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FURTHER STUDIES ON PYROCATECASE*

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In a previously reported experiment in this laboratory (*1*, *2*), a cell-free enzyme, pyrocatecase, was isolated and fairly purified from the acetone-dried cells of a strain of *Pseudomonas sp.*, which was adapted to anthranilic acid. This enzyme catalyzes the oxidative rupture of benzene ring of catechol, yielding *cis-cis* muconic acid as the end product. Stanier *et al.* (*3*) showed that the acetone powder of a strain of *Pseudomonas*, grown on benzoic acid, had the enzymatic activity on catechol oxidation, and they isolated and identified β-ketoadipic acid as the end product of this reaction. The enzyme preparation used by them was the suspension of the dried cells, while in our experiments a fairly purified enzyme was used. The oxygen uptake was the same in both cases (one mole of oxygen per mole of the substrate), while the end products were different.

It is reasonable to consider that catechol may be converted to β-ketoadipic acid via *cis-cis* muconic acid. For these reasons, we re-examined our earlier experiments and obtained some interesting results concerning the nature of pyrocatecase.

EXPERIMENTAL

Material—As reported in the previous paper (*1*), we used as the source of enzyme the acetone dried cells of *Pseudomonas* grown on media containing 0.2 per cent anthranylic acid for 12 hours.

Preparation of the enzyme—The purification of the enzyme was somewhat modified and simplified as compared with that reported previously. One gram of the acetone-dried cells was suspended in 50 ml. of *M*/15 phosphate buffer (pH 8.0), stored in an ice chest for 24 hours, and centri-

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fuged at 17000 r.p.m. for 20 minutes, the supernatant being used as the enzyme A (adjusted at pH 7.2). The enzyme A solution was then fractionated with 0.3 and 0.6 saturated ammonium sulfate and the precipitate formed was dissolved in 5 ml. of *M*/5 glycylglycine buffer (pH 7.4). This solution was used as the enzyme B. This enzyme solution was further dialyzed against distilled water for over 20 hours in a collodion bag. This solution was used as the enzyme C.

Measurement of the Enzymatic Reaction—Oxidation of the catechol was measured by the usual manometric technique in Warburg vessel at 38°. β-Ketoadipic acid was determined by the aniline citric acid method (*3*, *4*).

The Nature of Pyrocatecase—Ferrous ion as the components of this enzyme:

Both the enzymes A and B oxidize catechol consuming oxygen always in theoretical amount. But the enzyme C obtained does not oxidize catechol completely. This fact does not accord with our early report. (*1*)

It is, however, very intersting that, as shown in Fig. 1 the enzyme C restores its full activity in the presence of ferrous sulfate (end concentration $10^{-3}M$). Ferric ion and other metallic ions such as Zn^{++}, Mn^{++}, Co^{++}, and Mg^{++} were found to be incapable of replacing the ferrous ion in causing to the full restoration of the enzyme activity. These are shown in Figs. 1 and 2.

From the above experiments, it is realized that one of the components of pyrocatecase is ferrous ion. In respect to the necessity of ferrous ion for the enzyme action, the enzyme pyrocatecase is analoguous to the homogentisicase reported earlier in this laboratory (*5*). In the case of latter enzyme, it was very remarkable that αα'-dipyridyl inhibited the enzyme action immediately after the addition of this reagent. But in the case of pyrocatecase, αα'-dipyridyl exerted no inhibiting action at all in all the stages of enzyme purification, *i. e.* in the enzymes A, B, and C. If enzyme solution C was mixed previously with αα'-dipyridyl (end concentration, $3 \times 10^{-3}M$) and then supplied with both ferrous sulfate and substrate at the same time, total inhibition of enzyme reaction was observed.

It may be supposed that in this case the enzyme combines with ferrous ion forming a more hardly dissociable complex than in the case of homogentisicase, but a slowly dissociable one on long lasting dialysis. These are shown in Fig. 3 and Table I.

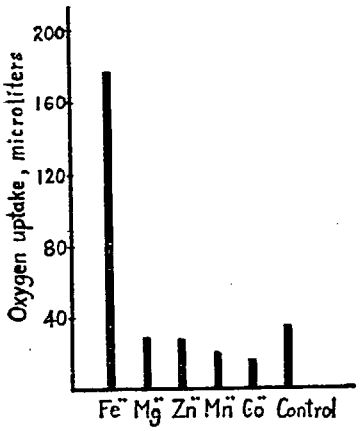

FIG. 1

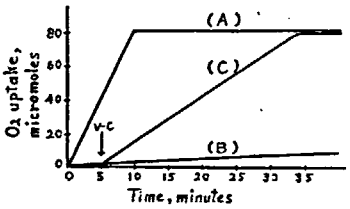

FIG. 2

FIG. 1. The effect of metallic ions on the catechol oxidation by enzyme C solution.

