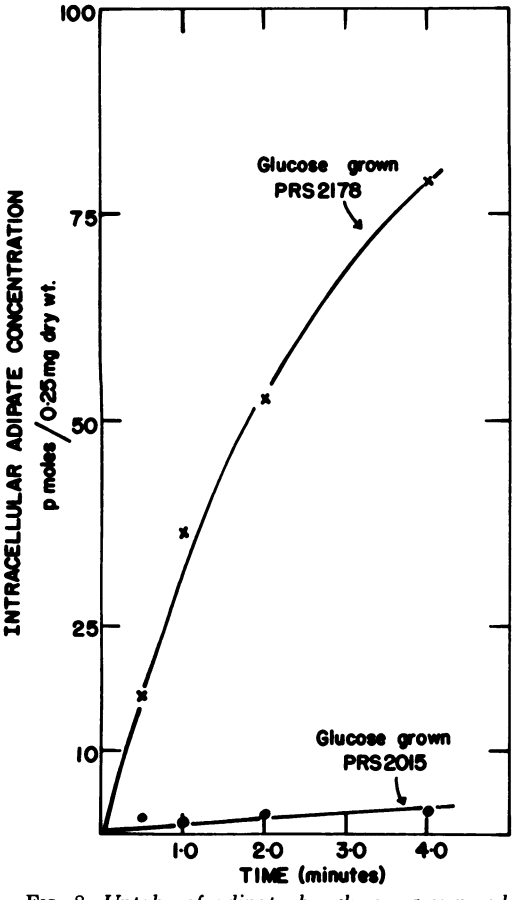

FIG. 8. *Uptake of adipate by glucose-grown cultures of strain PRS2178 (×), which forms the uptake system constitutively, and strain PRS2015 (●), which is wild type with respect to the uptake system. Strain PRS2178 was derived from strain PRS2015. Experimental procedures for the uptake experiments are summarized in the legend to Fig. 4.*

sion comes from the observation that the constitutive mutant strain transported adipate more rapidly after growth with glucose than after growth with *p*-hydroxybenzoate or β-ketoadipate (Fig. 11). Growth of the organism with succinate lowered the initial rate of adipate uptake to about half the level observed after growth of the organism with glucose.

The energy dependence of adipate uptake is shown by the fact that the process was completely inhibited by sodium azide (Fig. 12). Cells did not accumulate detectable amounts of adipate in the presence of sodium azide (Fig. 12A), and the addition of sodium azide to cells that had been pre-equilibrated with radioactive adipate led to a rapid loss of radioactivity from the cells (Fig. 12B).

Despite the apparent energy dependence of the uptake system, accumulation of radioactive adipate was inhibited by succinate (Fig. 13). One or more of three possible effects may be operative in the inhibition phenomenon. (i) Succinate, a decarboxylic acid, may act as a competitive inhibitor of adipate transport. Evidence that this might be the case comes from the observation that high concentrations (10 mM) of the dicarboxylic acids succinate, β-ketoadipate, adipate, and glutarate all cause the rapid release of radioactivity from cells that have been equilibrated with radioactive adipate (Fig. 14). Pimelate, the C_7 analogue of adipate, was less effective as an inhibitor of adipate transport (Fig. 14). (ii) Inhibition of adipate transport might be caused by a high energy charge in the cells. This effect is demonstrated by the observation that when cells that had been equilibrated with radioactive adipate were incubated with 10 mM glucose or 10 mM acetate, both of which are good sources of metabolic energy, a rapid loss of radioactivity from the cells was observed (Fig. 15). (iii) Levulinate, which is neither a source of metabolic energy nor an analogue of adipate, had an inhibitory effect on adipate transport (Fig. 15). This observation raises the possibility that the transport of any one of a number of compounds across the cell membrane inhibits the uptake of adipate.

Metabolism of [1,6-^{14}C]adipate. Like other representatives of *P. putida*, strains PRS2000 and PRS2178 do not grow at the expense of adipate (24). Radioactive adipate is taken up

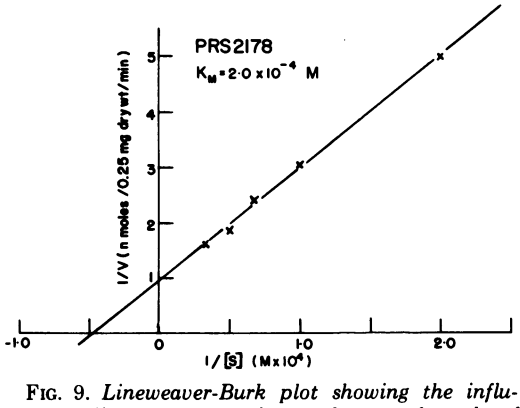

FIG. 9. *Lineweaver-Burk plot showing the influence of adipate concentration on the rate of uptake of adipate by glucose-grown cells of mutant strain PRS2178. This strain produces the uptake system constitutively. The initial rates of uptake were measured under conditions summarized in the legend to Fig. 4, except that the substrate concentration was varied.*

