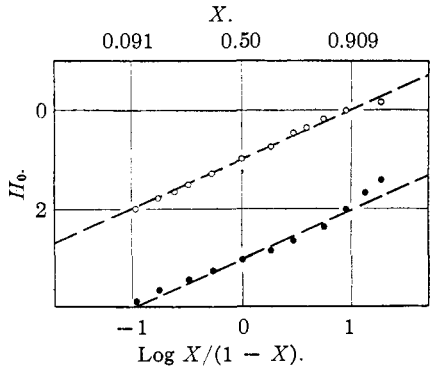

Fig. 5.—The data for urea are shown by the open circles; those for antipyrine, by the closed circles. Upper ordinate, X, the fraction of the base titrated; lower ordinate, log $X/(1 - X)$.

line drawn through the point of half-titration with the theoretical slope (the dashed lines in Fig. 5) does lie fairly close to the experimental points in both cases. This indicates that some of the errors inherent in the electrometric method such as the liquid junction potential and the change in activity coefficients with dilution tended to distort the results. It is of interest to note that the form of the antipyrine titration curve is somewhat abnormal. Hall[14] observed that the e. m. f. curve for this base was steeper than the curves for the titration of other bases of comparable strength, and that its strength in acetic acid appeared abnormally great. In these measurements, on the other hand, the abnormality is shown by a flatness of the curve and a considerable departure from the theoretical form on the *acid* side after about 80% titration.

Summary

The acidity of tenth molar urea–sulfuric acid solutions and antipyrine–sulfuric acid solutions in glacial acetic acid was studied at 25.0° with six indicators, *o*-nitraniline, *p*-nitraniline, *m*- and *p*-nitro-N,N-dimethylaniline, and *m*- and *p*-nitro-N,N-diethylaniline. The pK's of these indicators were determined in these buffers from the pK of *o*-nitraniline, taken as −0.17. The acidity function, H_0, was determined from these pK's for each solution used. The H_0 values for the points of half neutralization of urea and antipyrine were found to be 0.98 and 3.05, respectively. These values differ by about 1.7 from the pH(HAc) values found by Hall and his co-workers, and the value 1.7 for difference between the pH(HAc) scale and the H_0 scale is in fair agreement with the value 2 found by Hall and Spengeman.

MADISON, WISCONSIN RECEIVED JULY 19, 1940

[CONTRIBUTION FROM THE DEPARTMENT OF CHEMISTRY, COLUMBIA UNIVERSITY]

Further Studies on the Enzyme, Tyrosinase

BY DONALD C. GREGG AND J. M. NELSON

As is well known, the phenol oxidase, tyrosinase, has been regarded as an enzyme capable of catalyzing two types of aerobic oxidation. One of these is the introduction of a hydroxyl group ortho to the one already present in certain monohydric phenols. The other brings about the oxidation of certain *o*-dihydric phenols to their corresponding *o*-quinones. Since *p*-cresol has been widely used as a substrate for studying the first and catechol for the latter, the two activities will be termed cresolase and catecholase, respectively.

A convenient source for this enzyme is the common mushroom, *Psalliota campestris*. When aqueous extracts of the plant, containing the tyrosinase, are permitted to stand exposed to air, they darken and considerable enzymic activity is lost. A loss also occurs when the enzyme in the aqueous extract is subjected to the usual methods of purification, such as precipitation with cold acetone, ammonium sulfate, dialysis, etc. Upon comparing the ratio between the two final activities with that of the original fresh extract, it is found that the cresolase activity has suffered a greater loss than the catecholase activity. Thus preparations have been obtained very low in cresolase but high in catecholase. In highly purified preparations the latter activity has been found proportional to the copper content.[1,2] Due to this relatively greater loss in cresolase activity during the purification, and also due to only the catecholase activity being proportional to the copper content, certain workers[1] have concluded that the cresolase activity is not part of the en-

(1) D. Keilin and T. Mann, *Proc. Roy. Soc.* (London), **125B**, 187 (1938).

