impractical. β-Naphtholazobenzene-3-methyl-4-chloro-6-sodium sulfonate undergoes only a hydrogenolysis, and does not give up its chlorine at one atmosphere pressure. Since steric hindrance does not seem to be involved, and since 2-chloro-5-aminotoluene-4-sulfonic acid, the diazonium component, dehalogenates quite readily at one atmosphere, the behavior of this particular azo compound is rather peculiar. However, if the removal of halogen from the same compound is attempted in the presence of a chemically equivalent amount of α-amino-β-naphthol, a higher hydrogen pressure must be employed to obtain dehalogenation.

Another point of interest is the fact that no dehalogenation occurs when dioxane is the solvent. This is generally true, and it is believed that failure to obtain dehalogenation when employing dioxane is due to the insolubility of the alkali which forms an emulsion with this solvent, and then coats the Raney nickel and renders it inactive. If sodium ethylate, which is soluble in dioxane, is employed as a substitute for the alkali, only a hydrogenolysis of the azo group occurs, and no dehalogenation. It would appear, therefore, as though hydroxyl ion is indispensable for a successful dehalogenation by this method.

The Raney nickel catalyst should prove useful in the quantitative dehalogenation of organic compounds both in synthetic and analytical work. The reductive procedure employing this catalyst eliminates many of the undesirable features of the well-known Carius method for halogen determination. For example, the long heating period, the possibility of contaminating the reaction products with fragments of glass, and the many hazards are entirely avoided. In addition, the catalytic method yields results that are within the usual limits of error, and provides two different criteria for quantitative evaluation. The volume of hydrogen consumed serves as one, and an estimation of the alkali halide formed serves as the second.

Summary

The catalytic hydrogenation and dehalogenation of substituted azo compounds with Raney nickel, at room temperature and from 1 to 3 atmospheres absolute pressure, has been demonstrated. These reactions occur quantitatively, the hydrogen adding to and cleaving the compounds with greater ease in ethanol than in dioxane. For dehalogenation to occur, the presence of a stoichiometrical equivalent of alkali is indispensable in order to neutralize the hydrogen halide formed. In the presence of other substituents in the same benzene nucleus, the removal of halogen proceeds best at 3 atmospheres absolute pressure. Dioxane is not a satisfactory solvent for catalytic dehalogenation. This general method of reduction, employing the Raney nickel catalyst, should prove to be a useful tool in the analysis and estimation of the azo dyestuffs.

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[CONTRIBUTION FROM THE DEPARTMENT OF CHEMISTRY, COLUMBIA UNIVERSITY]

On the Nature of the Enzyme Tyrosinase. II

BY G. G. PARKINSON, JR., AND J. M. NELSON

One of the reasons why the phenolic oxidase, tyrosinase, has attracted attention is that it is supposed to catalyze two essentially different reactions: first, the oxidation of certain monohydric phenols by inserting a hydroxyl group ortho to the one already present, and, second, the oxidation of certain *o*-dihydric phenols to their corresponding *o*-quinones. *p*-Cresol and catechol have been used quite generally as substrates in the study of these two enzymic activities, which will therefore be referred to as cresolase and catecholase activities.

One of the convenient sources for tyrosinase is the common mushroom, *Psalliota campestris*. When a water extract of the ground mushroom is permitted to stand exposed to air, it darkens and gradually loses enzymic activity. This loss in activity also occurs when the enzyme in the water extract is purified by the usual methods, such as precipitation by cold acetone, ammonium sulfate, dialysis, etc. On comparing the losses of the two activities, it is found that the loss of cresolase activity is greater than that of the catecholase activity. In fact, often the major portion of the cresolase activity is lost. This loss in the latter activity, as the purification pro-

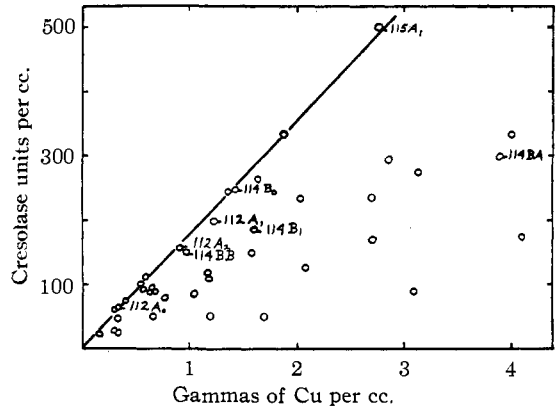

Fig. 1.—Diagram showing that cresolase units per γ of copper reach a maximum value in highly purified tyrosinase preparations high in cresolase activity.