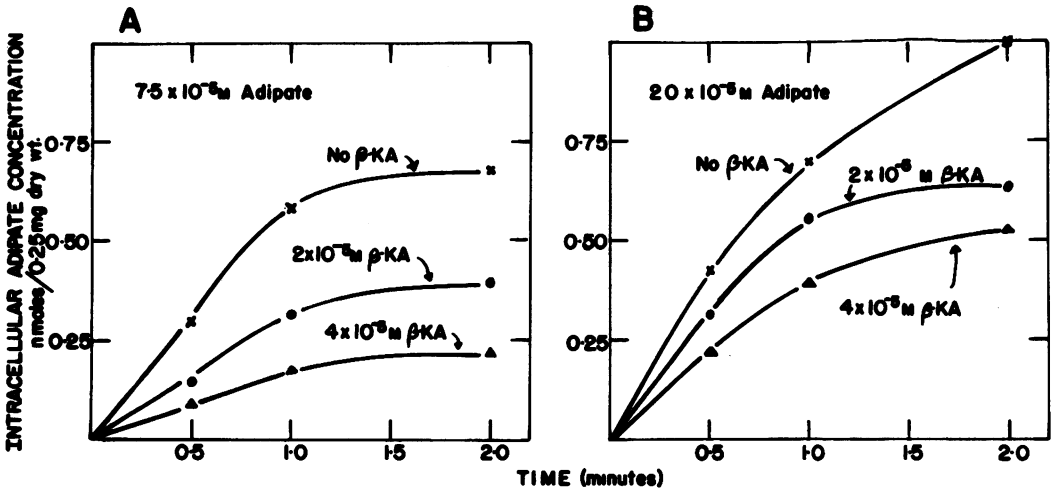

FIG. 10. *Inhibition of the uptake of adipate by β-ketoadipate (β-KA). Glucose-grown cultures of the adipate transport mutant strain PRS2178 were exposed to 7.5 × 10⁻⁵ M radioactive adipate (left) or 20 × 10⁻⁵ M radioactive adipate (right) in the presence of 4 × 10⁻⁵ M β-ketoadipate (Δ), 2 × 10⁻⁵ M β-ketoadipate (●), or no β-ketoadipate (×). The β-ketoadipate was not radioactive. Each experiment was initiated by the addition of a solution containing amounts of both adipate and β-ketoadipate necessary to achieve the designated concentration. Other experimental conditions are summarized in the legend to Fig. 4.*

rapidly by appropriately induced cultures of the wild-type strain or by the constitutive mutant strain PRS2178. When glucose-grown cultures of strain PRS2178 were equilibrated with 10^{-5} M adipate, over 25% of the radioactivity was inside the cells. The radioactive adipate was not metabolized to any great extent. Thirty minutes after incubation of glucose-grown PRS2178 with 10^{-5} M radioactive adipate, all detectable radioactivity could be removed from the cells by the addition of 10^{-2} M nonradioactive adipate. Furthermore, more than 75% of the radioactivity could be recovered in the incubation medium after 4 h.

Nevertheless, adipate did appear to undergo some slow metabolic conversion by the cells. Cells that had been incubated for 4 min with 10^{-5} M radioactive adipate contained a small amount of radioactive material that migrated more rapidly than adipate in ethanol-ammonia-water (Fig. 16). The rapidly migrating material represented a substantial fraction of the overall radioactivity after 4 h of incubation (Fig. 16). The chemical nature of the unknown metabolite(s) was not investigated further.

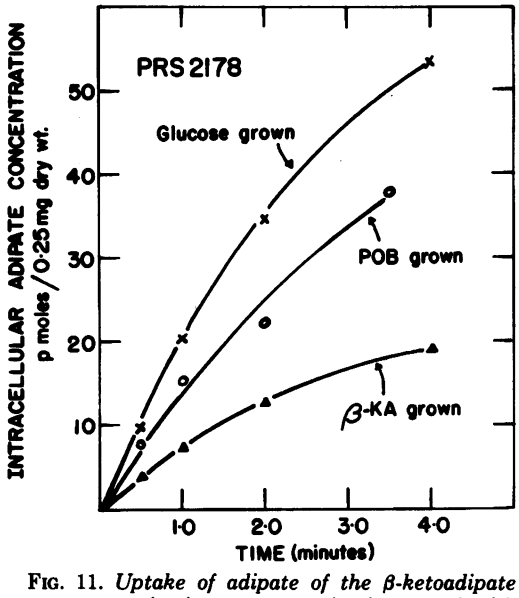

FIG. 11. *Uptake of adipate of the β-ketoadipate transport constitutive mutant strain after growth with glucose (×), p-hydroxybenzoate (POB, O), or β-ketoadipate (β-KA, Δ). Experimental conditions are summarized in the legend to Fig. 4.*

