Pathways for the Oxidation of Aromatic Compounds by *Azotobacter*

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SUMMARY

The metabolic pathways used by members of the genus *Azotobacter* for the oxidation of benzoate and *p*-hydroxy-benzoate have been investigated. *A. chroococcum*, *A. vinelandii* and *A. beijerinckii* oxidize benzoate *via* catechol, which is further metabolized by *meta* cleavage. The same species oxidize *p*-hydroxybenzoate *via* protocatechuate, which is dissimilated through the β -ketoadipate pathway.

INTRODUCTION

During recent years biochemical investigations of the oxidative degradation of aromatic compounds by several genera of aerobic bacteria (summarized by Ribbons, 1966) have revealed that many of these substrates are metabolized through the diphenols, protocatechuate and catechol. These intermediates are converted to aliphatic compounds by oxygenative ring cleavage. Two methods of *ortho*-diphenol cleavage have been demonstrated in bacteria (Dagley, Evans & Ribbons, 1960): (1) *ortho* cleavage, oxidative fission of the bond between carbon atoms bearing the hydroxyl groups; (2) *meta* cleavage, rupture of the aromatic structure at the C—C bond adjacent to the *ortho*-diphenol group. Completely different metabolic pathways result from these two modes of ring cleavage.

The ability of *Azotobacter* species to utilize aromatic compounds has been long known (Winogradsky, 1932; Guittonneau & Chevalier, 1939). However, besides a report on the metabolism of benzoate to catechol by *Azotobacter vinelandii* (Voets, 1958), the metabolic pathways utilized by *Azotobacter* species for the oxidation of aromatic substrates have not so far been investigated. We describe here the pathways used for the metabolism of benzoate and *p*-hydroxybenzoate by several *Azotobacter* species.

METHODS

Organisms. The strains utilized have the following origins and designations. *Azotobacter chroococcum* strain 203, *A. vinelandii* strain 206 and *A. agilis* strain 140 were derived from the Colección Española de Cultivos Tipo, C.S.I.C., Madrid, Spain. *A. chroococcum* strain v, *A. vinelandii* strains C-14 and C-15 and *A. beijerinckii* strain v were the gift of Professor J. P. Voets (Agricultural University, Ghent, Belgium). *A. chroococcum* strain IP and *A. vinelandii* strain IP were from the Department de

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Microbiologie du Sol, Institut Pasteur, Paris, France. *Beijerinckia derxii* strain B-30, *B. indica* strain B-17 and *B. fluminensis* were provided by Dr F. Hilger (Institut Agronomique de l'Etat, Gembloux, Belgium). *A. chroococcum*, strain P β -203, is a permeability mutant (able to grow on β -ketoadipate) isolated by spreading about 10^8 wild-type organisms of *A. chroococcum* strain 203 on plates of basal agar medium containing 5 mM- β -ketoadipate, as described by Ornston (1966c).

Chemicals and enzyme reagents. β -Carboxy-*cis,cis*-muconic acid, ($\pm$)-muconolactone, *cis,cis*-muconic acid and β -ketoadipic acid were synthesized by Dr M. Robert-Gero. Succinyl-CoA was synthesized by the method of Stadtman (1957). Purified preparations of β -ketoadipate enol-lactone hydrolase, muconolactone isomerase and muconate lactonizing enzyme, required for enzyme assays, were kindly provided by Dr G. D. Hegeman (Department of Bacteriology and Immunology, University of California, Berkeley, California, U.S.A.). All other reagents and chemicals were of the highest grade available commercially.

Growth media and culture conditions. A modification of Burk nitrogen-free medium (Burk & Lineweaver, 1930) was used for the cultivation of the *Azotobacter* strains. It had the following composition (mg./l.): KH_2PO_4 , 200; K_2HPO_4 , 800; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 200; CaCl_2 , 50; $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 10; $\text{MnSO}_4 \cdot \text{H}_2\text{O}$, 1; $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$, 2.5. To avoid precipitate formation, the solution of CaCl_2 was autoclaved separately and added immediately before inoculation. The final pH value of this medium was 7.3.

