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The Conversion of Catechol and Protocatechuate to β-Ketoadipate by *Pseudomonas putida*

III. ENZYMES OF THE CATECHOL PATHWAY*

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SUMMARY

Extracts of benzoate-grown *Pseudomonas putida* have high levels of β-carboxy-*cis*,*cis*-muconate-lactonizing enzyme and γ-carboxymuconolactone decarboxylase, even though these enzymes are uniquely associated with the protocatechuate pathway. Since these activities might result from nonspecific catalysis by the enzymes which catalyze the analogous reactions in the catechol pathway, *cis*,*cis*-muconate-lactonizing enzyme and muconolactone isomerase were purified extensively and their specificities were examined. Crystalline preparations had no detectable activity on the carboxylated substrate analogues of the parallel convergent pathway. The enzymes of the catechol pathway do, however, share some physical characteristics with the enzymes that catalyze the analogous reactions in the protocatechuate pathway.

Extracts of benzoate-grown *Pseudomonas putida* rapidly convert β-carboxy-*cis*,*cis*-muconate and γ-carboxymuconolactone to β-ketoadipate (1). These activities, which are uniquely associated with the protocatechuate pathway (2), might be attributed (*a*) to synthesis of β-carboxy-*cis*,*cis*-muconate-lactonizing enzyme and γ-carboxymuconolactone decarboxylase elicited by growth at the expense of catechol, or (*b*) to nonspecific catalysis by *cis*,*cis*-muconate-lactonizing enzyme and muconolactone isomerase, which mediate analogous reactions in the catechol pathway (2). The purpose of this paper is to present evidence which eliminates the latter interpretation. *cis*,*cis*-Muconate-lactonizing enzyme and muconolactone isomerase have been obtained in crystalline form and shown to have no activity on the carboxylated substrate analogues of the parallel catabolic pathway.

EXPERIMENTAL PROCEDURE

Materials and Methods

Biological and chemical materials, physical and chemical determinations, and molecular weight estimations have been previously described (3). Buffer D contained 5 mM disodium potassium phosphate, pH 6.8, and 5 μM Mg-EDTA.

Enzyme Assays

All enzymes were assayed spectrophotometrically at 25°. Reactions were initiated by addition of the enzyme the activity of which was to be determined. A unit is defined as the amount of enzyme necessary to cause disappearance of 1.0 μmole of substrate per min under the conditions of assay.

cis,*cis-Muconate-lactonizing Enzyme*—A modification of the procedure of Sistrom and Stanier (4) was used. Each cuvette received 0.3 μmole of *cis*,*cis*-muconate, 2 μmoles of $MnCl_2$, 100 μmoles of Tris-HCl (pH 8.0), and enzyme in a final volume of 3.0 ml. The blank cuvette contained no enzyme. A decrement of 5.75 absorbance units corresponds to the disappearance of 1.0 μmole of *cis*,*cis*-muconate per cuvette under these conditions.

Muconolactone Isomerase—Muconolactone isomerase catalyzes the migration of the endocyclic double bond of (+)-muconolactone to form β-ketoadipate enol-lactone (2). Neutral solutions of the substrate and the product of this enzyme absorb equally at 230 mμ. However, the isomerase can be measured in the presence of an excess of β-ketoadipate enol-lactone hydrolase because β-ketoadipate, the product of hydrolysis, is relatively transparent at 230 mμ.

A complication was introduced by the presence of *cis*,*cis*-muconate-lactonizing enzyme in crude extracts of benzoate-grown cells. This enzyme catalyzes the back-reaction of (+)-muconolactone to form *cis*,*cis*-muconate, which absorbs very strongly at 230 mμ. This difficulty was circumvented by adding pure *cis*,*cis*-muconate-lactonizing enzyme and measuring the rate of (+)-muconolactone disappearance from the equilibrium mixture. Muconolactone isomerase can be measured accurately as long as neither *cis*,*cis*-muconate-lactonizing enzyme nor β-ketoadipate enol-lactone hydrolase is limiting the rate of the disappearance of absorbance at 230 mμ.