The early stages of purification procedure for these preparations were similar to those for preparations C101A, C94, etc., described by Ludwig and Nelson. At this stage about 250,000 A. and N. catecholase and 100,000 cresolase units remained from 18 lb. (8 kg.) of mushrooms (*Psalliota campestris*). The volume was generally about two liters. Such solutions were made *p*H 5.0 with 0.1 *M* acetic acid and treated with 1 g. of kaolin for every 10–12 thousand catecholase units, stirred, and centrifuged. Elution of the adsorbed enzyme with 0.2 *M* disodium phosphate yielded dark brown solutions containing 80–90% of the cresolase and 15–25% of the catecholase activity in 500–700 cc. The eluates thus obtained were treated dropwise with *M* barium acetate until most of the color had been carried down by the freshly formed barium phosphate. The latter was centrifuged off, carrying down with it 50–75% of the enzyme content. (Elution of this barium phosphate with excess disodium phosphate gave solutions containing a considerable part of the adsorbed enzyme together with some color. These eluates were also used after further purification.) The material in the almost colorless supernatant liquid was precipitated with 0.6 saturated ammonium sulfate, taken up in 0.05–0.1 its original volume of water, and dialyzed in the refrigerator against glass distilled water. Such treatment as this, involving a total of 14 different steps, yielded Prepn. $114B_0$. To ensure that no higher value for cresolase units per γ of copper could be obtained by more extensive purification, Prepn. $115A_1$ was given, in addition to the treatment used in the case of Prepn. $114B_0$, seven further steps of purification as indicated hereafter. A solution which had had treatment similar to that of Prepn. $114B_0$ was treated with sufficient alumina to adsorb practically all the activity, and centrifuged. The adsorbed enzyme was eluted with 0.2 *M* disodium phosphate and dialyzed. This solution was treated dropwise with a 10% saturated solution of lead acetate until a slight cloudiness was noted. Then 5, 10, 25 and 50% by volume of cold acetone was added with intervening removal of the respective precipitates by centrifuging. The enzyme contained in the precipitates from the 10 and 25% acetone treatments was dissolved in 0.2 *M* disodium phosphate and the two fractions combined. This solution was treated with barium acetate again, dialyzed, adsorbed to alumina again, and finally dialyzed against glass distilled water. As is seen in the figure both Prepns. $114B_0$ and $115A_1$ had the same (maximum) cresolase activity per γ of copper. They were obtained from two different lots of mushrooms.

To show that repeated purifications of a given preparation also failed to increase the cresolase activity per γ of copper beyond the maximum value indicated by the straight line in the figure, Prepn. $112A_0$ was subjected to further purification yielding Prepn. $112A_1$. The latter was purified still further yielding Prepn. $112A_2$. As will be noted, all three of these preparations have values falling on the line.

The cresolase activity value of Prepn. $114B_0$ was on the line. Upon dialysis, for some unknown reason it became partially inactivated yielding Prepn. $114B_1$ which was low in cresolase activity per γ of copper. But by fractionation with the lead and acetone treatment described above, followed by dialysis, it was possible to remove the inactivated enzyme in the form of Prepn. 114BA, leaving Prepn. 114BB, the cresolase activity of which was again practically at the maximum value.

As further support for the conclusion that the value of 175 cresolase units per γ of copper is a true maximum, it may be mentioned that preparations which gave this value were obtained from two other lots of mushrooms in addition to those mentioned above. And in no case was a preparation obtained which gave a higher value.

All units were determined on the Warburg respirometer at 25° by the method of Adams and Nelson.[2] Copper was determined by the method of Warburg as applied by Kubowitz (F. Kubowitz, *Biochem. Z.*, **292**, 221 (1937)).

gresses, led Keilin and Mann[1] to conclude that it was probably some other factor, and that tyrosinase, itself, is only active toward polyhydric phenols, of which catechol is a representative. For this reason they appear to prefer the name polyphenol oxidase as a more suitable one than tyrosinase.

On the other hand, Dalton and Nelson,[2] working with tyrosinase preparations from the wild mushroom, *Lactarius piperatus*, found a constant relationship between the cresolase and catecholase activities. Heat, change in *p*H, adsorption, and precipitation treatments of the enzyme all were ineffective in changing this ratio of the two activities.