The organic carbon and energy sources were added to the mineral basal medium as concentrated sterile stock solutions, to yield the following final concentrations: disodium succinate, 20 mM; sodium *p*-hydroxybenzoate, 15 mM; sodium benzoate, 15 mM; sodium shikimate, 10 mM; disodium β -ketoadipate, 10 mM; sodium protocatechuate, 8 mM. For solid media, all organic substrates were used at a concentration of 10 mM and 1% (w/v) agar was added. Chemically unstable substrates (e.g. sodium protocatechuate, disodium β -ketoadipate) were sterilized by filtration through Millipore filters and used immediately. Stock cultures of *Azotobacter* strains were maintained at room temperature on agar slopes containing succinate 10 mM as the carbon source and were transferred monthly. Cultures for experimental work were incubated at 28°.

Liquid cultures were incubated aerobically with mechanical agitation. Small liquid cultures were grown in volumes of 50 ml. in 250 ml. conical flasks. When enzyme concentrations were to be determined in cell-free extracts, cultures were grown in volumes of 400 ml. in 2 l. Fernbach flasks. Fernbach flasks have a broad base and a long-narrow neck and are particularly suitable for growing aerobic organisms in liquid media. In liquid cultures a 10% (v/v) inoculum of a culture in the early log phase growing with succinate 20 mM was used. Growth was estimated by measuring the extinction of the culture at 660 m μ in a Bausch and Lomb Spectronic-20 Colorimeter. An extinction of 1.0 was equivalent, for an exponentially growing culture, to about 2×10^8 organisms/ml., for *Azotobacter chroococcum* strain 203; 3×10^8 organisms/ml., for *A. vinelandii* strain 206; 0.7×10^8 organisms/ml., for *A. beijerinckii* strain v.

Beijerinckia cultures were grown on the mineral basal medium described by De Ley & Park (1966), supplemented with an appropriate carbon source; stock cultures were maintained on slope of potato glucose agar.

Mechanism of ring fission. Organisms growing exponentially on *p*-hydroxybenzoate or benzoate were harvested by centrifugation, washed and resuspended in 20 mM-

tris + HCl buffer (pH 8.0). The method of Hosokawa as described by Stanier, Palleroni & Doudoroff (1966) was used with these suspensions to determine the mechanism of *ortho*-diphenol cleavage.

Manometric techniques. Vegetative organisms were harvested during exponential growth by centrifugation at 7000 *g* for 15 min. at 0°, washed twice in 20 mM-sodium potassium phosphate buffer (pH 7.0) and resuspended in the same buffer. The suspension was adjusted to give an extinction of approximately 0.3 at 660 m μ in the Spectronic-20 Colorimeter. Rates of oxygen uptake were determined at 30° with a Warburg type respirometer. The rates are expressed as Q_{O₂} (μ l. O₂/hr/mg. dry weight), corrected for endogenous respiration. Bacterial cell mass of the suspensions was determined from a standard curve relating extinction to dry weight. The contents of Warburg vessels were: 1 ml. (equivalent to 150–300 μ g. dry weight) of cell suspension in the main compartment; 0.2 ml. of 20% (w/v) NaOH in the centre well, and 10 μ mole substrate in the side arm. Sodium potassium phosphate buffer (20 mM, pH 7.0) to a total volume of 2 ml. was added to the main compartment. The gas phase was air.

Preparation of cell-free extracts. Rod-shaped organisms were removed from cultures by centrifugation at 7000 *g* for 15 min. at 0°. Cysts of *Azotobacter beijerinckii* strain v, were also harvested to compare enzymic activities of resting organisms with vegetative ones. The organisms were washed in chilled 20 mM-tris + HCl buffer (pH 8.0) containing 10 μ M-Mg-EDTA and centrifuged at 10,000 *g* for 45 min. at 0°. The yield of organisms was between 0.8 and 1.5 g. (wet weight)/l. culture, depending on substrate and organism. The pellets were resuspended as 25 to 30% (wet weight/volume) suspensions of organism in the same buffer and stored at –20°.

Preliminary observations showed that crude extracts of benzoate-grown organisms lost easily *m*-pyrocatechase activity, confirming the instability of this enzyme reported by Kojima, Itada & Hayaishi (1961). To stabilize this enzyme, 10% (v/v) of acetone as used by Nozaki, Kagamiyama & Hayaishi (1963) was added to 20 mM-sodium-potassium phosphate buffer containing 10 μ M-Mg-EDTA (pH 7.5), when harvesting organisms grown on benzoate for which *m*-pyrocatechase activity was to be determined.