DISCUSSION

Metabolism of adipate. Since natural isolates of *P. putida* do not grow at the expense of adipate or longer straight-chain dicarboxylic acids (24), it seemed unlikely that the bacteria would metabolize adipate to any great extent. In fact, adipate did appear to undergo a slow metabolic conversion in the constitutive mutant strain PRS2178 (Fig. 16): after 4 h about 20% of the adipate was converted to other metabolite(s). The slow metabolism of adipate is un-

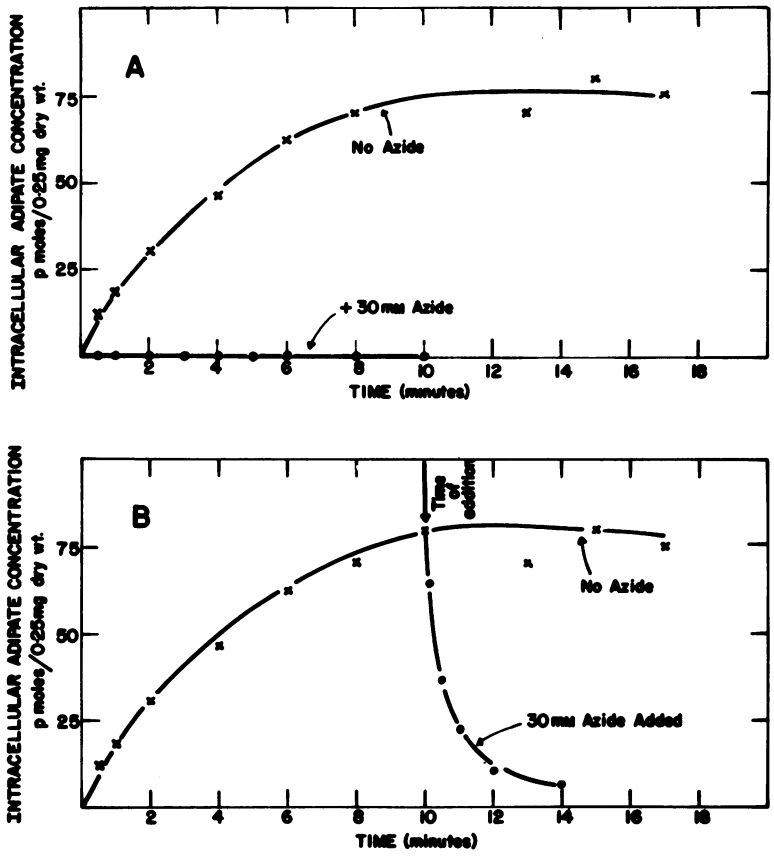

FIG. 12. *Inhibition of the uptake of adipate by sodium azide. (A) The uptake of adipate by glucose-grown cultures of the β-ketoadipate transport constitutive strain PRS2178 was measured in the presence (●) or absence (×) of 30 mM sodium azide. (B) The uptake experiment was conducted with two identical reaction mixtures containing glucose-grown cells of the constitutive mutant strain PRS2178. At 10 min, sodium azide was added to bring the final concentration in one of the mixtures (○) to 30 mM. Other experimental conditions are summarized in the legend to Fig. 4.*

likely to have introduced any major error into the uptake experiments since they normally were completed within a few minutes.

The fact that adipate can be metabolized by *P. putida* suggests that members of this species may have the genetic potential to grow at the expense of the compound. Indeed, this seems to be the case because we have isolated a spontaneous mutant *P. putida* strain that grows well at the expense of adipate. The nature of the mutation or mutations in this strain is unknown. Strains that are constitutive for the β-ketoadipate uptake system do not grow at the expense of the dicarboxylic acid; therefore, more than one mutation may be required for the acquisition by *P. putida* of the ability to utilize adipate as a growth substrate.

Evidence supporting the view that β-ketoadipate is the natural substrate of the uptake system. Three lines of evidence support the conclusion that the biological role of the uptake system is to transport β-ketoadipate rather than adipate. First, the uptake of adipate confers no known selective benefit upon representatives of *p. putida* because these organisms cannot grow at the expense of adipate (24). Second, the transport of radioactive adipate is severely inhibited by low concentrations of β-ketoadipate (Fig. 10), suggesting that the latter compound competes effectively with the former for the binding site of the uptake system. Third, the uptake system is induced by β-ketoadipate; adipate does not elicit the synthesis of the uptake system and therefore cannot be transported effectively by wild-type cells in the absence of an appropriate inducer. The specificity of induction of the uptake system has not been explored beyond the observation that