Each ion was used as sulfate.
Main compartment:
Enzyme C solution 1.0 ml.
M/15 phosphate buffer 1.3 ml.
M/100 metalic sulfate 0.3 ml.
(control: no addition of any above metallic ions.)
Side arm:
M/50 catechol 0.4 ml.
The columns show the amount of oxygen uptake in 30 minutes.

FIG. 2. The effect of iron ions on the catechol exidation by enzyme C.
Main compartment:
Enzyme C solution 1.0 ml.
M/15 phosphate buffer 0.6 ml.
M/100 ferrous sulfate 0.2 ml. (Curve A)
,, ferric sulfate 0.2 ml. (Curves B and C)
Side arm I
M/50 catechol 0.2 ml.
Side arm II
M/50 ascorbic acid 0.2 ml. (Curve C)
(This reagent was tipped in 5 minutes after the start.)

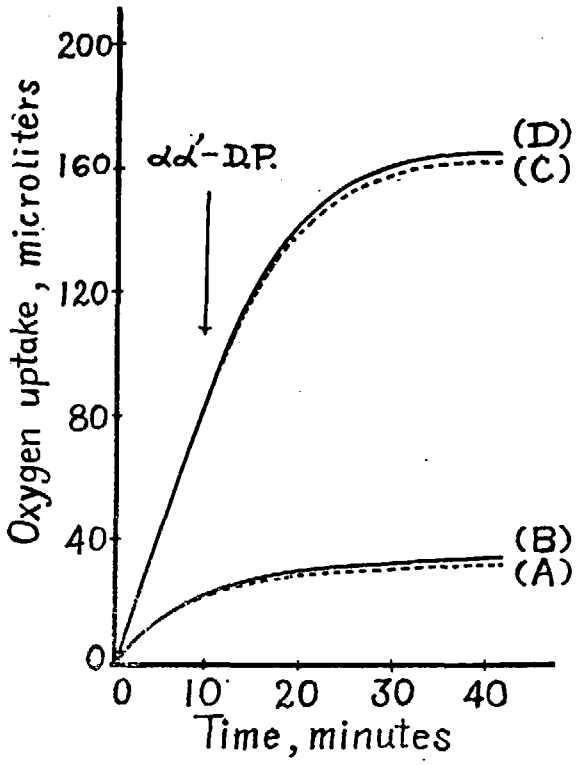

FIG. 3. The effect of $\alpha\alpha'$-dipyridyl (D. P.) on the pyrocatecase activity.

Enzyme C solution, 1.0 ml.; *M*/15 phosphate buffer (pH 7.4), 1.0 ml.; *M*/100 $FeSO_4$, 0.3 ml.; *M*/30 D. P., 0.3 ml.; *M*/50 catechol, 0.4 ml. Curve A: Enzyme, buffer, and substrate were mixed without ferrous sulfate. Curve B: Enzyme and buffer were previously mixed with D. P., and then substrate and ferrous sulfate were tipped in. Curve C: D. P. was added 10 minutes after the substrate was tipped. Curve D. (control). Enzyme, buffer, and ferrous sulfate were mixed previously, then substrate was tipped in at the start.

TABLE I

The Effect of Ferrous Ion on the Recovery of Enzyme Activity after Prolonged Dialysis

The volume of the enzyme solution was increased 4 to 4.8 times of the initial volume during prolonged dialysis, so the volumes of the enzyme solution used were corrected so as to have same amounts of enzyme protein to each other. With and without 0.3 ml. of *M*/100 ferrous sulfate. *M*/50 Catechol, 0.4 ml. (Side arm) Total volume was made to be 3.0 ml. with *M*/25 phosphate buffer (pH 7.2).

Time of dialysis	Volume of enzyme used	$FeSO_4$ added	O_2 uptake for 10 minutes
hours	*ml.*	*ml.*	*μl.*
0	0.25	0.3	115
	0.25	0	83
15	1.0	0.3	114
	1.0	0	41
30	1.25	0.3	112
	1.25	0	7

End products of the Enzyme Reaction on the Catechol Oxidation—Isolation of β-ketoadipic acid: β-ketoadipic acid was isolated according to the procedure of Stanier (*3*). We could thus obtain about 50 mg. of pure acid from 250 mg. of catechol. The properties of this crystal are identical with those of β-ketoadipic acid reported by Stanier. But when we used the fractionated and dialyzed enzyme, the end product was not identical with β-ketoadipic acid, but with *cis-cis* muconic acid.

Isolation of the end product of the purified enzyme reaction: The end product was isolated according to the method in our previous report (*1*) with slight modification. In this experiment the enzyme C was used. The composition of the reaction mixture was as follows:

Purified enzyme solution (enzyme C)	60 ml.
Catechol	250 mg.
M/15 phosphate buffer (pH 7.4)	60 ml.
M/50 $FeSO_4 \cdot 7H_2O$	8 ml.