For rough estimations, a crude extract of *p*-hydroxybenzoate-grown *P. putida* is a satisfactory source of β-ketoadipate enol-lactone hydrolase. Such extracts contain insignificant amounts

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TABLE I

Separation of enzymes of catechol and protocatechuate pathways from protamine sulfate supernatant of benzoate-grown P. putida by ammonium sulfate fractionation

Fraction	*cis,cis*-Muconate-lactonizing enzyme	Muconolactone isomerase	β-Carboxy-*cis,cis*-muconate-lactonizing enzyme	γ-Carboxymuconolactone decarboxylase	Protein	Volume
	µmoles/min				*g*	*ml*
Protamine sulfate supernatant	6,960	17,550	14,780	28,620	12.6	606
Ammonium sulfate (% saturated)						
30 to 40	5,230	414	2,110	326	0.75	68
40 to 50	382	2,040	8,180	10,630	1.82	91
50 to 60	8	12,600	3,850	13,650	3.73	175
60 to 70	<4	2,110	34	109	0.51	68
Total recovery (%)	81	98	96	86	54	

TABLE II

Purification of cis,cis-muconate-lactonizing enzyme

Step	Volume	Total activity	Protein	Specific activity	Recovery	Purification
	ml	*units*	*mg*	*units/mg protein*	*%*	*-fold*
1. Crude extract	282	10,300	15,800	0.64	100	1.0
2. Protamine sulfate supernatant	606	6,960	12,600	0.55	67.5	0.9
3. 30 to 40% saturated ammonium sulfate fraction	68	5,230	748	6.98	50.8	10.9
4. DEAE-cellulose eluate	102	1,560	20.4	76.6	15.2	120
5. Crystallization	8.2	1,051	12.4	84.5	10.2	132
6. Recrystallization	5.0	740	8.5	87.3	7.2	136

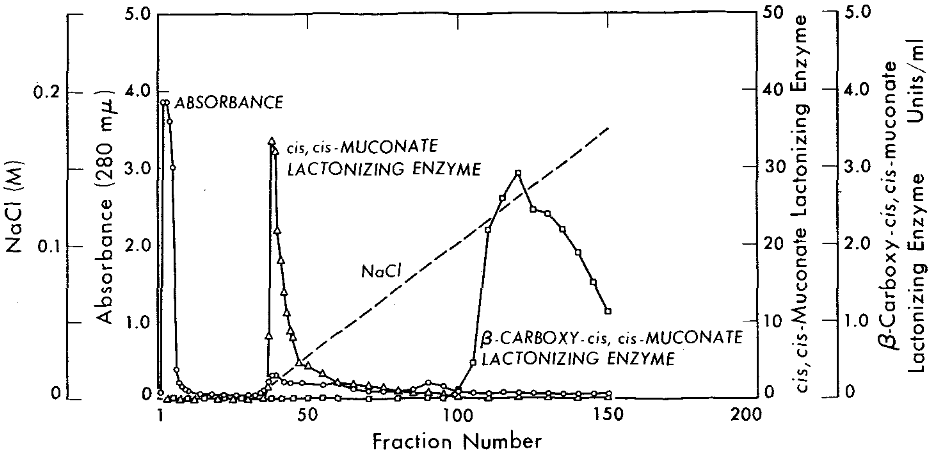

FIG. 1. DEAE-cellulose chromatography of *cis,cis*-muconate-lactonizing enzyme

of muconolactone isomerase (1). Accurate measurements, however, require the use of highly purified β-ketoadipate enol-lactone hydrolase, because crude extracts absorb strongly at 230 mµ. Enzyme levels of muconolactone isomerase reported here and in Paper IV (1) were determined with β-ketoadipate enol-lactone hydrolase which had been purified at least 200-fold (3).

Each cuvette received 100 µmoles of Tris-HCl, pH 8.0, 1.5 µmoles of (+)-muconolactone, 1.0 unit of crystalline *cis,cis*-muconate-lactonizing enzyme, and 1.0 unit of β-ketoadipate enol-lactone hydrolase in a final volume of 2.9 ml. The lactonization reaction was permitted to come to equilibrium and then 0.1 ml of the solution containing muconolactone isomerase was added; the blank cuvette received no muconolactone isomerase. Under these conditions, a change of 0.683 absorbance unit at 230 mµ corresponds to the conversion of 1.0 µmole of (+)-muconolactone to β-ketoadipate per cuvette. This assay was accurate for measurement of up to 0.2 unit of muconolactone isomerase. When *cis,cis*-muconate-lactonizing enzyme was not in the extract to be assayed, purified lactonizing enzyme was omitted from the reaction mixture. Under these circumstances a decrease of 0.476 absorbance unit at 230 mµ corresponds to the disappearance of 1.0 µmole of (+)-muconolactone per cuvette (4).