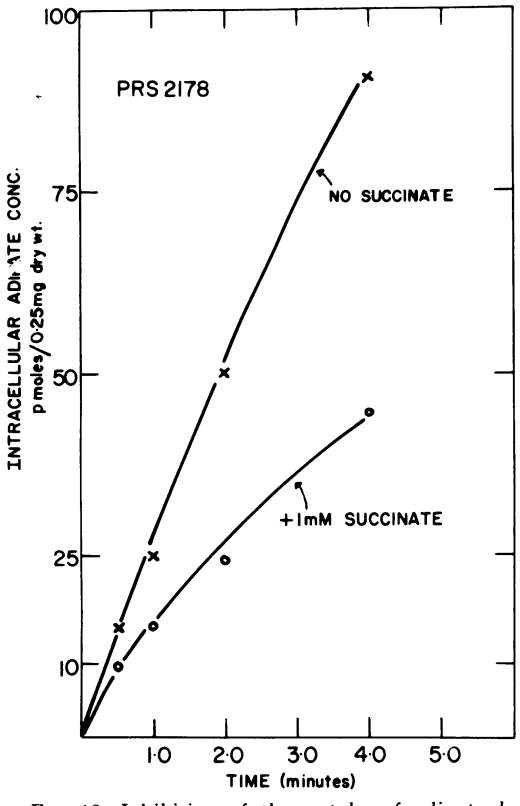

FIG. 13. *Inhibition of the uptake of adipate by succinate. The uptake of adipate by a glucose-grown culture of the constitutive uptake mutant strain PRS2178 was measured in the presence (O) or absence (×) of 1 mM disodium succinate. The succinate was mixed with the adipate that was used to initiate the experiment. Other experimental conditions are summarized in the legend to Fig. 4.*

adipate and another dicarboxylic acid, succinate, do not serve as its inducers.

Properties of the uptake system. The uptake system effectively concentrates adipate inside cells. The highest ratio of internal to external concentration of adipate was observed with glucose-grown cultures of the constitutive mutant strain PRS2178. When equilibrated with the external medium containing 10^{-6} M adipate, a cell suspension of 1.25 mg in 1 ml contained intracellularly about 500 pmol of adipate. If the intracellular volume in microliters is assumed to be about four times the dry weight in milligrams of the cells (12), the ratio of intracellular to extracellular adipate was about 200. Sodium azide, an inhibitor of energy metabolism, completely inhibited the uptake of radioactive adipate and caused a rapid loss of internally concentrated adipate from cells (Fig. 12).

The K_m for the transport of adipate was about 2×10^{-4} M with wild-type cells (Fig. 7) and with cells that produce the uptake system constitutively (Fig. 9). This value may be compared with the K_m of 3.3×10^{-5} M reported by Lawford and Williams (13) for the transport of citrate by the citrate uptake system in *Pseudomonas fluorescens* and the K_m's of 7×10^{-6} and 7×10^{-3} M reported by Guymon and Eagon (8)

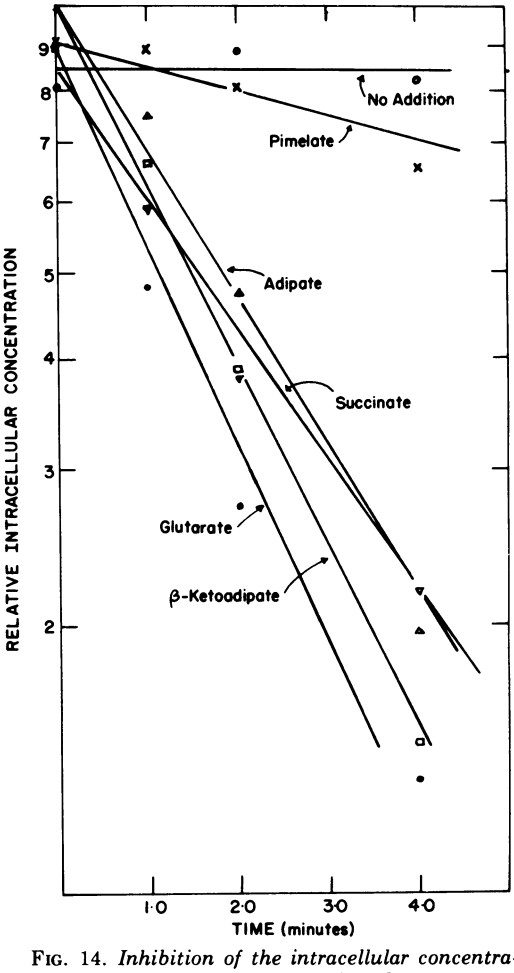

FIG. 14. *Inhibition of the intracellular concentration of adipate by dicarboxylic acids. Glucose-grown cultures of the uptake constitutive mutant strain were incubated for 30 min with 10^{-6} M adipate. At this time, when the intracellular and extracellular concentrations of adipate had equilibrated, the indicated dicarboxylic acids were added as disodium salts to a final concentration of 10 mM. The intracellular concentration of adipate was determined after 1, 2, and 4 min. The additions are symbolized as follows: no addition (O), pimelate (×), adipate (△), succinate (▽), β-ketoadipate (□), and glutarate (●). Other experimental conditions are summarized in the legend to Fig. 4.*