Frozen bacterial suspensions were thawed and broken by ultrasonic treatment for 4 min. with a 100 W Ultrasonic Disintegrator (Measuring & Scientific Equipment Ltd., London, England). The samples were cooled with ice water during this treatment. The extracts were centrifuged at 50,000 *g* for 45 min. at 0°, and the pellets discarded. The supernatant fluids were decanted and used as the crude extracts for measurement of enzyme activity. The protein concentration of the fluids so obtained was between 15 and 20 mg./ml.

Enzyme assays. All enzymic activities in crude extracts were assayed spectrophotometrically at room temperature (about 21°). The unit of activity is defined in all cases as the amount of enzyme necessary to convert one micromole substrate/min. under assay conditions, except the unit of β -ketoadipate succinyl-CoA transferase activity, which is defined as described by Cánovas & Stanier (1967).

The enzyme assays were performed by the following published procedures: *p*-hydroxybenzoate hydroxylase (Hosokawa & Stanier, 1966); protocatechuate-3,4-oxygenase (Stanier & Ingraham, 1954); carboxymuconate-lactonizing enzyme and β -ketoadipate enol-lactone hydrolase (Ornston, 1966*a*); β -ketoadipate succinyl-CoA transferase by a modification (Cánovas & Stanier, 1967) of the method of Katagiri &

Hayaishi (1957); *m*-pyrocatechase (Kojima *et al.* 1961); pyrocatechase (Hegeman, 1966); muconate lactonizing enzyme and (+)-muconolactone isomerase (Ornston, 1966*b*). The enzymic activity of each extract was always measured at several different concentrations to establish the linearity of the relationship between activity and concentration. Enzyme concentrations in crude extracts are expressed as units of enzyme/mg. protein.

Chemical measurements. Protein in crude extracts was determined by the method of Lowry, Rosebrough, Farr & Randall (1951), with crystalline bovine serum albumin (Nutritional Biochemicals Corporation, Cleveland, Ohio) as standard.

The quantity of α -hydroxymuconic semialdehyde formed in cell-free extracts was determined from the extinction at 375 m μ . The identity of the accumulated α -hydroxymuconic semialdehyde was established from the absorption spectrum.

RESULTS

Utilization of aromatic and hydroaromatic acids as sole sources of carbon and energy

Azotobacter and *Beijerinckia* strains were tested for ability to grow with benzoate, shikimate and *p*-hydroxybenzoate as carbon+energy sources. All strains of *Azotobacter chroococcum*, *A. vinelandii* and *A. beijerinckii* grew well with these substrates, whereas *A. agilis* did not. These results are in agreement with previous studies concerning the pattern of utilization of aromatic compounds by *Azotobacter* species (Tchan, 1953). None of the *Beijerinckia* strains was able to grow at the expense of these compounds.

Table 1. *Ring cleavage of catechol and protocatechuate by some strains of Azotobacter tested after growth on benzoate and p-hydroxybenzoate*

<i>Azotobacter</i> strains		Cleavage of catechol (organisms grown on benzoate)		Cleavage of protocatechuate (organisms grown on <i>p</i> -hydroxybenzoate)	
		<i>Ortho</i> cleavage	<i>Meta</i> cleavage	<i>Ortho</i> cleavage	<i>Meta</i> cleavage
<i>A. chroococcum</i>	strain 1P	—	+	+	—
<i>A. chroococcum</i>	strain v	—	+	+	—
<i>A. chroococcum</i>	strain 203	—	+	+	—
<i>A. vinelandii</i>	strain 1P	—	+	+	—
<i>A. vinelandii</i>	strain C-14	—	+	+	—
<i>A. vinelandii</i>	strain C-15	—	+	+	—
<i>A. vinelandii</i>	strain 206	—	+	+	—
<i>A. beijerinckii</i>	strain v	—	+	+	—

Mechanism of aromatic ring-cleavage

Qualitative tests for the mechanism of aromatic ring-cleavage were performed with the *Azotobacter* strains able to grow on benzoate and *p*-hydroxybenzoate. As shown in Table 1, all the strains examined de-aromatize protocatechuate by *ortho* cleavage after growth with *p*-hydroxybenzoate, and catechol by *meta* cleavage after growth with benzoate. Single strains representative of *Azotobacter chroococcum* (strain 203), *A. vinelandii* (strain 206) and *A. beijerinckii* (strain v) were selected for further study.

