[CONTRIBUTION FROM THE DEPARTMENT OF CHEMISTRY, COLUMBIA UNIVERSITY]

A Comparison of the Activities of Tyrosinase toward Phenol and *p*-Cresol

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When the tyrosinase contained in the aqueous extract from the common mushroom, *Psalliota campestris*, is subjected to the usual methods of purification, it suffers a greater loss in activity toward *p*-cresol than toward catechol. Due to this greater loss in activity toward *p*-cresol[1,2] and to the fact that only the activity toward catechol in such preparations is proportional to the copper content of the enzyme, certain workers are inclined to the view that the true enzyme is really a polyphenol oxidase and the monohydric phenol activity is due to some outside factor.

This conclusion appears to be too hasty. Recently Parkinson and Nelson, by altering the method of purification, have obtained highly purified preparations in which both the above-mentioned activities are proportional to the copper, and the ratio of the two activities resembles closely that of the fresh aqueous extract of the mushroom.

When phenol is used, instead of *p*-cresol, for following the monohydric phenol activity during the purification of the enzyme by the usual methods, then no greater loss in this activity occurs than the loss in activity toward catechol. Both the activity toward phenol and catechol run parallel and both are proportional to the copper content (Part B, Table I).

The tyrosinase preparations mentioned in Part A of Table I were like those described by Parkinson and Nelson, *i. e.*, both the activity toward *p*-cresol and toward catechol were proportional to the copper content. For convenience this kind of preparation has been termed a high cresolase preparation. On the other hand, the preparations mentioned in Part B were similar to those described by Keilin and Mann and by Ludwig and Nelson.[3] These preparations were low in *p*-cresol activity compared to the activity toward catechol and only the latter activity was proportional to the copper. Such preparations have been termed high catecholase preparations. As shown in Table I, the activity of both types of preparations toward phenol is proportional to the copper content. It is also interesting to note that the activity toward catechol is different in the two kinds of preparations. This activity in the case of the high cresolase preparations is only half of that in the high catecholase preparations, when based on the same amount of copper.

TABLE I[a]

SHOWING THAT THE PHENOLASE ACTIVITY OF THE TYROSINASE IS PROPORTIONAL TO THE CATECHOLASE ACTIVITY AND TO THE COPPER CONTENT OF THE ENZYME

Tyrosinase	Units Catecholase / Cresolase	Units Catecholase / Phenolase	Catecholase units per γ copper	Phenolase units per γ copper
		Part A		
$115A_0$	1.90	8.7	338	39.0
$114B_0$	2.00	8.9	350	39.4
$112B_2$	1.95	8.6	340	39.8
		Part B		
8–150	46.0	16.8	640	38.1
6–250	17.3	17.0	680	40.0
7– 50	12.2	16.9	710	42.0
9–100	7.0	17.1	695	40.6
110–150	4.8	16.8	650	38.7
113– 50	3.5	16.7	665	39.8

[a] The tyrosinase preparations $115A_0$, $114B_0$ and $112B_2$ (Part A) were high cresolase preparations described by Parkinson and Nelson. Those listed in Part B were high catecholase preparations similar to those described by Ludwig and Nelson. The method for the determination of copper was the same as that used by Parkinson and Nelson. The methods for determining the activities toward *p*-cresol and catechol were those described by Gregg and Nelson[6]. The activity toward phenol was determined by means of the Warburg respirometer. The reaction mixture was the same as that for *p*-cresol, except 4 mg. of phenol was used for high catecholase preparations and 20 mg. for high cresolase preparations, respectively.

Another difference between the high cresolase and the high catecholase preparations is the concentration of substrate necessary for the maximum rate of oxidation. As shown by the curves in Fig. 1, to permit a high cresolase preparation to attain its maximum activity, much higher concentrations of catechol or phenol are required than in the case of the high catecholase preparations. On the other hand, this difference between the two types of preparations does not occur when *p*-cresol is the substrate; 4 mg. of the latter was sufficient concentration, under the con-

(1) D. Keilin and T. Mann, *Proc. Roy. Soc.* (London), **B125**, 187 (1938).