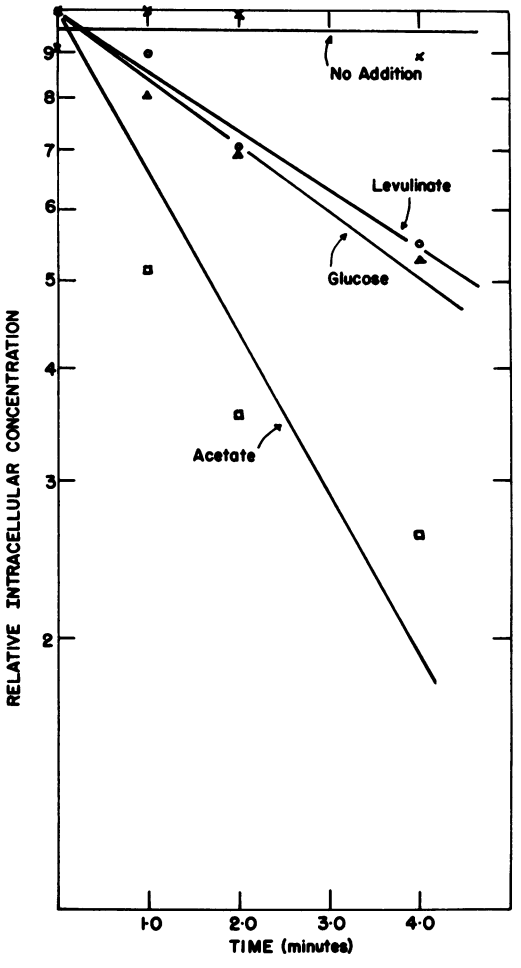

FIG. 15. *Inhibition of the intracellular concentration of adipate by compounds that are not dicarboxylic acids. The experimental procedure was identical to that described in the legend to Fig. 14. The following symbols are used: no addition (×), levulinate (○), glucose (△), and acetate (□). Levulinate and acetate were added as their sodium salts.*

for the transport of glucose and the glucose analogue methyl-α-glucoside, respectively, in *Pseudomonas aeruginosa*.

Inducible uptake systems for dicarboxylic acids that are intermediates in the tricarboxylic acid cycle have been described for *E. coli* (10) and *Bacillus subtilis* (7). The *B. subtilis* transport system has a K_m of about 10^{-4} M for succinate or fumarate and can concentrate these compounds intracellularly to levels 100- to 300-fold higher than the concentration in the external medium. The *P. putida* β-ketoadipate uptake system may resemble the *E. coli* and *B. subtilis* dicarboxylic acid uptake systems in some respects, but the β-ketoadipate uptake system differs in that it is not induced by growth with succinate.

Two other intermediates in the β-ketoadipate pathway of *P. putida* are known to be transported by inducible uptake systems. One of these is induced by and acts upon mandelate, an aromatic precursor of β-ketoadipate (9). Growth with β-carboxy-*cis,cis*-muconate induces the synthesis of a β-carboxy-*cis,cis*-muconate uptake system in mutant strains of *P. putida* that grow with this compound (15).

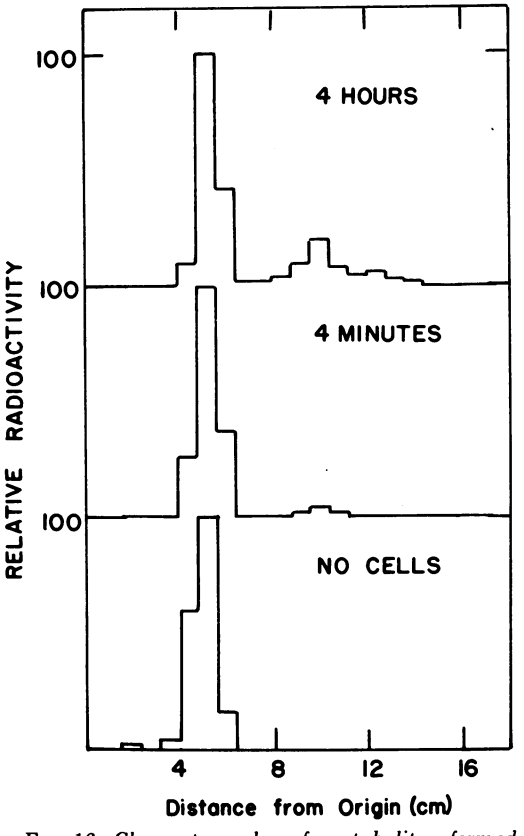

FIG. 16. *Chromatography of metabolites formed from adipate by glucose-grown cultures of the transport constitutive mutant strain PRS2178. Samples were removed after the cells had been exposed to 10^{-5} M adipate for 4 min (middle) or 4 h (top). The cells were washed and then toluenized in order to release radioactive material. The extracted material was subjected to ascending paper chromatography in a solvent containing 10 parts concentrated ammonium hydroxide, 160 parts ethanol, and 30 parts water. Chromatographic strips were cut into 0.5-cm portions, which were placed in 5 ml of Bray solution (1) for determination of their radioactivity. The bottom third of the figure shows the results of chromatography of a sample of 10^{-5} M adipate that was not exposed to cells. Details of the experimental procedure are presented in the text.*