Growth rates

The growth rates at 28° of these three strains with succinate, *p*-hydroxybenzoate, benzoate and shikimate are shown in Table 2. In every case, growth at the expense of the aromatic and hydroaromatic substrates was considerably slower than growth with succinate.

Table 2. Generation times of *Azotobacter* strains studied with various compounds as sole sources of carbon and energy

Azotobacter strains		Growth substrate			
		Succinate	<i>p</i> -Hydroxybenzoate	Shikimate	Benzoate
			Generation times (min.)		
<i>Azotobacter chroococcum</i>	strain 203	110	200	205	180
<i>A. vinelandii</i>	strain 206	135	240	220	180
<i>A. beijerinckii</i>	strain v	180	215	205	190

Table 3. Rates of oxygen uptake (Q_{O_2}) at the expense of various compounds by *Azotobacter chroococcum* strain 203*

Organisms grown with	Rate of oxygen uptake (Q_{O_2}) with							
	Succinate	<i>p</i> -Hydroxybenzoate	Shikimate	Protocatechuate	β -Carboxy- <i>cis,cis</i> -muconate	Benzoate	Catechol	β -Ketoadipate
Succinate	1400	6	45	—	—	38	—	60
<i>p</i> -Hydroxybenzoate	190	1660	210	1080	60	—	—	105
Shikimate	165	28	940	1480	—	—	—	—
Benzoate	185	—	—	—	—	1900	1680	75

* All data corrected for endogenous oxygen uptake.

Influence of growth substrates on the respiration of aromatic compounds

The attack on benzoate, *p*-hydroxybenzoate and shikimate by all three *Azotobacter* species was more or less strictly inducible. Succinate-grown organisms respired with these compounds at negligible rates, whereas organisms grown with any one of the three compounds in question oxidized it at a high rate. Growth with shikimate or *p*-hydroxybenzoate induced a high rate of protocatechuate oxidation. β -Ketoadipate and β -carboxy-*cis,cis*-muconate were not respired at significant rates by organisms grown with *p*-hydroxybenzoate or shikimate, suggesting that the whole organism^a were impermeable to these later intermediates in the metabolism of protocatechuate. Benzoate-grown organisms respired both benzoate and catechol at high rates. Data for *Azotobacter chroococcum* strain 203 are shown in Table 3; essentially similar results were obtained with *A. vinelandii* strain 206 and *A. beijerinckii* strain v.

Enzymic activities of cell-free extracts

The activities of certain enzymes in cell-free extracts prepared from *Azotobacter chroococcum*, *A. vinelandii* and *A. beijerinckii* after growth with succinate, *p*-hydroxybenzoate, shikimate, protocatechuate and benzoate are shown in Tables 4, 5 and 6.

All three strains behaved in a similar manner. Most of the enzymes assayed were not detected in extracts of succinate-grown organisms; however, carboxy-muconate lactonizing enzyme and β -ketoadipate succinyl CoA transferase were present at very low but detectable concentrations. The values given for the other enzymes assayed in extracts of succinate-grown organisms represent the smallest concentrations which could have been detected by the assay methods used.

Table 4. *The effect of different growth substrates on the specific activities of the β -ketoadipate pathway enzymes in cell-free extracts of Azotobacter chroococcum strain 203*

Enzyme	Substrate used for growth				
	Succinate	<i>p</i> -Hydroxybenzoate	Protocatechuate	Shikimate	Benzoate
	Enzyme units/mg. protein in cell-free extracts				
<i>p</i> -Hydroxybenzoate hydroxylase	< 0.001	0.94	< 0.001	< 0.001	< 0.001
Protocatechuate 3,4-oxygenase	< 0.003	5.29	2.32	2.22	< 0.003
Carboxymuconate-lactonizing enzyme	0.009	0.58	0.52	0.49	0.009
Enol-lactone hydrolase	< 0.02	0.88	0.94	1.03	< 0.02
Transferase	0.002	0.39	0.41	0.36	0.002
Pyrocatechase	< 0.008	0.092	0.042	0.040	0.01
<i>m</i> -Pyrocatechase	< 0.004	< 0.004	—	< 0.004	0.38
Muconate-lactonizing enzyme	< 0.002	< 0.002	—	< 0.002	< 0.002
Muconolactone isomerase	< 0.01	< 0.01	—	< 0.01	< 0.01