(2) G. G. Parkinson and J. M. Nelson, THIS JOURNAL, **62**, 1693 (1940).

(3) B. J. Ludwig and J. M. Nelson, *ibid.*, **61**, 2601 (1939).

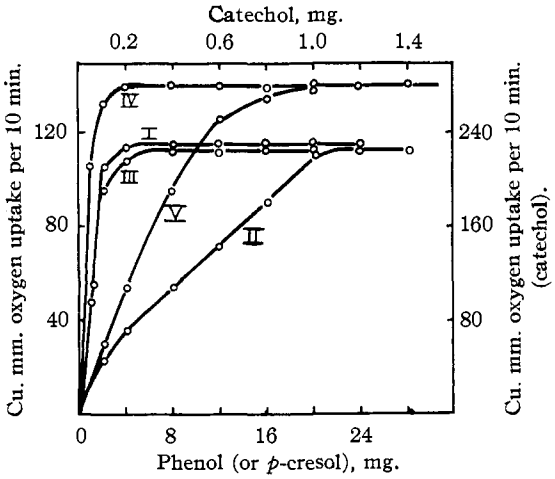

Fig. 1.—Showing the effect of substrate concentration on the rate of oxidation of phenol, *p*-cresol and catechol by given amounts of high cresolase and high catecholase preparations of tyrosinase. The rates of oxygen uptake in the oxidation of *p*-cresol and phenol were followed by means of the Warburg respirometer, using flasks of 30 cc. capacity; temp., 25°. Curve I: Reaction vessels contained 1 cc. of a high catecholase prepn. (1.15 phenolase, 1.6 cresolase and 19.5 catecholase units), 2 cc. (0.2 *M*) citrate–(0.4 *M*) phosphate buffer (*p*H of final mixture 7.1), 1 cc. gelatin soln. (5 mg.), aqueous phenol solutions of indicated concentrations and sufficient water to make the final volume 8 cc. Curve II: Same reaction mixture as for Curve I except 1 cc. of high cresolase prepn. (1.15 phenolase, 5.25 cresolase and 10.0 catecholase units) was used in place of the high catecholase preparation. Curve III: Same reaction mixture as for Curve I except 1 cc. high cresolase prepn. (1.15 cresolase and 2.20 catecholase units) used as the enzyme and solutions of *p*-cresol of the indicated concentrations replaced the phenol solutions. When a high catecholase prepn. (1.15 cresolase and 5.6 catecholase units) was used in this experiment, practically the same Curve III was obtained. The points on the two curves were well within 5% of each other. Curve IV: Barcroft respirometer used. Reaction vessels contained 1 cc. high catecholase prepn. (0.23 cresolase and 2.8 catecholase units), citrate–phosphate buffer, (*p*H 7.1), 1 cc. gelatin solution (5 mg.), aqueous solutions of catechol of indicated concentrations, 5–10 mg. imino-*d*-gluco-ascorbic acid (used to reduce *o*-quinone and thereby maintaining a constant concentration of catechol. The imino-*d*-gluco-ascorbic acid is not affected by the tyrosinase and exerts no inhibiting action) and sufficient water to make the final volume 8 cc. Curve V: Reaction mixture the same as for Curve IV except a high cresolase prepn. (1.5 cresolase and 2.8 catecholase units) was used in place of the high catecholase preparation.

dition of the experiments, to yield the maximum activity, when either a high cresolase or high catecholase preparation was used.

