From the data in Table II it will be observed that the change in rate of oxygen uptake is quite marked in the more acid range of *p*H but approaches a maximum around *p*H 7. Beyond *p*H 7 there is danger of autoxidation and for this reason *p*H 7 is the better hydrogen ion concentration for determining activity of tyrosinase toward *p*-cresol, rather than *p*H 6.2 originally used by Graubard and Nelson. Furthermore, the above data indicate no optimum *p*H value for the enzymatic oxidation of *p*-cresol, which is not in line with results reported by Graubard and Nelson. Due to an oversight the last-named investigators failed to mention the use of borate buffer in the more alkaline range. Since then this buffer has been shown to exert a retarding influence on the rate of oxygen uptake, and thus explains the apparent optimum *p*H. Narayanamurti and Ramaswami Ayyar[7] also reported an optimum *p*H value of 6.5 in the oxidation of tyrosine by tyrosinase (from the bean *Dolichos lab lab*). They also used borate buffer for the more alkaline *p*H range.

The data in Table III show that there is a gradual increase in oxygen uptake with increase in concentration of *p*-cresol used. Changing the rate of oscillation of the Warburg apparatus to more than 90 complete oscillations did not influence the rate of oxygen uptake.

(7) D. Narayanamurti and Ramaswami Ayyar, *J. Indian Inst. Science*, **12**, 109 (1929).

TABLE III

SHOWING THE INFLUENCE OF CONCENTRATION OF *p*-CRESOL ON THE RATE OF OXYGEN UPTAKE

Temperature 25°. *p*H of reaction mixture 7.0. Same amount of enzyme used in all experiments. 5 mg. gelatin. Reaction volume 8 cc.

p-Cresol, mg.	0.0	1.0	2.0	3.0	4.0	5.0
Activity, units per cc.	.0	0.36	0.58	0.72	0.83	0.94

In the light of the results obtained in this study it was decided to modify the method of Graubard and Nelson as follows. Reaction mixture: 1 cc. of an aqueous solution containing 4 mg. of *p*-cresol, added to the reaction mixture from the side-arm of the reaction flask at zero time; 2 cc. of (0.1 *M*) citrate–(0.2 *M*) phosphate buffer solution (*p*H of reaction mixture, 7.0); 1 cc. of an aqueous solution containing 5 mg. of gelatin; 1 cc. of enzyme solution and sufficient volume of water to make total reaction volume 8 cc.

Summary

1. The importance of protein concentration in the determination of the activity of highly purified preparations of tyrosinase has been pointed out.

2. The method of Graubard and Nelson for determining the activity of tyrosinase toward *p*-cresol has been modified to conform to the influence of protein.

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[CONTRIBUTION FROM THE DEPARTMENT OF CHEMISTRY, COLUMBIA UNIVERSITY]

On the Nature of the Enzyme Tyrosinase

BY MARK H. ADAMS AND J. M. NELSON

In the literature on oxidases from the time of Bourquelot to the present, tyrosinase has been generally considered capable of performing two quite distinct oxidative functions: one, the introduction of a hydroxyl group ortho to the already existing hydroxyl in certain monohydric phenols; and two, the oxidation of certain ortho-dihydric phenols to the corresponding ortho-quinones. Several theories have been proposed to account for these two types of activity. Onslow[1] favored the view that tyrosinase is really a catechol oxidase, and that the oxidation of the monohydric phenols is due to a secondary reaction in which *o*-quinones serve as the oxidizing agent. Richter[2] states "The oxidation of *p*-cresol therefore appears to be essentially a secondary oxidation in which the formation of an *o*-quinone is involved, rather than a direct oxidation of the cresol." Keilin and Mann[3] also believe that the monophenolase activity is due to the catechol oxidase (polyphenolase) plus some "factor" probably of the *o*-quinone type as suggested by Onslow.

Against Onslow's theory may be cited the

(1) M. W. Onslow, "The Principles of Plant Biochemistry," University Press, Cambridge, 1931, p. 138.

(2) D. Richter, *Biochem. J.*, **28**, 901 (1934).