(2) B. J. Ludwig and J. M. Nelson, THIS JOURNAL, **61**, 2601 (1939).

zyme, but an outside factor, and that tyrosinase when pure is only active toward polyhydric phenols, such as catechol. For this reason the term polyphenol oxidase has been suggested as a more suitable name for the enzyme.

Recently Parkinson and Nelson[3] have shown that by starting with the above preparation, at a partially purified stage, it is possible to recover by adsorption to kaolin, etc., a preparation possessing a high activity toward *p*-cresol as well as toward catechol. Due to both activities of this new preparation being proportional to the copper content and to the fact that the ratio of the two activities corresponds closely to that of the activities of the fresh water extracts, this preparation of Parkinson and Nelson seems to resemble more closely the enzyme as it occurs in the mushroom.

If this is the case, then the question again arises as to whether the true tyrosinase is one enzyme possessing two different activities, or is a polyphenol oxidase, only active toward polyphenols, such as catechol, associated in its crude form with another enzyme or factor, responsible for the activity toward monohydric phenols.[4]

It was found, in the present study, that the rates of oxygen uptake were practically the same and independent of the catechol present in the reaction mixture when either a high cresolase or a high catecholase preparation, each containing the same amount of activity toward *p*-cresol (1.1 cresolase unit but 2.1 and 50.5 units of catecholase, respectively),[5] were permitted to act on separate mixtures consisting of 4 mg. of *p*-cresol and 4 mg. of catechol (Curves II and IV Fig. 1).[6] It would have been expected, if the two activities were separate enzymes, that the high catecholase preparation, containing 23 times more catecholase units than the high cresolase preparation, would have catalyzed the oxidation of the catechol, present in the reaction mixture, 23 times faster than the high cresolase preparation. Since this was not the case, it appears that the cresolase activity, which was the same in both experiments, in some way controlled the rates of oxidation and prevented the large excess of catecholase in the high catecholase preparation from acting on the catechol.

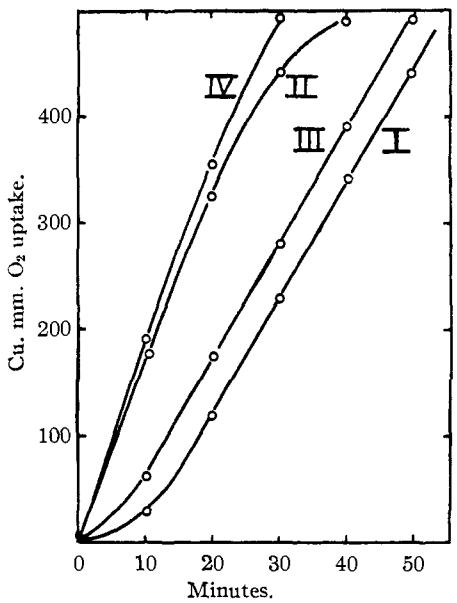

Fig. 1.—Showing the effect of added catechol on the oxidation of *p*-cresol by tyrosinase: Warburg respirometer used; temp. 25°; capacity of reaction flasks 30 cc. Curve I.—Reaction mixture contained 2 cc. of (0.2 *M*) citrate–(0.4 *M*) phosphate buffer (*p*H 7.1); 1 cc. of gelatin solution (5 mg.); 1 cc. of enzyme solution (1.1 cresolase and 2.1 catecholase units); 1 cc. of *p*-cresol solution (4 mg.) added from side arm at zero time; and sufficient water to make the total volume 8 cc. Curve II.—Reaction mixture same as that for Curve I except 1 cc. of catechol solution (4 mg.) was mixed with the *p*-cresol and added to the reaction flask from the side arm at zero time. Curve III.—Reaction mixture same as for Curve I except that 1 cc. of enzyme solution used contained 1.1 cresolase and 50.5 catecholase units. Curve IV.—Same as for Curve III except 1 cc. of catechol solution (4 mg.) was mixed with the *p*-cresol solution and added from the side arm to the reaction flask at zero time. Curves I and III serve to show that the activity toward *p*-cresol was the same for both the high cresolase and high catecholase preparations used in experiments corresponding to Curves II and IV, respectively.