Regulation of the uptake of the inducing metabolite β-ketoadipate. In addition to eliciting the synthesis of the uptake system in *P. putida*, β-ketoadipate induces the formation of at least four enzymes of the protocatechuate pathway: β-carboxy-*cis*,*cis*-muconate lactonizing enzyme, γ-carboxymuconolactone decarboxylase, β-ketoadipate enol-lactone hydrolase, and β-ketoadipate succinyl CoA transferase (16; Fig. 3). Therefore, by controlling the internal concentration of β-ketoadipate, cells regulate the synthesis of many proteins. This control may be exercised by regulating the uptake system by induction, repression, and inhibition.

The biosynthesis of the uptake system is induced by β-ketoadipate and appears to be repressed by metabolites derived from the compound. This repression is relieved by a mutation that blocks the conversion of β-ketoadipate to β-ketoadipyl CoA and thence to tricarboxylic acid cycle intermediates (Fig. 6). Additional evidence that metabolites derived from β-ketoadipate repress synthesis of the uptake system comes from the observation that glucose-grown cultures of the constitutive mutant strain PRS2178 take up adipate about twice as rapidly as cultures of the same strain that have been grown with *p*-hydroxybenzoate or β-ketoadipate (Fig. 11). The role of transport systems in the regulation of the biosynthesis of enzyme systems was recognized by Kepes and Cohen (12). The utilization of malate by *Streptococcus faecalis* provides an example of regulatory control that is somewhat analogous to that of β-ketoadipate utilization. The induction of malic enzyme synthesis by malate was demonstrated to depend upon an uptake system for the accumulation of the inducer; the biosynthesis of the malate uptake system was induced by malate and subject to catabolite repression by glucose (14).

Regulation of the internal concentration of inducing metabolites may also be effected by the inhibition of uptake systems. The uptake of radioactive adipate is inhibited by high levels of the dicarboxylic acids succinate and glutarate, which may compete with adipate for the binding site of the transport system. Inhibition of adipate transport by acetate and glucose is unlikely to be competitive, and the mechanism of the inhibition may be quite indirect. For example, the metabolism of the two compounds may increase the concentration of a metabolite that inhibits the activity of the adipate uptake system. A possible basis for such an effect has been described recently by Romano et al. (21), who showed that the glucose uptake system of *Thiobacillus intermedius* is inhibited by thiosulfate. It seems likely that the thiosulfate exerts its inhibitory effect by increasing the energy charge of the cells; the inhibiting metabolite may be either a high-energy compound like adenosine 5′-triphosphate or a compound that serves as an effective reductant for the electron transport chain (21). It is conceivable that the inhibition of adipate transport in *P. putida* is due to a regulatory interaction among different transport systems. Interactions of this type have been reported, but to date little is known about their mechanism (26, 27).

β-Ketoadipate as a selective nutrient in the natural environment. The fact that wild-type *P. putida* strain PRS2000 elaborates an uptake system that is induced by and appears to act upon β-ketoadipate strongly suggests that this compound can serve as a growth substrate for representatives of the species in the natural environment. Under laboratory conditions, wild-type cultures do grow, albeit slowly, at the expense of β-ketoadipate at concentrations ranging from 5 to 10 mM. Manometric experiments of Stanier (22) using approximately 1 mM β-ketoadipate indicated that the compound was oxidized at a slow rate by cultures of *P. putida* strain 90 that were induced to oxidize benzoate or *p*-hydroxybenzoate rapidly. Similar observations have been made with strain PRS2000 (15). These observations have suggested that β-ketoadipate entered the cells slowly by passive diffusion.

The slow growth rate with β-ketoadipate and the limited oxidation of the compound by wild-type strains of *P. putida* are not necessarily inconsistent with the existence of an inducible uptake system for β-ketoadipate in the species. The concentrations of β-ketoadipate used in conventional growth and manometric experiments may be several orders of magnitude above the levels of β-ketoadipate that normally exist in the natural environment. The K_m of the uptake system for adipate is 2×10^{-4} M; the K_m for β-ketoadipate may be well below this value. At the high concentrations of β-ketoadipate used in the manomeric experiments, the uptake system for this compound probably is saturated with substrate. Thus at high substrate concentrations there may be greater permeability barriers for β-ketoadipate than for its aromatic precursors benzoate or *p*-hydroxybenzoate. We conclude that manometric evidence indicating the existence of permeability barriers to a compound does not rule out the possibility that it is a potential growth substrate in the natural environment.