Other assays were performed according to procedures described in Paper II (3).

RESULTS AND DISCUSSION

Extracts of benzoate-grown *P. putida* were prepared, treated with protamine sulfate, and fractionated with ammonium sulfate according to previously described procedures (3). The fractionation of *cis,cis*-muconate lactonizing enzyme, muconolactone isomerase, β-carboxy-*cis,cis*-muconate lactonizing enzyme and γ carboxymuconolactone decarboxylase from the protamine sulfate supernatant of benzoate-grown *P. putida* is shown in Table I. The 30 to 40% saturated ammonium sulfate fraction, which contained almost all of the *cis,cis*-muconate-lactonizing activity, was used for further purification of this enzyme. The 50 to 60% saturated ammonium sulfate fraction was used for further purification of muconolactone isomerase. It should be noted that since β-carboxy-*cis,cis*-muconate-lactonizing enzyme and γ-carboxymuconolactone decarboxylase are present in high levels in the 40 to 50% saturated ammonium sulfate fraction, this fraction can serve as a satisfactory source of these enzymes for further purification.

Purification of cis,cis-Muconate-lactonizing Enzyme (EC 5.5.1.1, 4-Carboxymethyl-4-hydroxyisocrotonolactone Lyase (Decyclizing))—Sistrom and Stanier (4) accomplished a 15-fold purification of this enzyme by protamine sulfate treatment and ammonium sulfate fractionation. Introduction of diethylaminoethyl cellulose chromatography into their procedure permitted crystallization of the enzyme.

The results of the purification procedure are summarized in Table II. The 30 to 40% saturated ammonium sulfate fraction (Step 3) was dialyzed against Buffer D overnight and placed on a DEAE-cellulose column, 2.5 × 35 cm, which had been preequilibrated with the same buffer. The column was washed with 300 ml of Buffer D; the enzyme was eluted with 1600 ml of the same buffer which contained NaCl in a linear gradient from 0.00 to 0.20 M. The phosphate concentration and the pH were kept constant during the gradient elution. Fractions of 11.8 ml were collected. Fig. 1 shows that *cis,cis*-muconate lactonizing enzyme was completely separated from β-carboxy-*cis,cis*-muconate-lactonizing enzyme by this treatment. Fractions 37 through 45, which contained most of the *cis,cis*-muconate-lactonizing activity were pooled (DEAE-cellulose eluate, Step 4).