It was pointed out by Bordner and Nelson[4] that tyrosinase can only bring about the oxidation of *p*-cresol if at the same time some *o*-dihydric phenol, such as catechol, is being oxidized by the enzyme. This same, so to speak priming effect, caused by the simultaneous oxidation of an *o*-dihydric phenol, is also necessary when tyrosinase acts on phenol. Curve II in Fig. 2 represents the rate of oxygen uptake when phenol was oxidized by 0.7 unit of phenolase, in the form of a high cresolase preparation, and Curve III by 0.7 unit of phenolase in the form of a high catecholase preparation.[5] Both curves show induction periods, which were removed (Curve I) by the addition of small amounts of catechol, just as in the oxidation of *p*-cresol. As in the oxidation of *p*-

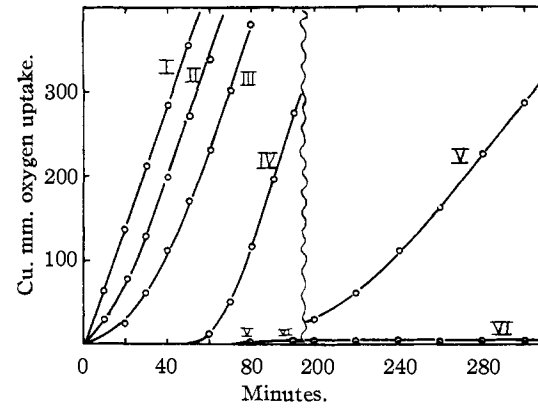

Fig. 2.—Showing the effect of substances which decrease the concentration of catechol, as it is formed, in the oxidation of phenol by tyrosinase. The rate of oxygen uptake was followed by means of the Warburg respirometer; temp. 25°. Curve II: Reaction flasks contained 2 cc. of citrate–phosphate buffer (*p*H of final mixture 7.1), 1 cc. of gelatin solution (5 mg.), 1 cc. of aqueous phenol solution (20 mg.), 1 cc. of high cresolase prepn. (0.7 phenolase, 6.2 catecholase and 3.1 cresolase units) and sufficient water to make the final volume 8 cc. Curve III: Reaction mixture same as for Curve II except 1 cc. of a high catecholase prepn. (0.7 phenolase, 12.0 catecholase and 0.7 cresolase units) was used. Curve I: Reaction mixture same as for Curve II except 0.5 cc. of catechol solution (0.1 mg.) was added. Adding this amount of catechol to the reaction mixture for Curve III also gave results corresponding to Curve I. Curve IV: Reaction mixture same as for Curve II except 0.5 cc. of a solution of potassium ferricyanide (1 mg.) was added, replacing part of the water in the reaction mixture. Curve V: Reaction mixture same as for Curve II except 3 cc. of 0.2 *M* sodium borate–boric acid buffer replaced the citrate–phosphate buffer (*p*H of final solution 7.1). Curve VI: Reaction mixture same as for Curve III except 3 cc. of 0.2 *M* borate buffer replaced the citrate–phosphate buffer. Final *p*H was 7.1.

(4) C. A. Bordner and J. M. Nelson, THIS JOURNAL, **61**, 1507 (1939).

(5) For description of enzyme units see legend of Table I.

cresol by tyrosinase, the addition of an oxidizing agent to the reaction mixture, when phenol is oxidized by the enzyme, lengthens the induction period. Thus the addition of 1 mg. of potassium ferricyanide to the reaction mixture corresponding to Curve II gave results shown by Curve IV. Most likely a trace of catechol, or substance similar to catechol in rendering the tyrosinase active toward monohydric phenols, is formed through autoxidation as the reaction mixture, containing phenol and enzyme, is shaken with air in the Warburg reaction flask. The presence of potassium ferricyanide in the reaction mixture removes, by oxidation, this catechol as it is formed, thereby preventing the oxidation of the phenol from starting until all the ferricyanide has been reduced.