The flask was shaken in a water bath at 38°. To avoid shifting of pH of the liquid towards acid side, diluted NaOH solution was pipetted in at intervals of 10 minutes. After the phenolic reaction in the small sample of fluid had decreased to a minimum value, the flask was removed from the water bath. The reaction mixture was shaken with chloroform several times, then conccented H_2SO_4 was added to bring

the pH of the fluid to 2.8, and the flask was cooled immediately with ice. The reaction mixture was extracted with ether and the etheral extract was treated as reported earlier. Fine needles appeared. Melting point: 174°.

This substance was hardly soluble in water, easily in alkali solution, soluble in ether and in alcohol. Phenolic reaction and Rothera's reaction were negative. No CO_2 was evolved by catalytic decarboxylation with aniline citrate. It may be supposed that it is not β-ketoadipic acid as in Stanier's report (*3*), but is *cis-cis* muconic acid as already reported.

Biochemical Behavior of the End Product Obtained and β-Ketoadipic Acid—β-Ketoadipic acid was not oxidized further by living cells adapted to catechol. Also the preparation (*cis-cis* muconic acid) obtained above was not further oxidized by the same living cells. (With living cell suspension seven or more atoms of oxygen per mole of substrate were measurable for the complete oxidation of catechol).

But it was remarkable that our preparation (*cis-cis* muconic acid) was converted to β-ketoadipic acid without consumption of molecular oxygen by the action of the acetone-dried preparation of adapted cells. This transformation could not be brought about by the acetone-dried preparation of non adaptive cells. This is shown in Table II.

It is, however, not known at present why these substances, such as β-ketoadipic acid and *cis-cis* muconic acid, are not oxidized by living cells adapted to catechol. One of the tentative suppositions may be the impermeability of the cell membranes.

DISCUSSION

It has been reported by Stanier as well as by us that catechol is an intermediate in the oxidation of benzoic acid or tryptophane and anthranilic acid, and in the course of the reaction benzene ring is ruptured, with the formation of dicarboxylic acid.

As the intermediate, Stanier obtained the same compound as synthesized β-ketoadipic acid with a crude enzyme, while we obtained *cis-cis* muconic acid with a purified enzyme.

It is natural to consider that *cis-cis* muconic acid lies on the metabolic pathway from catechol to β-ketoadipic acid. We can separate these enzyme reaction into two parts, one is the course from catechol to *cis-cis*-muconic acid, another is that from *cis-cis* muconic acid to β-ketoadipic acid.

TABLE II

The Formation of β-Ketoadipic Acid from cis-cis Muconic Aacid by Acetone Dried Preparation of Catechol Adapted and Non Adapted Cells.

A. For substrate oxidation—
Main compartment: 0.1 ml. of acetone-dried cell suspension (10 mg./1 ml. H_2O) or enzyme A solution; 1.0 ml. of *M*/15 phosphate buffer (pH 7.4).
Center cup: 0.2 ml. of 10 per cent KOH.
Side arm I: 0.2 ml. of *M*/50 catechol or isolated *cis-cis* muconic acid.
These were incubated for 30 minutes at 38° for the oxidation of the substrates, and then the formation of β-ketoadipic acid was determined as follows.

B. For catalytic decarboxylation by anilin citrate method (*4*)—
Main compartment: 0.3 ml. of 50 per cent citric acid was added.
Side arm II: 0.4 ml. of anilin citrate mixture.
(β-Ketoadipic acid produced was determined by CO_2 released.)

Enzyme	Substrate	O_2 uptake	CO_2 released
		μl.	*μl.*
Acetone-dried prepn. (adapted)	(1) catechol	86.4	81.0
	(2) *cis-cis* muconic acid	1.8	83.0
Cell-free prepn. (adapted)	(1) catechol	88.0	85.0
	(2) *cis-cis* muconic acid	0	88.5
Acetone dried prepn. (non adapted)	(1) catechol	7.0	0
	(2) *cis-cis* muconic acid	2.0	0

Theoretical value for the complete oxidation of catechol or the complete decarboxylation of β-ketoadipic acid is 90 μl.

In the process of purification of pyrocatecase, which comprises an exhaustive dialysis, we found that our earlier experiment had been incomplete. As reported above the enzyme "pyrocatecase" is one of the specific enzymes, having ferrous ion as an important component of the enzyme like "homogentisicase" (*5*) or "aconitase" (*6*).

The enzyme which catalyzes the conversion *cis-cis* muconic acid into β-ketoadipic acid is obtained in the cell-free state, but the nature of this enzyme is not known precisely at present except the facts that the enzyme reaction can be carried out without oxygen uptake and is inhibited by the presence of excess iron sulfate and by silver nitrate (end concentration, $10^{-4}M$).

SUMMARY

1. The further study of the pyrocatecase catalyzing breakdown of catechol has shown that one of the essential compentents of this enzyme is ferrous ion.

2. The end product of pyrocatecase reaction may be *cis-cis* muconic acid as reported earlier. By use of dried cells adapted to catechol, and cell extracts, *cis-cis* muconic acid was shown to be converted to β-ketoadipicacid.

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