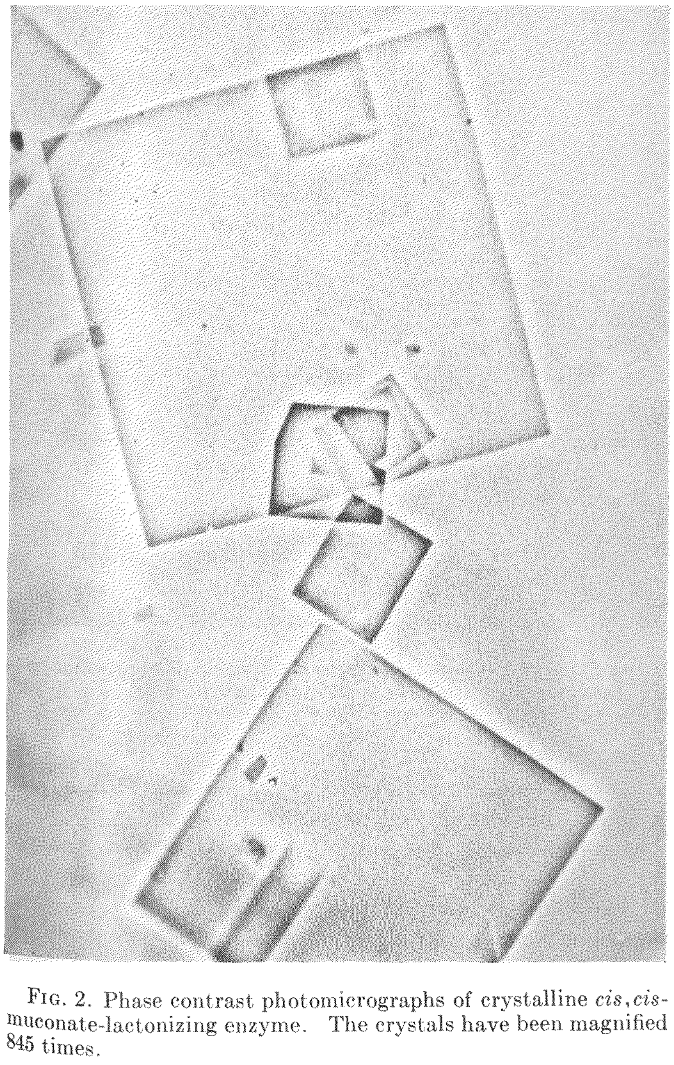

FIG. 2. Phase contrast photomicrographs of crystalline *cis,cis*-muconate-lactonizing enzyme. The crystals have been magnified 845 times.

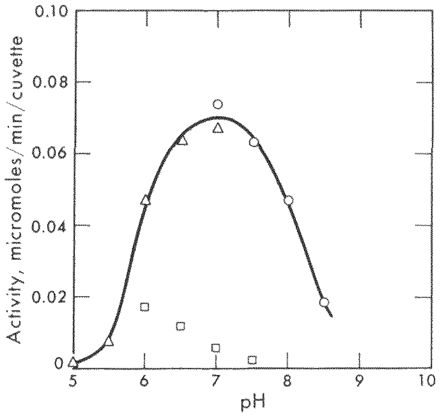

FIG. 3. The effect of pH and buffer on the activity of *cis,cis*-muconate-lactonizing enzyme. Buffers are indicated as follows: ○, 0.033 M Tris-HCl; □, 0.033 M disodium potassium phosphate; △, 0.033 M DL-histidine-HCl.

A 35 to 50% saturated ammonium sulfate fraction of the DEAE-cellulose eluate was prepared and suspended in 8.2 ml of Buffer D. The protein did not dissolve completely, but the suspension became extremely birefringent after about 20 min. When a drop of this suspension was mixed with a drop of saturated ammonium sulfate, pH 7.5, the formation of thin square plates was observed microscopically. The plates were not observed in the absence of ammonium sulfate, probably because the crystals dissolved when they were warmed on the microscope stage.

The crystals were dissolved in 10.0 ml of Buffer D and an equal volume of saturated ammonium sulfate was added to precipitate the enzyme. The precipitated protein was birefringent when suspended in 4.0 ml of Buffer D. After addition of 1.0 ml of saturated ammonium sulfate, pH 7.5, the suspension was allowed to stand overnight at 0°. The plates which were formed by this treatment (Step 6) were free of amorphous material. A photomicrograph, showing the shape and the range of sizes of recrystallized *cis,cis*-muconate-lactonizing enzyme is shown in Fig. 2. Starch gel electrophoresis of the recrystallized material in the discontinuous buffer system of Poulick (5) revealed a single band of protein.

Properties of cis,cis-Muconate-lactonizing Enzyme—The influence of pH on the activity of *cis,cis*-muconate-lactonizing enzyme, shown in Fig. 3, is similar to the influence of pH on the activity of β-carboxy-*cis,cis*-muconate-lactonizing enzyme (3). At pH 6.5, both enzymes are considerably less active in phosphate buffer than in histidine buffer. *cis,cis*-Muconate-lactonizing enzyme, like β-carboxy-*cis,cis*-muconate-lactonizing enzyme, is not detectably affected by heating for 10 min at 60° in 20 mM disodium potassium phosphate buffer, pH 6.8. The molecular weight of *cis,cis*-muconate-lactonizing enzyme was estimated by the method of Whitaker (6) to be 220,000.

Purification of Muconolactone Isomerase—Sistrom and Stanier

TABLE III

Purification of muconolactone isomerase

Step	Volume	Total activity	Protein	Specific activity	Recovery	Purification
	ml	*units*	*mg*	*units/mg protein*	*%*	*-fold*
1. Crude extract	282	20,000	15,800	1.26	100	1.0
2. Protamine sulfate supernatant	606	17,550	12,600	1.39	87.8	1.1
3. 50 to 60% saturated ammonium sulfate fraction	175	12,600	3,730	3.38	63.0	2.7
4. Heat-treated supernatant	140	11,200	2,190	5.12	56.0	4.1
5. Second 50 to 60% saturated ammonium sulfate fraction	15.6	9,590	289	33.1	48.0	26.3
6. DEAE-cellulose eluate	46.3	5,270	27.8	189.5	26.4	150.3
7. Crystallization	2.0	4,224	6.0	707	21.1	562
8. Recrystallization	2.0	3,860	5.6	695	19.3	552