Table 5. *The effect of different growth substrates on the specific activities of the β -ketoadipate pathway enzymes in cell-free extracts of Azotobacter beijerinckii strain V*

Enzyme	Substrate used for growth				
	Succinate	<i>p</i> -Hydroxybenzoate	Protocatechuate	Shikimate	Benzoate
	Enzyme units/mg. protein in cell-free extracts				
<i>p</i> -Hydroxybenzoate hydroxylase	< 0.001	0.79	< 0.001	< 0.001	< 0.001
Protocatechuate 3,4-oxygenase	< 0.003	5.44	2.44	2.36	< 0.003
Carboxymuconate-lactonizing enzyme	0.009	0.44	0.46	0.46	0.009
Enol-lactone hydrolase	< 0.02	0.81	0.84	0.93	< 0.02
Transferase	0.003	0.46	0.52	0.48	0.003
Pyrocatechase	< 0.008	0.093	0.047	0.042	0.01
<i>m</i> -Pyrocatechase	< 0.004	< 0.004	—	—	0.34
Muconate-lactonizing enzyme	< 0.002	< 0.002	—	—	< 0.002
Muconolactone isomerase	< 0.01	< 0.01	—	—	< 0.01

Growth at the expense of *p*-hydroxybenzoate induced high degrees of activity of *p*-hydroxybenzoate hydroxylase, protocatechuate oxygenase, carboxymuconate lactonizing enzyme, enol-lactone hydrolase and β -ketoadipate succinyl-CoA transferase. This confirms that the dissimilation of *p*-hydroxybenzoate proceeded through the β -ketoadipate pathway. The patterns of enzymic activity of organisms grown with shikimate and protocatechuate were qualitatively similar, except that *p*-hydroxy-

benzoate hydroxylase was not induced. The specific activities of the enzymes of the β -ketoadipate pathway in shikimate-grown and in protocatechuate-grown organisms were also similar except for the protocatechuate-3,4-oxygenase and pyrocatechase values which were about half those found in *p*-hydroxybenzoate-grown organisms. Growth at the expense of these three substrates also induced a measurable pyrocatechase activity; the product of this reaction, as determined by measurement of its absorption spectrum, was *cis,cis*-muconate. It is probable that this activity reflected the fact that the protocatechuate oxygenase of these bacteria is non-specific, and can also perform an *ortho* cleavage of catechol at a very low rate. *m*-Pyrocatechase, which was present at high values in benzoate-grown organisms, was not measurably induced by growth with *p*-hydroxybenzoate, shikimate or protocatechuate.

Table 6. *The effect of different growth substrates on the specific activities of the β -ketoadipate pathway enzymes in cell-free extracts of Azotobacter vinelandii strain 206*

Enzyme	Substrate used for growth				
	Succinate	<i>p</i> -Hydroxybenzoate	Protocatechuate	Shikimate	Benzoate
	Enzyme units/mg. protein in cell free extracts				
<i>p</i> -Hydroxybenzoate hydroxylase	< 0.001	0.48	< 0.001	< 0.001	< 0.001
Protocatechuate 3,4-oxygenase	< 0.003	5.33	2.21	2.40	< 0.003
Carboxymuconate-lactonizing enzyme	0.009	0.91	0.92	0.89	0.009
Enol-lactone hydrolase	< 0.02	0.78	0.82	0.96	< 0.02
Transferase	0.002	0.20	0.23	0.19	0.002
Pyrocatechase	< 0.008	0.086	0.038	0.042	0.02
<i>m</i> -Pyrocatechase	< 0.004	< 0.004	—	—	0.57
Muconate-lactonizing enzyme	< 0.002	< 0.002	—	—	< 0.002
Muconolactone isomerase	< 0.01	< 0.01	—	—	< 0.01

Of the enzymes assayed, the only one present at high values in benzoate-grown organisms was *m*-pyrocatechase. It should be noted that muconate-lactonizing enzyme, muconolactone isomerase, enol-lactone hydrolase and transferase, all required for the dissimilation of catechol through the β -ketoadipate pathway, were not measurably induced by growth with benzoate; this showed that benzoate dissimilation must proceed, in all three species, exclusively through the *meta* cleavage pathway.