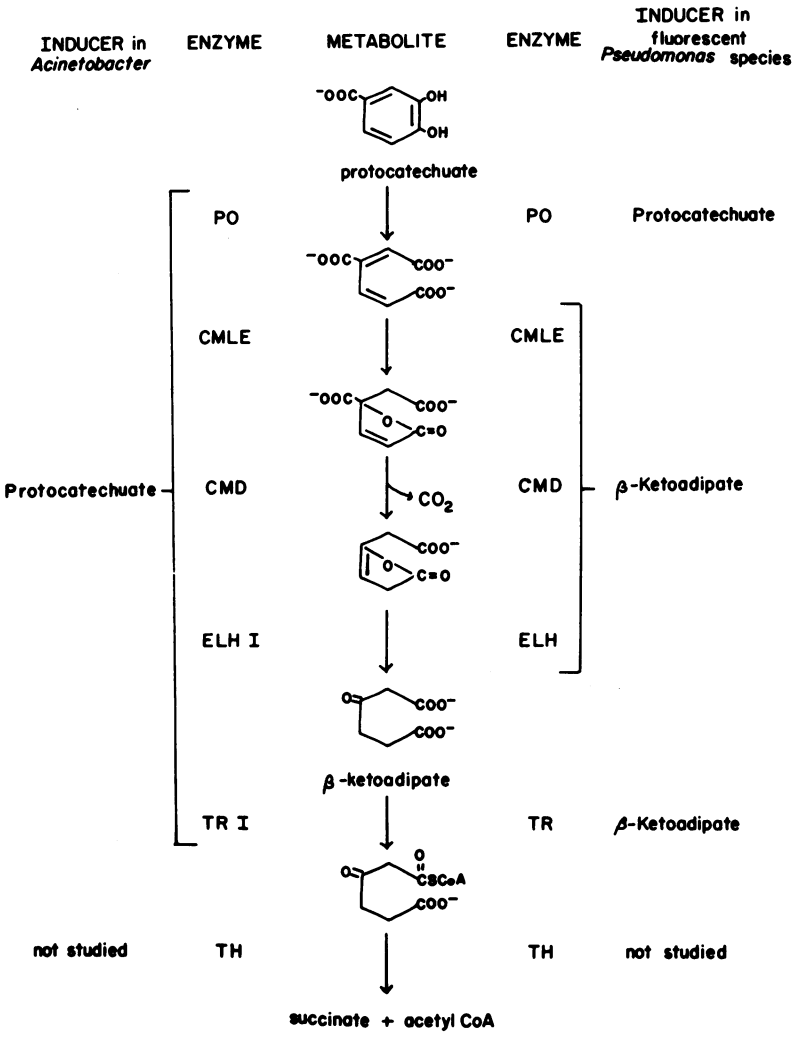

FIG. 17. *Direct comparison of the induction mechanisms governing the synthesis of the enzymes of the protocatechuate pathway in Acinetobacter and in fluorescent Pseudomonas species. β-Ketoadipate is not an inducer in Acinetobacter, whereas it induces most of the enzymes of the pathway in the Pseudomonas species.*

The inference that β-ketoadipate occurs at low levels in the natural habitat of *Pseudomonas* suggests that the compound may have exerted a selective pressure favoring the evolution of the regulatory mechanisms used by members of the genus to govern the synthesis of enzymes of the β-ketoadipate pathway. The use of β-ketoadipate as an inducer for the enzymes that mediate its dissimilation permits the compound to be utilized as a growth substrate. In addition, product induction of the three enzymes that convert β-carboxy-*cis*,*cis*-muconate to β-ketoadipate by β-ketoadipate in fluorescent pseudomonads (Fig. 17) may permit these organisms to grow with β-carboxy-*cis*,*cis*-muconate. This may be contrasted with the induction mechanism used to govern the synthesis of the isofunctional enzymes in *Acinetobacter:* use of protocatechuate as an inducer for all of the enzymes that mediate the conversion of protocatechuate to β-ketoadipyl CoA (Fig. 17) precludes the utilization of β-carboxy-*cis*, *cis*-muconate or β-ketoadipate as a growth substrate by wild-type members of this genus. Thus examination of uptake systems may provide clues to selective pressures that operate in the evolution of induction mechanisms.