Adams and Nelson[3] noticed that when solutions of tyrosinase preparations, from *Psalliota campestris*, which had lost considerable activity, especially cresolase activity, during partial purification of the enzyme, were treated with kaolin, more of the remaining cresolase than catecholase activity was adsorbed. Because of this difference

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

(2) H. R. Dalton and J. M. Nelson, THIS JOURNAL, **61**, 2946 (1939).

(3) M. H. Adams and J. M. Nelson, *ibid.*, **60**, 2472, 2474 (1938).

in ease of adsorption between the two activities, it has been possible to obtain, by procedure described in the legend of Fig. 1, from the common mushroom highly purified preparations of tyrosinase possessing high cresolase activity. In fact, the ratios of the catecholase to cresolase activity, in these high cresolase preparations, have been found to resemble closely those of the original fresh water extracts of the plant.

In Fig. 1 are plotted cresolase units[4] against γ's of copper for forty quite highly purified preparations of high cresolase activity. About a third of these preparations show a constant value of about 175 cresolase units per γ of copper. In other words, there seems to be a limit to the activity toward *p*-cresol, when based on the copper content. All the preparations for which the copper content was too high and the values fall below the line in Fig. 1, can be accounted for by the instability of these highly purified preparations.

TABLE I

Prepn.	Cresolase units per γ Cu	Catecholase units (A and N) per γ Cu
X11B	162	35
$112A_0$	191	191
$112A_1$	165	208
$112B_1$	187	91
$112C_1$	190	47
$112A_2$	173	250
$112B_2$	173	95
113B	178	56
$114B_0$	175	59
114BB	157	48
$113M_1$	186	182
$113M_2$	163	153
$115A_0$	178	47
$115A_1$	181	48
	Av. 175.6	

Dry weight determinations were made by the method of Lutz and Nelson[5] on several high cresolase preparations. Four of these preparations, showing the lowest dry weight values, are given in Table II.

TABLE II

Preparation	X11B	$112A_2$	113B	$115A_1$
Cresolase units per mg. dry weight	161	160	175	145
Copper, %	0.10	0.09	0.10	0.08

(4) One unit of cresolase is the amount of enzyme required to cause the uptake of 10 c. mm. of oxygen per minute, when acting on 4 mg. of *p*-cresol. One Adams and Nelson catecholase unit is the amount of enzyme required to cause the uptake of 10 c. mm. of oxygen per minute when acting on a mixture of 0.1 mg. of catechol and 5 mg. of hydroquinone (for further details see Adams and Nelson).

(5) J. B. Lutz and J. M Nelson, *J. Biol. Chem.*, **107**, 169 (1934).

Table I contains the number of cresolase and Adams and Nelson catecholase units per γ of copper, for fourteen high cresolase preparations, whose copper values lie close to the straight line in Fig. 1.

The apparent non-proportionality of the catecholase activity to copper in the case of these high cresolase preparations, as shown by the values in the table, will be shown below to be due to the Adams and Nelson method for measuring catecholase activity. This method is not applicable to these high cresolase preparations.

Ludwig and Nelson working with preparations high[6] in catecholase activity, in which the activity (A. and N. units) was proportional to copper, found that one A. and N. catecholase unit, when allowed to act on sufficient catechol, became inactive when the reaction had reached the point where[7] 100 c. mm. of oxygen had been taken up. In fact, this value of 100 c. mm. of oxygen uptake per unit held for so many of their high catecholase preparations that they suggested the value as a possible quantitative measure of catecholase activity.

After the method had been developed for obtaining high cresolase preparations, Wisansky (unpublished) working in these laboratories determined the catecholase units by the inactivation method and compared them with the values obtained by the Adams and Nelson method. In Fig. 2 are shown the number of c. mm. of oxygen uptake required for the inactivation of one Adams and Nelson catecholase unit for several preparations high in cresolase activity. Obviously, the observation of Ludwig and Nelson that the inactivation of one Adams and Nelson catecholase unit corresponds to an oxygen uptake of 100 c. mm. does not hold in the case of the high cresolase preparations. For example, when an amount of a high cresolase preparation containing 3 cresolase units and one Adams and Nelson catecholase unit was permitted to act on an excess of catechol, 900 instead of 100 c. mm. of oxygen uptake was required to bring about the inactivation of the one unit of catecholase. It is evident, therefore, that either the inactivation method of

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

(7) A high cresolase preparation is any preparation in which the cresolase activity is proportional to copper and the A. and N. catecholase units are not (0.5 to 5 cresolase units to one A. and N. catecholase unit). A high catecholase preparation is one in which the catecholase activity (A. and N. units) is proportional to copper but the cresolase activity is not (0.0 to 0.3 cresolase units to one A. and N. catecholase unit).