This same influence of the oxidation of *p*-cresol on the oxidation of catechol is also shown by some of the data represented in Fig. 2. Curve I represents the oxidation of *p*-cresol by a high catecholase preparation of tyrosinase. It is therefore similar to Curve III in Fig. 1, except the zero time reading was started about ten minutes after the reaction had begun in order to avoid the initial lag period shown in Curve III in Fig. 1. When the reaction in a duplicate experiment had been in

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

(4) For convenience the Parkinson and Nelson preparations (1 cresolase to 2 catecholase units) will be referred to as "high cresolase" preparations and the preparations low in cresolase (less than 1 cresolase to 2 catecholase units) but high in catecholase activity as "high catecholase" preparations.

(5) See determination of activities.

(6) Four different high cresolase preparations and six different high catecholase preparations, the latter varying from 3.5 to 46 catecholase to one cresolase unit, were compared with respect to their behaviors toward *p*-cresol and toward mixtures of *p*-cresol and catechol. The results obtained were similar to those indicated in Fig. 1.

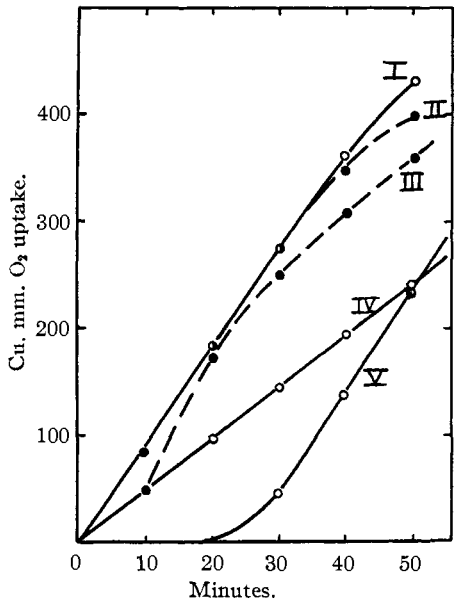

Fig. 2.—Showing the influence of *p*-cresol on the oxidation of catechol by the catecholase component of tyrosinase. Also other catechol oxidases, such as that from the sweet potato and the one from the wild mushroom, *Russula foetens*, possessing no cresolase activity, are contrasted in this respect with the catecholase in tyrosinase. Warburg respirometer used; temp. 25°. Curve I.—Reaction mixture contained 2 cc. (0.2 *M*) of citrate–(0.4 *M*) phosphate buffer (*p*H of final reaction mixture 6.3); 1 cc. of gelatin (5 mg.); 1 cc. of *p*-cresol solution (4 mg.); 1 cc. of enzyme solution (1.2 cresolase and 7.5 catecholase units), and sufficient water to make the total volume 8 cc. Curve II.—Reaction mixture same as for Curve I except 0.5 cc. of catechol solution (1 mg.) was added from the side arm at ten minutes beyond zero time. Curve IV.—Same as Curve I except 0.5 cc. of a solution of catechol oxidase from the sweet potato (2 units) was added to the 1 cc. of the tyrosinase solution. Curve III.—Same as Curve IV except 0.5 cc. of catechol solution (1 mg.) was added from the side arm at ten minutes beyond zero time. Curve V.—Same as Curve I except 0.5 cc. of laccase solution (*Russula foetens*) (0.02 unit) was added to the 1 cc. of tyrosinase solution. All reactions had been in progress for about fifteen minutes before zero time was started.

progress ten minutes beyond zero time, one mg. of catechol was added to the reaction mixture, and the resulting rate of oxygen uptake observed is represented by Curve II. Since the latter curve is superimposable for a considerable time on Curve I, it is evident that no extra oxidation of the added catechol occurred even though a high catecholase preparation, containing an excess of catecholase activity, was present.