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

experience of Pugh,[4] who obtained from slugs (*Arion ater*) an oxidase which catalyzes the oxidation of catechol but not *p*-cresol. If the oxidation of *p*-cresol is dependent on *o*-quinones, then a mixture of this enzyme and homoquinone should bring about the oxidation of the *p*-cresol. This however, was found not to be the case. Workers in these Laboratories have had similar experience. When the oxidase from sweet potatoes (*Batatas batatas*), which also brings about the oxidation of catechol but not *p*-cresol, was added to a mixture of catechol and *p*-cresol, only the catechol was oxidized.

The oxidation of catechol when catalyzed by tyrosinase is very rapid, and when the rate of oxygen uptake is measured by means of the Warburg form of respirometer, difficulty is often encountered in securing accurate readings. If the rate of oxidation is reduced by using less enzyme, then considerable inactivation of the enzyme is apt to occur, especially in the case of the more highly purified preparations. Richter and Kubowitz[5] as well as others have ascribed the inactivation of the enzyme to presence of *o*-benzoquinone formed in the oxidation of the catechol. To avoid inactivation, Richter carried on the oxidation of the catechol in the presence of reagents such as aniline which react with the quinone as it formed and thereby prevent its action on the enzyme. Kubowitz, on the other hand, oxidizes the catechol in the presence of a reducing system, thereby changing the quinone as it is formed back to catechol. An objection to Kubowitz's reducing system, which consists of hexosemonophosphate, Negelein's Zwischen Ferment and Coferment II, is that very few laboratories have it on hand. Richter's use of aniline is not satisfactory since Wagreich and Nelson[6] have shown that even in the presence of aniline considerable inactivation is apt to occur.

Graubard and Nelson (unpublished) have shown that although tyrosinase is without action on hydroquinone, it can be made active if a small amount of catechol is added. This is readily understood since the oxidation–reduction system of catechol–*o*-benzoquinone has a higher potential than the system hydroquinone–*p*-benzoquinone. The *o*-benzoquinone being reduced by the hydroquinone back to catechol, causes the latter to play the role of an oxygen carrier. In a sense the hydroquinone can be looked upon as serving the same purpose as the reducing system used by Kubowitz, but having the advantage of being more generally available. Furthermore, the enzyme seems to suffer less inactivation when the catechol–hydroquinone system is used. Judging from Kubowitz's data, his rates of oxygen uptake remained constant for only ten to fifteen minutes, while when catechol–hydroquinone is used the rates remain constant for over half an hour.

The activity of tyrosinase preparations with respect to catechol was determined by the use of the Warburg form of respirometer[7] using 50-cc. flasks and 90 complete oscillations per minute; temperature 25°. Reaction mixtures consisted of 1 cc. of an aqueous solution containing 5 mg. of hydroquinone and 0.1 mg. of catechol (added from the side-arm of the flask at zero time). In the flask were: 2 cc. of (0.1 *M*) citrate–(0.2 *M*) phosphate buffer (*p*H of reaction mixture 7.0); 1 cc. of aqueous solution containing 5 mg. of gelatin; enzyme solution and sufficient water to make the total volume equal to 8 cc. The amount of enzyme required to cause the rate of oxygen uptake to equal 10 cu. mm. per minute was defined as one "catechol unit." The rate of oxygen uptake was usually directly proportional to the amount of oxidase used, provided the enzyme had been purified sufficiently to remove the naturally occurring substrates, and the amount of enzyme was less than two catechol units. As would be expected, the amount of catechol used affects the rate of oxygen uptake. The data represented in Fig. 1 were obtained by using 5 mg. of hydroquinone and amounts of catechol varying from 0.01 to 0.3 mg. It will be noted that beyond 0.1 mg. of catechol the increase in the rate of oxygen uptake tends toward a maximum, and explains why the mixture of 0.1 mg. of catechol and 5 mg. of hydroquinone was selected for use in the method described above.

When a tyrosinase preparation also contains laccase, then obviously this method for determining the activity of tyrosinase toward catechol is no longer applicable. The presence of laccase can be detected readily since such preparations catalyze the oxidation of hydroquinone when no catechol is present.

(4) C. E. M. Pugh, *Biochem. J.*, **24**, 1442 (1930).

(5) Kubowitz, *Biochem. Z.*, **292**, 221 (1937).

(6) H. Wagreich and J. M. Nelson, *J. Biol. Chem.*, **115**, 459 (1936).