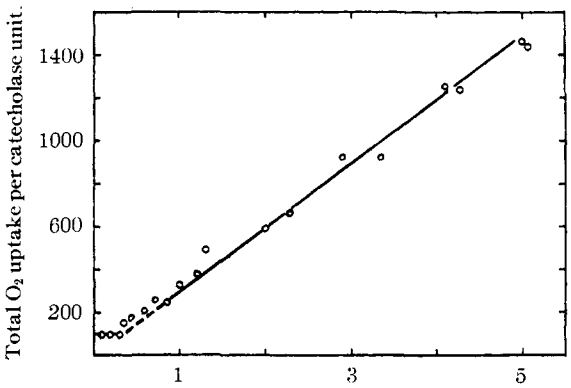

Fig. 2.—Showing that for tyrosinase preparations high in cresolase activity the total oxygen uptake corresponding to the inactivation of one A. and N. catecholase unit: first increases as the ratio of cresolase to A. and N. catecholase units increases and, second, this increase is constant and amounts to 300 c. mm. per unit increase in the cresolase to A. and N. catecholase ratio. The figure also shows that the inactivation value per A. and N. catecholase unit is constant in the region from 0 to 0.3 cresolase per A. and N. catecholase unit, which region corresponds to high catecholase preparations.

The values shown in the figure were determined on the Warburg respirometer at 25° in the manner described by Ludwig and Nelson, the reaction mixture consisting of 1 cc. of gelatin solution (5 mg.), 2 cc. of 0.2 *M* phosphate buffer (*p*H of final reaction mixture 6.9), 1 cc. of enzyme solution, and sufficient water to make the final volume 8 cc., including 1 cc. of catechol solution (4 mg.) added from the side arm. A second vessel was always used with 2 cc. of catechol solution (8 mg.). The control vessel received water in place of enzyme solution.

The three constant values shown for high catecholase preparations were taken from the data of Ludwig and Nelson, being representative of nine values given by them and confirmed in connection with this work. Of the values shown for high cresolase preparations, eight were obtained by Wisansky in these Laboratories and the remaining six in connection with this work.

Ludwig and Nelson, or the catechol–hydroquinone method of Adams and Nelson for determining catecholase units, does not apply in the case of high cresolase preparations.

It has been found possible to decide between these two methods in favor of the inactivation method in the following way. Keilin and Mann, as well as Ludwig and Nelson, showed that the catecholase activity, in the case of high catecholase preparations, was proportional to the copper content of the enzyme. Thirteen[8] different highly purified high catecholase preparations from five different lots of mushrooms showed an average of 351 (a. d. ± 5%) Adams and Nelson catecholase units per γ of copper. Consequently the inactivation of an amount of high catecholase preparation containing $^1/_{350}$ γ of copper (one A. & N. catecholase unit) corresponds to an oxygen uptake of 100 c. mm. When, however, an amount of a high cresolase preparation containing the same amount of copper, $^1/_{350}$ γ, was allowed to act on an excess of catechol, 150 c. mm. of oxygen was taken up before inactivation occurred. The catecholase in the high cresolase preparations appears to be more stable than that in the high catecholase preparations.

If 150 c. mm. of oxygen uptake is taken as the measure of one unit of catecholase activity in the high cresolase preparations, instead of the A. and N. catecholase unit, then by dividing the oxygen uptake values indicated in Fig. 2 by 150, it turns out that all the high cresolase preparations mentioned in the Figure are alike, *i. e.*, they all contain one cresolase unit to two of the new catecholase units.