Since the results presented so far indicate that the catecholase component of tyrosinase is not a separate enzyme, it could be expected that a catechol oxidase which was known to be independent of the cresolase component would behave differently. This has been found to be the case. Curve IV in Fig. 2 represents the oxidation of *p*-cresol by the same amount of tyrosinase as in the case of the experiments represented by Curve I, except after the reaction had gotten under way 2 units of a catecholase from the sweet potato were added to the reaction mixture. The latter enzyme is known to have no activity toward *p*-cresol.[7] The decrease in the rate of oxygen uptake shown by Curve IV when the latter is compared to Curve I is very likely due to the catecholase from the sweet potato competing with the catecholase component of the tyrosinase for the homocatechol formed in the oxidation of the *p*-cresol by the tyrosinase.

As shown by Bordner and Nelson[8] the oxidation of *p*-cresol by tyrosinase requires the simultaneous oxidation of catechol or homocatechol by tyrosinase. The induction period shown in Curve I in Fig. 1 is due to the lack of the *o*-dihydric phenol at the start of the reaction, when *p*-cresol is being oxidized by tyrosinase. The addition of a trace of catechol at the start removes this induction period. On the other hand, the addition of a trace of an oxidizing agent, such as potassium ferricyanide, which would remove homocatechol from the reaction mixture, lengthens the induction period.

Returning to Curve IV in Fig. 2, the presence of the foreign catechol oxidase from the sweet potato tends to remove homocatechol, much like the potassium ferricyanide, leaving less of the *o*-dihydric phenol to be oxidized by the catecholase oxidase component of the tyrosinase. As already stated, for tyrosinase to oxidize *p*-cresol, it is necessary that the enzyme be primed, so to speak, by the simultaneous oxidation of an *o*-dihydric phenol by tyrosinase. The oxidation by any other catechol oxidase such as that from sweet potato or an oxidizing agent such as potassium ferricyanide does not prime tyrosinase so that the latter can oxidize *p*-cresol. This priming ability is characteristic of the catechol oxidase part of tyrosinase.

This close relationship between the two activities of tyrosinase, as, for example, the control of the oxidation of the catechol by the *p*-cresol oxi-

(7) M. H. Adams and J. M. Nelson, THIS JOURNAL, **60**, 2472 (1938).

(8) C. A. Bordner and J. M. Nelson, *ibid.*, **61**, 1507 (1939).

TABLE I

SHOWING THE EFFECT OF INACTIVATION OF TYROSINASE IN THE ENZYMATIC OXIDATION OF CATECHOL AND OF *p*-CRESOL[a]

Expt.	Substrate	Units of enzyme before treatment: Catecholase	Units of enzyme before treatment: Cresolase	Ratio of activities Catecholase / Cresolase	Units remaining after treatment: Catecholase	Units remaining after treatment: Cresolase	Ratio of activities after treatment Catecholase / Cresolase	% of enzyme inactivated (based on catecholase activity)
1	Catechol (50 mg.)	1100	200	5.50	775	138	5.61	29.5
2		950	122	7.80	595	74	8.05	37.5
3		750	360	2.08	518	244	2.12	31.0
4		545	275	1.98	333	165	2.02	39.0
5	*p*-Cresol (100 mg)	1100	200	5.50	885	157	5.64	19.5
6		950	122	7.80	712	88.5	8.05	25.0
7		750	360	2.08	590	277	2.13	21.4
8		545	275	1.98	392	191	2.05	28.0

[a] Experiments 1, 2, 5 and 6 were high catecholase preparations, and 3, 4, 7 and 8 were high cresolase preparations.