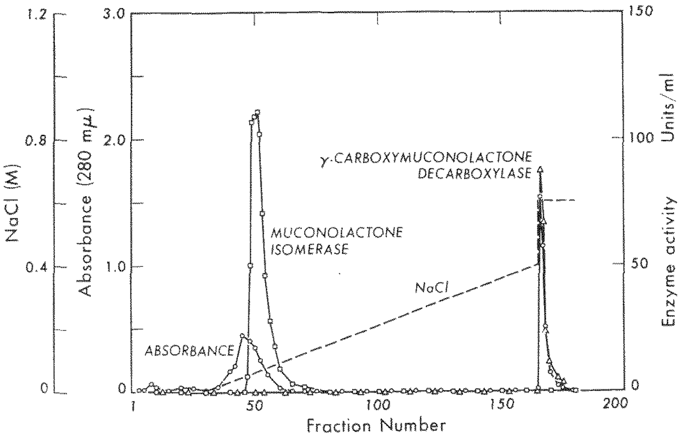

FIG. 4. DEAE-cellulose chromatography of muconolactone isomerase.

(4) purified muconate-delactonizing enzyme (EC 3.1.1.16, 4-carboxymethyl-4-hydroxyisocrotonolactone hydrolase) from *P. putida* 5.1-fold by use of protamine sulfate and ammonium sulfate fractionation. Subsequent studies (2) showed that both muconolactone isomerase and β-ketoadipate enol-lactone hydrolase (3) are essential for the "delactonizing activity" observed by Sistrom and Stanier. Application of heat treatment and DEAE-cellulose chromatography permitted the crystallization of the first enzyme of this two-enzyme system.

The results of muconolactone isomerase purification are summarized in Table III. The 50 to 60% ammonium sulfate fraction (Step 3) was further purified by heat treatment. A beaker containing this fraction was placed in a water bath at 60° and heated, with continuous stirring, until the temperature of the preparation reached 55°. The preparation was maintained at this temperature for 30 min and then rapidly chilled in an ice bath. The suspension was centrifuged at 35,000 × *g* for 15 min. The supernatant from this centrifugation (Step 4) was treated with solid ammonium sulfate; the 50 to 60% saturated ammonium sulfate fraction was dissolved in Buffer D and dialyzed against this buffer overnight (Step 5). The dialyzed material was placed on a DEAE-cellulose column, 2.5 × 21 cm, which had been pre-equilibrated with Buffer D. The column was washed with 300 ml of this buffer and then the muconolactone isomerase was eluted with 1600 ml of Buffer D which contained NaCl in a linear gradient from 0.00 to 0.40 M. The pH and phosphate concentration were kept constant during the gradient elution. After the gradient elution was completed, γ-carboxymuconolactone decarboxylase was eluted from the column with Buffer D containing 0.60 M NaCl.