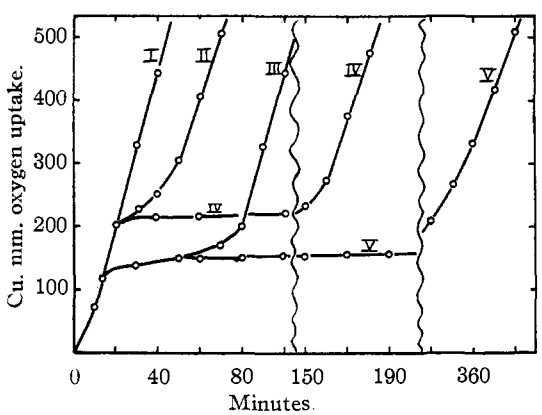

Fig. 3.—Showing the effect of potassium ferricyanide on the rate of oxidation of phenol and of *p*-cresol. Warburg respirometer used; temp. 25°. Curve I represents both the oxidation of *p*-cresol and of phenol by tyrosinase. Amounts of enzyme were chosen so that the rates of oxygen uptake for both monohydric phenols were the same. The reactions in both cases were allowed to proceed for a short time before readings were taken so that the differences in the length of the induction periods would be avoided.

In the case of the *p*-cresol oxidations the reaction flasks contained 2 cc. of citrate–phosphate buffer (*p*H of final solutions 7.1), 1 cc. of gelatin solution (5 mg.), 1 cc. of a solution of *p*-cresol (4 mg.), 1 cc. of a high cresolase prepn. (1.2 cresolase and 2.4 catecholase units) and sufficient water to make the final volume 8 cc. When phenol was being oxidized, the reaction flasks contained as the enyzme 1 cc. of a high cresolase prepn. (1.2 phenolase, 10.5 catecholase and 5.5 cresolase units) and 1 cc. of phenol solution (20 mg.) in place of the *p*-cresol used when the latter served as the substrate. Curves II and III: Reaction mixtures same as for Curve I, when *p*-cresol was the substrate, except 1 cc. of a potassium ferricyanide solution (10 mg.) was added from the side arm at the twenty and thirteen minute intervals, respectively. Curves IV and V: Reaction mixtures same as for Curves II and III except 20 mg. of phenol used in place of 4 mg. of *p*-cresol.

Hence a long induction period occurs due to the presence of potassium ferricyanide at the beginning of the reaction, as shown by Curve IV.

It is known that borates form complexes with *o*-dihydric phenols, such as catechol. Therefore, if the simultaneous oxidation of catechol is necessary for tyrosinase to oxidize phenol, it would be expected that the presence of borates would tend to prevent the accumulation of sufficient free catechol in the beginning of the reaction to render the enzyme active toward the phenol. This seems to be the case. The reaction mixtures corresponding to Curves V and VI in Fig. 2 only differed, respectively, from those corresponding to Curves II and III in that 0.2 *M* sodium borate replaced the citrate–phosphate buffer used.

Just as Gregg and Nelson[6] found in the oxidation of *p*-cresol by tyrosinase, only enough catechol is oxidized by the enzyme, when phenol is present in the reaction mixture, to keep the enzyme primed so that it can introduce a hydroxyl in the phenol. Hence as the oxidation of the phenol progresses, more and more catechol accumulates, until most of the phenol has been oxidized. Toward the end of the reaction when almost all of the phenol has been oxidized, then the accumulated catechol is oxidized. Curve 1 in Fig. 3 represents the rates of oxygen uptake when 4 mg. of *p*-cresol or 20 mg. of phenol was oxidized by means of amounts of tyrosinase so chosen that the two rates were the same. By the addition of 10 mg. of potassium ferricyanide to the reaction mixtures at thirteen and twenty minutes after the oxidations had started, the reactions became interrupted for certain lengths of time, after which they again continued at practically the original rate. The reason why the oxidations of the *p*-cresol and the phenol were interrupted for a shorter length of time when the ferricyanide was added twenty minutes after the reaction had started instead of thirteen minutes, was because more catechol had had time to accumulate. The greater the amount of accumulated catechol the greater the amount of ferricyanide reduced and hence less of the latter remained to give rise to an induction period like that shown by Curve IV in Fig. 2.