dation, shown by Curves I and II in Fig. 2, is also emphasized when the actions of tyrosinase and sweet potato catecholase on catechol in the presence of *p*-cresol are compared. After the reaction in which *p*-cresol was being oxidized by a mixture of tyrosinase (high catecholase preparation) and sweet potato oxidase (Curve IV, Fig. 2) had been in progress for ten minutes, 1 mg. of catechol was added. An immediate rise in the rate of oxygen uptake occurred (Curve III), showing that the sweet potato catecholase was free to act on the added catechol. This experiment was similar to that represented by Curve II, except in the latter no sweet potato oxidase was present. Since a high catecholase preparation of tyrosinase was used in both experiments, the reaction mixture (Curve II) also contained an excess of catecholase, but due to the presence of *p*-cresol the tyrosinase catecholase could not act on the added catechol as in the case of the sweet potato oxidase (Curve III).

An oxidase from the wild mushroom, *Russula foetens*, called laccase by the workers in these laboratories,[9] has the ability to oxidize catechol but cannot oxidize *p*-cresol in the same manner as tyrosinase. Therefore, here is another catechol oxidase, which like the sweet potato oxidase is free of cresolase action, and like the latter interferes with the oxidation of *p*-cresol by tyrosinase. Even when a small amount of the laccase (0.02 unit) was added to the reaction mixture, corresponding to Curve I, Fig. 2, a long interruption of the oxidation of the *p*-cresol occurred (Curve V). When 7.5 units of laccase, the same amount as the tyrosinase catecholase present in the reaction mixture, was used, then no oxidation of the *p*-cresol by the tyrosinase took place after several hours. This interruption of the oxidation of the *p*-cresol by the tyrosinase present in the reaction mixture is, as pointed by Bordner and Nelson, very likely due to the laccase preventing, by oxidation, the accumulation of any homocatechol essential for the oxidation of the *p*-cresol by the tyrosinase.

If the cresolase and catecholase activities of tyrosinase from *Psalliota campestris* are intimately associated, it might be expected that any inactivation which affected the activity of the enzyme as a whole, such as the copper in the molecule, would affect both activities in the same ratio.

Ludwig and Nelson have shown that the oxidation of catechol by tyrosinase gradually brings about the inactivation of the enzyme. The amount of work done by the enzyme, as measured by the total oxygen uptake, was found proportional to the amount of enzyme present, based on the copper content. This was true, of course, only in the case of the enzyme preparation being free of extraneous copper. Their preparations were high in catecholase. Since then Parkinson and Nelson have shown that high cresolase preparations are also inactivated in the oxidation of catechol and that in this case also the inactivation was proportional to the amount of enzyme, based on the copper content. If both the cresolase and catecholase activities are dependent directly or indirectly on the same copper in the two types of tyrosinase preparations, it seems probable that this type of inactivation would affect the two activities in the same ratio. The experiments described in Table I indicate this to be the case.

A 200-cc. reaction mixture containing 1100 catecholase and 200 cresolase units, 20 mg. of gelatin, citrate–phosphate buffer of *p*H 7.1, and 50 mg. of catechol was agitated by bubbling air slowly through the solution for about one hour at room temperature. The mixture then was filtered and

(9) D. C. Gregg and W. H. Miller, THIS JOURNAL, **62**, 1374 (1940).

TABLE II[a]

SHOWING THE EFFECT OF 4-NITROCATECHOL, POTASSIUM CYANIDE AND SODIUM DIETHYLDITHIOCARBAMATE AS INHIBITORS OF THE ENZYMIC ACTIVITIES OF TYROSINASE

Ratio of activity, Catecholase/ Cresolase	Units in reaction Catecholase	Cresolase	Substrate (4 mg.)	Inhibitor	Inhibition, %
42	42	1.00	*p*-Cresol	4-Nitrocatechol, 1 mg.	28
42	0.90	0.021	Catechol		22
2	2.40	1.20	*p*-Cresol		60
2	1.30	0.65	Catechol		56
20	36	1.80	*p*-Cresol	KCN, 0.065 mg.	60
20	2.20	0.11	Catechol		58
2	4.80	2.40	*p*-Cresol	KCN, 0.037 mg.	50
2	2.10	1.05	Catechol		46
20	36	1.80	*p*-Cresol	Sodium diethyldithiocarbamate, 0.050 mg.	62
20	2.20	0.11	Catechol		57
2	4.80	2.40	*p*-Cresol	Sodium diethyldithiocarbamate, 0.025 mg.	51
2	2.10	1.05	Catechol		46