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

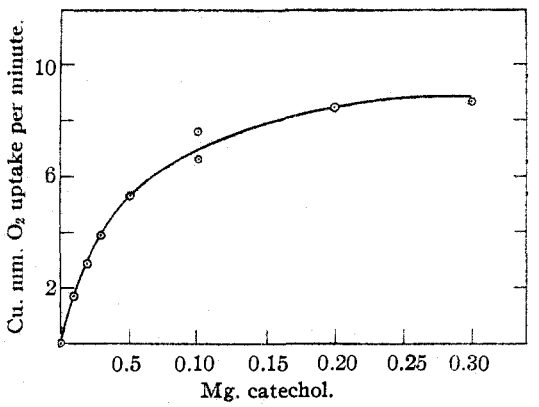

Fig. 1.—Showing the change in rate of oxygen uptake when varying amounts of catechol were used in the presence of 5 mg. of hydroquinone.

The addition of gelatin was found to be necessary in measuring the activity of tyrosinase preparations toward catechol, just as it was found necessary in the *p*-cresol method described in the previous paper. In one instance the addition of gelatin caused a 50% increase in the rate of oxidation of the catechol–hydroquinone reaction mixture. The effect of the gelatin varies with the purity and concentration of the enzyme.

Since the use of the catechol–hydroquinone mixture as a substrate depends on the oxidation of catechol, while the method described in the previous paper involves the oxidation of *p*-cresol, it was decided to compare the activities of different tyrosinase preparations with respect to the two substrates. In Table I are given the relative catechol and *p*-cresol activities for preparations of tyrosinase obtained from different sources. The activities toward catechol were obtained by using that amount of tyrosinase solution equal to 1 unit when *p*-cresol served as the substrate.

TABLE I

Source	Activity toward *p*-cresol	Activity toward catechol–hydroquinone
Lactarius piperatus	1 unit	0.08 unit
Irish potato	1 unit	.67 unit
Puff ball	1 unit	.05 unit
Psalliota campestris	1 unit	.64 unit
Sweet potato	0.0 unit	1.00 unit

It is evident from the values given in the table that the tyrosinases from the various sources are not identical. On the basis of one *p*-cresol unit, the potato and *Psalliota campestris* enzymes are roughly eight to ten times as active toward catechol as are the enzymes of the puff ball and *Lactarius piperatus*.

Several possible explanations for these differences in activities toward the two substrates suggest themselves: first, that each source yields a tyrosinase which is unique (if this were the case then the relative activity of the enzyme toward *p*-cresol as compared with that toward catechol should remain unchanged during any number of purification processes); second, that the tyrosinase preparations in reality consist of a mixture of two enzymes, one acting on *p*-cresol, or perhaps on both *p*-cresol and catechol, the other acts exclusively on catechol; third, that some accompanying substance, non-enzymatic in nature, as suggested by Keilin and Mann, enables the catechol oxidase to bring about the oxidation of the *p*-cresol.

To test the first and third possibilities a tyrosinase preparation (*Psalliota campestris*) was subjected to series of purification operations: precipitation with acetone, precipitation with ammonium sulfate, adsorption to kaolin and alumina, foreign proteins removed by gentle heat, and thorough dialysis against distilled water at frequent stages during the process. The activities with respect to the catechol–hydroquinone mixture and to *p*-cresol were measured at the different stages of purification and the ratios of the two activities are given in Table II.

TABLE II

Showing loss in catechol activity in contrast to loss in *p*-cresol activity when a tyrosinase preparation from the common mushroom was subjected to the following purification.

Stage of purification	Catechol units/*p*-cresol units
After first acetone precipitation	3.2
After second acetone precipitation	2.2
After ammonium sulfate precipitation	2.5
After adsorption to kaolin	1.6
After adsorption to alumina	1.5
Highly purified preparation	0.64

The data in Table II show that by this particular method of purification it is possible to increase relatively the ratio of the *p*-cresol activity to the catechol activity about five times. Keilin and Mann, on the other hand, using a somewhat different procedure in purifying their tyrosinase (polyphenolase) preparation (also from *Psalliota campestris*), obtained a highly purified preparation very low in *p*-cresol activity compared to its catechol activity.