Properties of Azotobacter chroococcum p β -203, a permeability mutant

Wild-type organisms of the three *Azotobacter* strains studied cannot grow with β -ketoadipate, and respirometric data showed that *p*-hydroxybenzoate-grown organisms were impermeable to β -ketoadipate and β -carboxy-*cis,cis*-muconate. Spontaneous mutants with increased permeability to β -ketoadipate were obtained from *Azotobacter chroococcum* strain 203. The respiratory behaviour of one of these mutants, *p β -203*, showed that mutation had rendered it permeable to β -ketoadipate but not to β -carboxy-*cis,cis*-muconate (Fig. 1).

Table 7 shows the values for protocatechuate pathway enzymes in crude extracts of mutant *p β -203* grown with succinate and with β -ketoadipate. The enzymic constitution of *p β -203* after growth with succinate was identical with that of the wild type

grown with the same substrate. Growth of mutant $p\beta$ -203 with β -keto adipate elicited high transferase and enol-lactone hydrolase values. The value of carboxymuconate-lactonizing enzyme increased about 3-fold over the basal value, but this increase was probably not significant. p -Hydroxybenzoate hydroxylase and protocatechuate-3,4-oxygenase were not induced by growth with β -keto adipate.

Fig. 1. The rates of oxidation of p -hydroxybenzoate, protocatechuate, β -carboxy-*cis,cis*-muconate and β -keto adipate by cells of *Azotobacter chroococcum* strain 203 (a) and a permeability mutant, strain $p\beta$ -203 (b) both grown on p -hydroxybenzoate.

Table 7. Enzyme values in cell-free extracts of *Azotobacter chroococcum* strain $p\beta$ 203, a permeability mutant, after growth on β -keto adipate and succinate

Enzyme	Substrate used for growth	
	Succinate (20 mM)	β -Keto adipate (10 mM)
Enzyme units/mg. protein in cell-free extracts		
p -Hydroxybenzoate hydroxylase	< 0.001	< 0.001
Protocatechuate 3,4-oxygenase	< 0.003	< 0.003
Carboxymuconate lactonizing enzyme	0.009	0.027
Enol-lactone hydrolase	< 0.02	1.17
Transferase	0.0025	0.30

Induced enzymic activities in vegetative forms and cysts

Both *Azotobacter chroococcum* and *A. beijerinckii* produce the specialized resting forms known as cysts (Winogradsky, 1938). A culture of *A. beijerinckii* strain v, growing with p -hydroxybenzoate, was harvested after massive cyst formation had occurred, and the activities of four inducible enzymes were assayed in parallel with assays of the same enzymes in vegetative forms (Table 8). It can be seen that relatively high values of these four enzymes (about half the values characteristic of vegetative forms) were found in the cysts.

Co-factor requirements of p-hydroxybenzoate hydroxylase

Experiments made with crude extracts of *p*-hydroxybenzoate-grown organisms (Table 9) suggested that the *p*-hydroxybenzoate hydroxylase of the three *Azotobacter* species is a NADPH-dependent oxygenase. The enzyme was wholly inactive with NADH. As in the case of the *p*-hydroxybenzoate hydroxylase of *Pseudomonas putida* (studied by Hosokawa & Stanier, 1966), FAD was a co-factor and seemed to be tightly bound to the enzyme; FMN was inhibitory. In tris HCl buffer (pH 8.0) was the optimal value for enzyme activity.

Table 8. *Relative activities of the enzymes of the p-hydroxybenzoate pathway in cell-free extracts of rod-shaped vegetative forms and cysts of Azotobacter beijerinckii strain v grown on p-hydroxybenzoate*

Activities in cell-free extracts of vegetative forms are taken as 100

Enzyme	Relative activity in crude extract of:	
	Vegetative forms	Cysts
<i>p</i> -Hydroxybenzoate hydroxylase	100	48
Protocatechuate 3,4-oxygenase	100	56
Carboxymuconate lactonizing enzyme	100	60
Enol-lactone hydrolase	100	54

Table 9. *Effect of reduced pyridine nucleotide and flavine derivatives on activity of p-hydroxybenzoate hydroxylase in crude extracts of p-hydroxybenzoate-grown organisms**

Azotobacter strains		Relative activities† of <i>p</i> -hydroxybenzoate hydroxylase under the standard assay conditions with					
		NADPH	NADH	NADPH + FAD	NADH + FAD	NADPH + FMN	NADH + FMN
<i>A. chroococcum</i>	strain 203	84	6	100	9	13	0
<i>A. vinelandii</i>	strain 206	85	4	100	5	17	0
<i>A. beijerinckii</i>	strain v	96	3	100	3	17	0

* Assay conditions were the same as described by Hosokawa & Stanier (1966).