[a] Barcroft differential and Warburg respirometers were used in determining the catecholase and cresolase activities, respectively; temp. 25°; reaction mixtures *p*H 7. Solutions containing inhibitor, buffer, gelatin and enzyme were shaken 10–15 minutes before the substrate was added from the side-arm of the reaction flask. As a check on the reliability of the rates of oxygen uptake,[10] when catechol was the substrate, total oxygen uptakes before inactivation occurred were also noted. The changes in the total oxygen uptakes due to the presence of the inhibitors, potassium cyanide and sodium diethyldithiocarbamate, agreed closely with the changes in the corresponding rates of oxygen uptake caused by these inhibitors. Sodium diethyldithiocarbamate[11,12] is often used as a reagent in the quantitative estimation of minute quantities of copper.

the filtrate dialyzed overnight, and the two activities determined. About 30% of the enzyme had been inactivated, but the ratio of the two activities was still the same as before the inactivation.

The above experiment conducted with a high catecholase preparation was repeated using a high cresolase preparation (750 catecholase and 360 cresolase units). The amount of enzyme inactivated was about the same as in the previous experiment and the ratio of the two activities remaining was the same as before the inactivation.

Ludwig and Nelson also found that inactivation occurred in the oxidation of *p*-cresol by tyrosinase but to a less extent than in the case where catechol was used as the substrate. Two experiments, the same as those described above, were performed using 100 mg. of *p*-cresol instead of the catechol. About 20% of the enzyme was inactivated but the ratio of the final two activities was the same as those before the treatment.

The results of the inactivation experiments appear to support the view that the two activities of the tyrosinase are intimately associated and that the activity toward *p*-cresol is dependent on the same enzyme copper as the activity toward catechol, and that this is true irrespective of the ratio of the two activities in the tyrosinase preparation.

If the cresolase and catecholase activities of tyrosinase are closely associated, the action of certain inhibitors with the enzyme might indicate the close relationship of these two activities. An inhibitor which affected the enzymic activity toward catechol might also be expected to affect the enzymic activity toward *p*-cresol. The effects produced by 4-nitrocatechol, potassium cyanide and sodium diethyldithiocarbamate (Table II) appear to indicate that this view is correct.

Using a typical high cresolase tyrosinase preparation and also a high catecholase preparation the effect of the above inhibitors was observed.

4-Nitrocatechol was found to decrease the enzymatic activity of the tyrosinase. This catechol derivative (using 4 mg.) is not oxidized by tyrosinase even when 50–100 catecholase units are employed. It seems reasonable to expect, since 4-nitrocatechol is similar to catechol, that it forms like catechol an enzyme–substrate complex. This inhibitor affected both the cresolase and catecholase activity of the tyrosinase, irrespective of the ratio of the two activities in the preparation.

The data also show that potassium cyanide and sodium diethyldithiocarbamate decreased the cresolase and the catecholase activities to the same

(10) The amount of total oxygen uptake in the oxidation of catechol before inactivation of the enzyme occurs has been shown by Ludwig and Nelson to be proportional to the activity, based on the copper content.

(11) T. Callan and J. A. R. Henderson, *Analyst*, **54**, 650 (1929).

(12) L. W. Conn, A. H. Johnson, H. A. Trebler and V. Karpenko, *Ind. Eng. Chem., Anal. Ed.*, **7**, 15 (1935).

extent. These inhibitors also affected the rate of oxidation of catechol by the enzyme and the amount of oxygen uptake before inactivation, in the oxidation of catechol, to the same extent.