In adsorbing the tyrosinase to alumina, mentioned above, an insufficient amount of the latter was used, thereby leaving some of the enzymatic activity still remaining in the residual solution.

The activity in the latter solution was found to have five catechol units to each *p*-cresol unit, instead of 1.5 catechol to one *p*-cresol unit in the case of the material adsorbed to the alumina. In other words, the activity toward the *p*-cresol appears to be more readily adsorbed to the alumina than the part which brings about the oxidation of the catechol. By repeating the treatment with insufficient alumina, it was possible to obtain, as shown by the data in Table III, preparations very high in catechol activity compared to the activity toward *p*-cresol.

TABLE III

Showing the preferential adsorption of the activity toward *p*-cresol by alumina at *p*H 5.

		p-Cresol units per cc.	Catechol units per cc.	Catechol units / *p*-cresol units
Orig. crude prepn.		43	53	1.3
After adsorptions	first	13.5	30	2.2
	second	5.3	22.5	4.3
	third	2.0	17.4	8.7
	fourth	0.58	5.4	9.3

Likewise, it was possible to increase the activity toward *p*-cresol more than toward catechol by eluting that part of the tyrosinase preparation which was adsorbed to alumina. For this purpose a series of adsorptions, followed by elutions by means of secondary sodium phosphate, were performed and solutions were obtained having as high as 3 *p*-cresol units to one catechol unit, whereas the original solution contained about 4 *p*-cresol units to 5 of the catechol units. Furthermore, the substance responsible for the *p*-cresol activity did not pass through a dialyzing membrane, and was completely precipitated from solution when the latter was made 0.6 saturated with ammonium sulfate.

The facts that the activity toward *p*-cresol can be concentrated by absorption techniques relatively more rapidly than the activity toward catechol, that it does not dialyze through semi-permeable membranes, and that it is completely precipitated by ammonium sulfate, all point toward the probability that the aerobic oxidation of *p*-cresol is catalyzed by an enzyme different from the one which brings about the oxidation of catechol.

As further support for this view may be cited the effect of gentle heat. A tyrosinase preparation containing 73 catechol units and 11.5 *p*-cresol units, or 6.4 catechol to 1 *p*-cresol unit, was heated for five minutes at 60°. The catechol activity dropped to 6.5 units and the *p*-cresol activity to 0.3 unit, making the ratio of the two activities 22 catechol units to 1 *p*-cresol unit, thus showing that activity toward *p*-cresol suffered more inactivation than the activity toward catechol.

If the catalytic activity toward *p*-cresol were due to some non-enzymatic factor plus catechol oxidase, as is believed to be the case by some, then the addition of catecholase to a solution of tyrosinase preparation from the wild mushroom, *Lactarius piperatus*, which is rich in *p*-cresol activity and poor in catechol oxidase, thereby containing a relatively high concentration of the factor, should cause an increase in the *p*-cresol activity greater than the sum of *p*-cresol activities of the two preparations. The data given in Table IV, however, show that the activities of the two preparations were just additive and mutually independent.

TABLE IV

Units	1 cc. *Lactarius piperatus* prepn.	1 cc. *Psalliota campestris* prepn.	1 cc. each prepn.
Catechol	0.10	1.47	1.56
p-Cresol	1.05	0.23	1.26

To make certain that the lack of increase in the *p*-cresol activity could not be ascribed to the *Lactarius piperatus* preparation containing a "factor" not suitable for enabling the catechol oxidase, obtained from *Psalliota campestris*, to oxidize *p*-cresol, experiments like those corresponding to the data in Table IV, were repeated using two preparations both from *Psalliota campestris*. Again the two activities were additive and independent of each other.

TABLE V

Units	1 cc. *Psalliota campestris* No. L	1 cc. *Psalliota campestris* No. 70	1 cc. of each prepn.
Catechol	0.52	1.44	1.90
p-Cresol	1.03	0.24	1.22
	Prepn. No. L heated at 60° for 5 min.		
Catechol	0.27	1.44	1.65
p-Cresol	.15	0.24	0.43
	Prepn. No. L acidified to below *p*H 3.5 for 5 min.		
Catechol	0.0	1.44	1.40
p-Cresol	.0	0.24	0.28

Furthermore, if the activity toward *p*-creso is due to some "factor" plus catechol oxidase then a preparation that had been inactivated should regain its activity toward *p*-cresol on the

addition of some active catechol oxidase. To test this possibility preparation No. L was inactivated both by heat and with acid, after which it was added to preparation No. 70 and the activities again measured. In neither instance was there any indication of a non-enzymatic factor which operated with the catechol oxidase in bringing about the oxidation of *p*-cresol.