† Activities under standard assay conditions with NADPH + FAD are taken as 100.

Table 10. *The stoichiometry of α -hydroxymuconic semialdehyde formation from catechol by cell-free extracts of Azotobacter strains grown with benzoate*

Experimental conditions were the same as described in Methods except that 100 mM-sodium potassium phosphate buffer (pH 7.5) was used; 5 μ mole catechol and 0.1 ml. of cell-free extract (equiv. 10 mg. protein/ml.) were used. The quantity of product formed was determined from absorbance at 375 m μ .

Azotobacter strains		Micromoles		
		Catechol provided	Oxygen consumed	α -Hydroxymuconic semialdehyde formed
<i>A. chroococcum</i>	strain 203	5.0	4.6	4.8
<i>A. vinelandii</i>	strain 206	5.0	4.7	5.04
<i>A. beijerinckii</i>	strain v	5.0	4.5	4.7

Stoichiometry of the reaction catalyzed by m-pyrocatechase

Crude cell-free extracts of all three *Azotobacter* strains grown with benzoate catalysed a quantitative *meta* cleavage of catechol, with accumulation of α -hydroxymuconic semialdehyde, the immediate product (Table 10). We did not attempt to detect or assay any subsequent enzymes of the *meta* cleavage pathway.

DISCUSSION

The results reported here show that the three species of the *Azotobacter* group capable of utilizing benzoate and *p*-hydroxybenzoate metabolize these two acids through completely different metabolic pathways. The β -ketoadipate pathway, used by fluorescent pseudomonads (Stanier *et al.* 1966) and oxidase-negative moraxellas (Cánovas & Stanier, 1967) for the dissimilation of both these substrates, serves in the *Azotobacter* group only for the dissimilation of *p*-hydroxybenzoate and of the metabolically related hydro-aromatic acid, shikimate, which is also dissimilated through the protocatechuate branch of the β -ketoadipate pathway. Benzoate, on the other hand, is metabolized, after conversion to catechol, exclusively through the *meta* cleavage pathway. None of the enzymes operative in catechol dissimilation through the β -ketoadipate pathway is induced in benzoate-grown *Azotobacter* organisms. This metabolic pattern has not been reported in any other group of aerobic bacteria capable of growth at the expense of benzoate and *p*-hydroxybenzoate, and therefore appears to constitute a taxonomically significant biochemical property of the *Azotobacter* group.

As in all other bacteria so far examined, the utilization by the *Azotobacter* group of aromatic compounds is mediated by strictly inducible enzymes. Our observations on the patterns of induction, though preliminary, suggest that the regulation of the enzymes of the β -ketoadipate pathway in *Azotobacter* may be distinct from the patterns of regulation described in fluorescent pseudomonads (Ornston, 1966*c*; Kemp & Hegeman, 1968) and in *Acinetobacter* (*Moraxella*) *calcoacetica* (Cánovas & Stanier, 1967). The properties of a permeability mutant of *Azotobacter chroococcum* suggest that β -ketoadipate can act as an inducer of β -ketoadipate enol-lactone hydrolase and β -ketoadipate succinyl-CoA transferase, but not of carboxymuconate lactonizing enzyme. In *Acinetobacter calcoacetica* β -ketoadipate induces none of these enzymes; in *Pseudomonas putida* it induces all three.

Our data indicate that the ability to utilize benzoate, *p*-hydroxybenzoate and shikimate as sources of carbon + energy is a useful nutritional character for distinguishing *Azotobacter chroococcum*, *A. vinelandii* and *A. beijerinckii* from *A. agilis*, which can utilize none of them. We have also found that the *Beijerinckia* strains examined are unable to use these aromatic and hydro-aromatic components as growth substrates.

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