Determination of Activities.—The determination of the activities of the various tyrosinase preparations was made using Barcroft differential manometers.[13] The reaction flasks were of 50 cc. capacity, temp. 25°. Reaction mixtures contained 1 cc. of an aqueous solution of catechol (4 mg.) added from the side arm at zero time; 2 cc. of (0.2 *M*) citrate–(0.4 *M*) phosphate buffer (*p*H of reaction mixture 7.1); 1 cc. of an aqueous solution of gelatin (5 mg.); enzyme solution and sufficient water to make the total volume equal to 8 cc. A rate of oxygen uptake equal to 10 cmm. per minute was defined as one catecholase unit. The rapid inactivation of the enzyme in the oxidation of the catechol necessitated the taking of readings every minute for the first five minutes. Usually two or three constant values for the oxygen uptake per minute were obtained. However, three or four sets of readings were taken and an average value of these was used in calculating the catecholase activity.

A Warburg respirometer, having 30-cc. reaction flasks, was used in determining the activities toward *p*-cresol; temp. 25°. Reaction mixtures contained 1 cc. of an aqueous solution of *p*-cresol (4 mg.), added from the side arm at zero time; 2 cc. of (0.2 *M*) citrate–(0.4 *M*) phosphate buffer (*p*H of reaction mixture 7.1); 1 cc. of an aqueous solution of gelatin (5 mg.); enzyme solution and sufficient water to make the final volume 8 cc. A rate of oxygen uptake equal to 10 cmm. per minute during the oxidation of the *p*-cresol was taken as one cresolase unit.

Summary

1. The amount of the catecholase compared to that of the cresolase component in a tyrosinase preparation has no influence on the rate of oxidation of *p*-cresol. Curves I and III, Fig. 1, have the same slope beyond the induction period.

2. The influence on the rate of oxygen uptake when *p*-cresol is oxidized by tyrosinase in the presence of catechol is practically independent of the amount of the catecholase component of the enzyme present in the reaction mixture (Curves II and IV, Fig. 1).

3. The addition of catechol to the reaction mixture in which *p*-cresol is being oxidized by tyrosinase is without influence on the rate of oxygen uptake (Curves I and II, Fig. 2). This shows that only enough catechol is being oxidized, when *p*-cresol is present, to prime the enzyme so that it can bring about the oxidation of the *p*-cresol.

4. The addition of a foreign catecholase, which is not a component of tyrosinase, to a reaction mixture in which *p*-cresol is being oxidized by tyrosinase tends to lower the rate of oxygen uptake (Curves I and IV, Fig. 2). This suggests that homocatechol, formed in the first step in the oxidation of the *p*-cresol, is being oxidized and removed by the foreign catecholase to such an extent that the rate of oxidation of the *p*-cresol by the tyrosinase is decreased. It also shows that the foreign catecholase is different in its action from the catecholase component of the tyrosinase.

5. A foreign catecholase added to the reaction mixture in which *p*-cresol is being oxidized by tyrosinase, differs from the catecholase component of the tyrosinase in that it is free to bring about the oxidation of catechol added to the reaction mixture (Curves II and III, Fig. 2).

6. The following three facts appear to be significant: first, tyrosinase cannot oxidize *p*-cresol without the simultaneous oxidation of an *o*-dihydric phenol (Bordner and Nelson); second, in the presence of *p*-cresol, tyrosinase cannot oxidize catechol any faster than the rate required for priming the enzyme toward *p*-cresol; third, in the case of the high cresolase preparations of tyrosinase, both the activity toward catechol and toward *p*-cresol are proportional to the same copper content (Parkinson and Nelson). These three facts taken together suggest, so to speak, an overlapping of the two activities. This overlapping is a strong indication that the two activities belong to the same enzyme complex.

(13) M. Dixon, "Manometric Methods," University Press, Cambridge, 1934.

NEW YORK, N. Y. RECEIVED JUNE 6, 1940