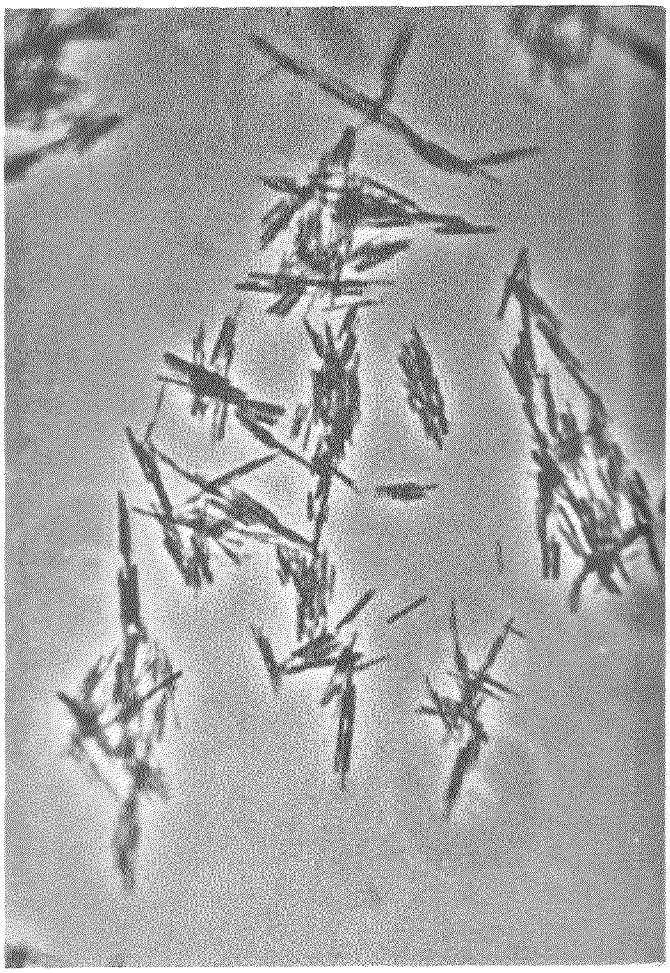

FIG. 5. A phase contrast photomicrograph of crystalline muconolactone isomerase. The crystals have been magnified 1900 times.

Muconolactone isomerase and γ-carboxymuconolactone decarboxylase were completely separated from each other by DEAE-cellulose column chromatography (Fig. 4). Fractions containing most of the muconolactone isomerase activity (Fractions 49 through 52 in Fig. 4) were pooled (DEAE-cellulose eluate, Step 6). The 50% saturated ammonium sulfate fraction of the DEAE-cellulose eluate was suspended in 2.0 ml of Buffer

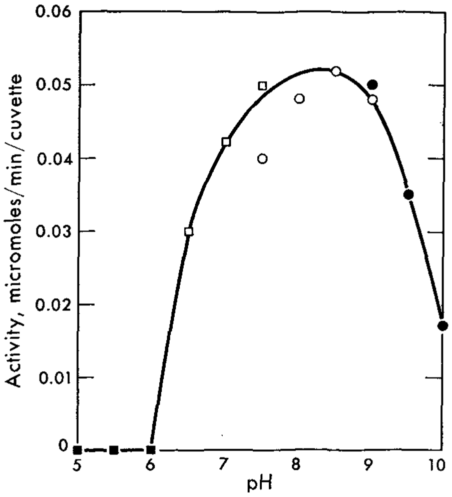

FIG. 6. The effect of pH and buffer on the activity of muconolactone isomerase. Buffers are indicated as follows: ●, 0.033 M sodium glycine; ○, 0.033 M Tris-HCl; □, 0.033 M disodium potassium phosphate; ■, 0.033 M sodium succinate.

D. After 30 min, the suspension was extremely birefringent. When a drop of the suspension was mixed with an equal volume of saturated ammonium sulfate, pH 7.5, needles were observed microscopically. The crystalline preparation (Step 7) was dissolved by addition of 4.0 ml of Buffer D, and 6.0 ml of saturated ammonium sulfate, pH 7.5, were added. The birefringent suspension was allowed to stand for 1 hour and was then recentrifuged. The pellet was resuspended in 2.0 ml of Buffer D which was 50% saturated in ammonium sulfate and allowed to stand overnight. A photomicrograph of the product of this step (Step 8) is shown in Fig. 5. A single band of protein was observed after starch gel electrophoresis of the recrystallized material in the discontinuous buffer system of Poulick (5).

Properties of Muconolactone Isomerase—Muconolactone isomerase has a half-life of about 2½ hours at 55° in 20 mM disodium potassium phosphate buffer, pH 6.8. In this respect the enzyme is similar to γ-carboxymuconolactone decarboxylase of the protocatechuate pathway (3). Both enzymes are rapidly inactivated at 65°. The relationship between pH and the activity of muconolactone isomerase is shown in Fig. 6. The molecular weight of muconolactone isomerase was estimated by the method of Whitaker (6) to be 93,000.

Specificity of cis,cis-Muconate-lactonizing Enzyme and Muconolactone Isomerase—Crystallization of *cis,cis*-muconate-lactonizing enzyme and muconolactone isomerase permitted a rigorous examination of their specificities. Under standard assay conditions, 1 unit of *cis,cis*-muconate-lactonizing enzyme caused disappearance of no more than 0.05 mμmole of β-carboxy-*cis,cis*-muconate per min; 1 unit of muconolactone isomerase caused disappearance of no more than 1.0 mμmole of γ-carboxymuconolactone per min. These facts show that β-carboxy-*cis,cis*-muconate-lactonizing enzyme and γ-carboxymuconolactone decarboxylase activities observed in extracts of benzoate-grown cells (1) cannot be attributed to nonspecific catalysis by *cis,cis*-muconate-lactonizing enzyme and muconolactone isomerase.

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