Ludwig and Nelson showed that the presence of sodium benzoate exerts a retarding action when catechol is oxidized by means of tyrosinase. This retardation is very likely due to a displacement of

(6) Gregg and J. M. Nelson, THIS JOURNAL, **62**, 2500 (1940).

the catechol on the enzyme by the benzoate. Since the oxidation of a monohydric phenol by the enzyme is dependent on the simultaneous oxidation of an *o*-dihydric phenol, it follows that if the latter reaction is retarded, then the oxidation of the monohydric phenol should also be affected. Curve I in Fig. 4 represents the rates of oxygen uptake when 4 mg. of *p*-cresol or 4 mg. of phenol was oxidized by amounts of tyrosinase so chosen that the rates in the two cases were the same. When the same amounts of sodium benzoate were added to each of the above reaction mixtures, the rate of oxidation of the *p*-cresol was retarded to the extent shown by Curve II and that of the oxidation of the phenol by Curve III.

The authors are indebted to Dr. W. A. Wisansky for part of the data given in Table I.

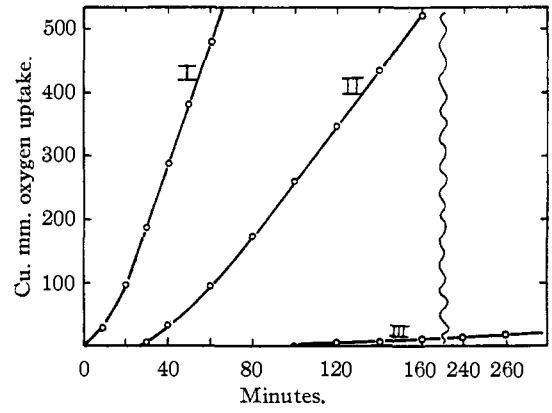

Fig. 4.—Showing the effect of sodium benzoate on the enzymic oxidation of phenol and *p*-cresol. The rate of oxygen uptake was followed by means of the Warburg respirometer; temp. 25°. Curve I: Reaction mixture was 1 cc. of *p*-cresol (4 mg.), 1 cc. of high catecholase prepn. (1.0 cresolase and 17.3 catecholase units), citrate-phosphate buffer, *p*H 7.1, 1 cc. of gelatin soln. (5 mg.) and sufficient water to make the final volume 8 cc. In another experiment 4 mg. of phenol was used in place of the *p*-cresol, and the 1 cc. of high catecholase enzyme solution used contained 1.0 phenolase, 17 catecholase and 1.0 cresolase units. Both rates of oxygen uptake fell on the same Curve I. Curve II: Same reaction mixture as for the *p*-cresol experiment represented by Curve I, except 0.25 cc. of *M* sodium benzoate solution replaced part of the water. Curve III: Same reaction mixture as for the phenol experiment represented by Curve I, except 0.25 cc. of *M* sodium benzoate replaced part of the water.

Summary

1. The loss in activity toward *p*-cresol, occurring when tyrosinase, from *Psalliota campestris*, is purified by certain methods, was not observed when phenol was used in place of *p*-cresol as the substrate. This observation is contrary to the claim that the enzyme, in the pure state, is only a polyphenol oxidase and that the activity toward monohydric phenols is due to some outside factor.

2. The activity of tyrosinase toward phenol is proportional to the copper content for both types of tyrosinase preparations, high catecholase and high cresolase.

3. The activity of high cresolase preparations toward *p*-cresol is some 4–5 times greater than toward phenol.

4. The maximum activities of high cresolase preparations toward phenol and catechol require higher concentrations of these phenols than do the high catecholase preparations.

5. The activity of high catecholase preparations toward catechol is practically twice that of the high cresolase preparations, when based on the copper content of the highly purified enzyme.

6. The oxidation of phenol by means of tyrosinase, like the oxidation of *p*-cresol by the enzyme, requires the simultaneous oxidation of an *o*-dihydric phenol.

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