If there is a "factor" which together with catecholase catalyzes the aerobic oxidation of monohydric phenols, such as *p*-cresol, it is not nonenzymatic, since it has so many properties common to enzymes. Thus it is adsorbable to alumina and kaolin, completely precipitated by ammonium sulfate, does not pass through semipermeable membranes, is heat labile, readily inactivated by acid, and by methods generally employed in the purification of enzymes it has been possible to separate it partially from catechol oxidase.

It should be mentioned that so far the adsorption to alumina and kaolin has failed to affect the ratio of catechol oxidase to the activity responsible for the oxidation of *p*-cresol in the case of tyrosinase preparations obtained from the wild mushrooms, *Lactarius piperatus* and the common pasture puff ball, *Calvatia cyathiformis*. Both of these preparations were rich in activity toward *p*-cresol and poor in catechol oxidase.

Summary

1. The ratio of the activities toward catechol and *p*-cresol in tyrosinase preparations from the common mushroom, *Psalliota campestris*, can be varied by adsorption of the enzymes to alumina and kaolin.

2. By means of partial adsorption it is possible to obtain preparations in the residual solution rich in catechol oxidase and poor in activity toward *p*-cresol.

3. The ratio of the activity toward *p*-cresol to that toward catechol was greater in the elutate from the adsorption than in the original solution.

4. The activity toward *p*-cresol possesses properties common to enzymes in general. It is heat labile, inactivated by dilute acids, does not pass through semi-permeable membranes, and can be precipitated from solution by ammonium sulfate.

5. The catechol activity and the activity toward *p*-cresol are mutually independent.

6. So far attempts to affect the ratio of the catechol and *p*-cresol activities of tyrosinase preparations from the mushroom, *Lactarius piperatus* and the puff ball, *Calvatia cyathiformis*, have been unsuccessful.

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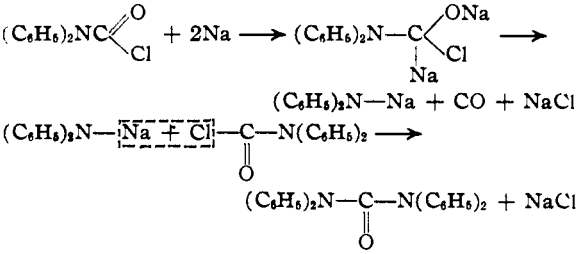
[CONTRIBUTION FROM THE CHEMISTRY LABORATORY OF THE UNIVERSITY OF WASHINGTON]

Reactions of Sodium and Potassium on Acid Chlorides

BY IRWIN A. PEARL, THEODORE W. EVANS AND WILLIAM M. DEHN

In a prior article[1] it was reported that diphenylcarbamine chloride treated with sodium in boiling toluene yielded tetraphenyloxamide. Further work now has shown conclusively that the reaction follows the more interesting course to form tetraphenylurea and carbon monoxide.[2] That tetraphenyloxamide was not first formed in the reaction was proved by the following experiments. Tetraphenyloxamide, prepared from diphenylamine and oxalyl chloride by the method of Stolle,[3] was treated with atomized sodium in boiling toluene under the same conditions as was the diphenylcarbamine chloride. No tetraphenylurea could thus be found, or from tetraphenyloxamide by heating for two hours at 195°. The reaction can be explained in the following manner



Proof of this type of mechanism was obtained by treating diphenylcarbamine chloride with an excess of sodium in toluene and obtaining sodium diphenylamine in the residue. These observa-

(1) Evans and Dehn, THIS JOURNAL, **52**, 3645 (1930).

(2) Hammerich [*Ber.*, **25**, 1826 (1892)] reported the reaction of ditolylcarbamine chloride with sodium in ether to give the oxamide derivative.

(3) Stolle, *ibid.*, **46**, 3916 (1913).