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LITERATURE CITED

1. Bray, G. A. 1960. A simple efficient liquid scintillator for counting aqueous solutions in a liquid scintillation counter. Anal. Biochem. **1**:279-285.
2. Cánovas, J. L., B. F. Johnson. 1968. Regulation of the enzymes of the β-ketoadipate pathway in *Moraxella calcoaceticus*. 4. Constitutive synthesis of β-ketodipate succinyl-CoA transferases II and III. Eur. J. Biochem. **3**:312-317.
3. Cánovas, J. L., L. N. Ornston, and R. Y. Stanier. 1967. Evolutionary significance of metabolic control systems. Science **156**:1695-1699.
4. Cánovas, J. L., and R. Y. Stanier. 1967. Regulation of the enzymes of the β-ketoadipate pathway in *Moraxella calcoacetica* (*Acinetobacter*). 1. General aspects. Eur. J. Biochem. **1**:289-300.
5. Cánovas, J. L., M. L. Wheelis, and R. Y. Stanier. 1968. Regulation of the enzymes of the β-ketoadipate pathway in *Moraxella calcoaceticus*. 2. Role of protocatechuate as inducer. Eur. J. Biochem. **3**:293-304.
6. Cohen-Bazire, G., and M. Jolit. 1953. Isolement par sélection de mutants d'*Escherichia coli* synthétisant spontanement l'amylomaltase et la β-galactosidase. Ann. Institut Pasteur Paris **84**:937-945.
7. Ghei, O. K., and W. W. Kay. 1973. Properties of an inducible C_4-dicarboxylic acid transport system in *Bacillus subtilis*. J. Bacteriol. **114**:65-79.
8. Guymon, L. F., and R. G. Eagon. 1974. Transport of glucose, gluconate, and methyl α-D-glucoside by *Pseudomonas aeruginosa*. J. Bacteriol. **117**:1261-1269.
9. Higgins, S. J., and J. Mandelstam. 1972. Evidence for induced synthesis of an active transport factor for mandelate in *Pseudomonas putida*. Biochem. J. **126**:917-922.
10. Kay, W. W., and H. L. Kornberg. 1971. The uptake of C_4-dicarboxylic acids by *Escherichia coli*. Eur. J. Biochem. **18**:274-281.
11. Kemp, M. B., and G. D. Hegeman. 1968. Genetic control of the β-ketoadipate pathway in *Pseudomonas aeruginosa*. J. Bacteriol. **96**:1488-1499.
12. Kepes, A., and G. N. Cohen. 1962. Permeation, p. 179-221. *In* I. C. Gunsalus and R. Y. Stanier (ed.), The bacteria, vol. 4. The physiology of growth, Academic Press Inc., New York.
13. Lawford, H. G., and G. R. Williams. 1971. The transport of citrate and other tricarboxylic acids in two species of *Pseudomonas*. Biochem. J. **123**:571-577.
14. London, J., and E. Y. Meyer. 1970. Malate utilization by group D streptococcus: regulation of malic enzyme synthesis by an inducible malate permease. J. Bacteriol. **102**:130-137.
15. Meagher, R. B., G. M. McCorkle, M. K. Ornston, and L. N. Ornston. 1972. Inducible uptake system for β-carboxy-*cis,cis*-muconate in a permeability mutant of *Pseudomonas putida*. J. Bacteriol. **111**:465-473.
16. Ornston, L. N. 1966. The conversion of catechol and protocatechuate to β-ketoadipate by *Pseudomonas putida*. IV. Regulation. J. Biol. Chem. **241**:3800-3810.
17. Ornston, L. N., and R. Y. Stanier. 1966. The conversion of catechol and protocatechuate to β-ketoadipate by *Pseudomonas putida*. I. Biochemistry. J. Biol. Chem. **241**:3776-3786.
18. Palleroni, N. J. 1975. General properties and taxonomy of the genus *Pseudomonas*, p. 1-36. *In* P. H. Clarke and M. H. Richmond (ed.), Genetics and biochemistry of Pseudomonas. John Wiley and Sons, New York.
19. Patel, R. N., R. B. Meagher, and L. N. Ornston. 1973. Relationships among enzymes of the β-ketoadipate pathway. II. Properties of crystalline β-carboxy-*cis,cis*-muconate-lactonizing enzyme from *Pseudomonas putida*. Biochemistry **12**:3531-3537.
20. Patel, R. N., R. B. Meagher, and L. N. Ornston. 1974. Relationships among enzymes of the β-ketoadipate pathway. IV. Muconolactone isomerase from *Acinetobacter calcoaceticus* and *Pseudomonas putida*. J. Biol. Chem. **249**:7410-7419.
21. Romano, A. H., N. J. Van Vranken, P. Preisand, and M. Brustolon. 1975. Regulation of the *Thiobacillus intermedius* glucose uptake system by thiosulfate. J. Bacteriol. **121**:577-582.
22. Stanier, R. Y. 1950. Problems of bacteriol oxidative metabolism. Bacteriol. Rev. **14**:179-191.
23. Stanier, R. Y., and L. N. Ornston. 1973. The β-ketoadipate pathway, p. 89-151. *In* A. H. Rose and D. W. Tempest (ed.), Advances in microbial physiology, vol. 9. Academic Press Inc., New York.
24. Stanier, R. Y., N. J. Palleroni, and M. Doudoroff. 1966. The aerobic pseudomonads: a taxonomic study. J. Gen. Microbiol. **43**:159-271.
25. Wheelis, M. L., and L. N. Ornston. 1972. Genetic control of enzyme induction in the β-ketoadipate pathway on *Pseudomonas putida*: deletion mapping of *cat* mutations. J. Bacteriol. **109**:790-795.
26. Wilson, D. B. 1974. The regulation and properties of the galactose transport system in *E. coli* K12. J. Biol. Chem. **249**:553-558.
27. Winkler, H. H., and T. H. Wilson. 1967. Inhibition of β-galactoside transport by substrates of the glucose transport system in *Escherichia coli*. Biochim. Biophys. Acta **135**:1030-1051.