It will be recalled that $^1/_{175}$ γ of copper corresponds to one unit of cresolase, and the same amount of copper, $^2/_{350}$ γ, corresponds to two of the new catecholase units. Hence it follows that if the catecholase values per γ of copper mentioned in Table I had been determined by the inactivation method instead of the Adams and Nelson rate method, there would have been no appreciable variation in the amount of oxygen uptake corresponding to the inactivation of one unit (new) of catecholase. This constant relationship between cresolase and catecholase activity is also supported by direct determinations of catecholase activity in the case of the high cresolase preparations. These direct determinations (Table III) were made by observing the rate of oxygen uptake when the enzyme acted on 4 mg. of catechol alone, instead of on a mixture of 0.1 mg. of catechol and 5 mg. of hydroquinone, as in the method of Adams and Nelson. The objection to this direct method, as pointed out by Adams and Nelson, is that the rapid inactivation of the enzyme makes accurate rate measurements of oxygen uptake very difficult. In spite of the 20–30% error involved, if the rate unit is defined as the uptake of 10 c. mm. of oxygen per minute,

TABLE III

Preparation	115C	115D	115F	114M	C9
Catecholase units (direct) / Cresolase unit	1.86	1.96	1.75	1.60	1.70

(8) Included the six preparations with values above 300 mentioned by Ludwig and Nelson in their Table I.

the ratio of catecholase to cresolase units was found to approach the value of 2 to 1 for a series of high cresolase preparations.

Facts obtained in the study of high cresolase preparations such as: (1) the constant ratio between the cresolase and catecholase activities; (2) the limit of cresolase activity when based on the amount of catecholase activity; (3) the dependency of both activities on the copper content; (4) the ratio of the two activities resembling closely that in the fresh water extract of the plant, all point to the conclusion that tyrosinase as it occurs in the common mushroom is one enzyme complex rather than two separate independent factors.

Summary

1. A method is described for preparing tyrosinase from the common mushroom, *Psalliota campestris*, high in cresolase activity.

2. In the preparations high in cresolase activity the latter is proportional to copper, and to catecholase activity, both activities being dependent on the same copper content.

3. The Adams and Nelson method of determining catecholase activity does not apply in preparations high in cresolase activity.

4. In high cresolase preparations the volume of oxygen uptake corresponding to the inactivation of an amount of catecholase containing $\gamma/350$ of copper, was found to be 150 c. mm. instead of 100 c. mm. as found by Ludwig and Nelson in the case of high catecholase preparations.

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[CONTRIBUTION FROM THE DEPARTMENT OF CHEMISTRY, NEW YORK UNIVERSITY]

The Reaction of 2-Chloro-5-nitropyridine and Thiourea

BY ALEXANDER R. SURREY AND H. G. LINDWALL

In a recent paper[1] a description of a method of preparation of 5,5′-dinitro-2,2′-dipyridylsulfide (I) is included. A better method of obtaining this compound has now been found, using the reaction of 2-chloro-5-nitropyridine and thiourea in aqueous solution. This new procedure results in a better yield and a high degree of purity for compound I.

When these two reagents are allowed to react in absolute alcohol, an addition product (II) results which can be decomposed with sodium carbonate to form 2-thiol-5-nitropyridine.[2]

That certain halogen compounds and thiourea can produce either thiols or sulfides has been demonstrated by others. Depending upon the conditions of the experiment, 2-chlorobenzothiazole and thiourea react in aqueous solution[3] to give predominantly either the sulfide or thiol; in this case no addition compound could be isolated. Also, the reaction of 4-chloroquinaldine and thiourea[4] in alcoholic solution gives an addition compound which can be decomposed by sodium carbonate to yield either the sulfide or the thiol.

Inasmuch as an addition compound could be isolated in this present investigation, several reactions involving compound II were studied in order to determine a possible mechanism for the formation of sulfide (I) in aqueous solution. (a) It was found that upon heating the addition compound (II) in aqueous solution, it decomposed slowly to form the sulfide. At the end of one hour, 20% of I was formed, at the end of three and one-half hours, 60%. The decomposition was complete after eight hours. (b) When compound II was allowed to react with 2-chloro-5-nitropyridine (III) more sulfide was obtained than could be expected if only compound II had decomposed. (c) With compound II and 2-thiol-5-nitropyridine (IV) under the same conditions, as much sulfide was formed in one hour as in (b) in four hours. (d) In the preparation of the addition compound, if any water was present in the alcohol, the reaction did not proceed as expected, but rather the sulfide was obtained.

From the experimental data, it seemed reasonable to assume that the addition compound, in aqueous solution, decomposed to form some thiol (IV) which then reacted with II to yield the sulfide. The reaction of II and III could be explained on this basis since some thiourea would be expected to be liberated from the reaction of the

(1) THIS JOURNAL, **62**, 173 (1940).
(2) Rath and Binz, *Ann.*, **487**, 105 (1931).
(3) Watt, *J. Org. Chem.*, **4**, 436 (1939).
(4) Rosenhauer, Hoffman and Heuser, *Ber.*, **62**, 2730 (1929).