[CONTRIBUTION FROM THE DEPARTMENT OF CHEMISTRY, COLUMBIA UNIVERSITY]

The Action of Tyrosinase on Hydroquinone

BY DONALD C. GREGG AND J. M. NELSON

Bertrand[1] pointed out as early as 1896 that tyrosinase when added to an aqueous solution of hydroquinone failed to bring about any aerobic oxidation of the latter. This inability of tyrosinase to catalyze the oxidation of hydroquinone often has been used as a means of distinguishing between tyrosinase and laccase, a phenolase, or possibly a group of phenolases, which possess great activity toward hydroquinone. Graubard[2] showed that if a small amount of catechol is added to an aqueous solution of hydroquinone and tyrosinase, then the hydroquinone is oxidized. He suggested that probably the catechol, which is known to be oxidized by the tyrosinase, functions so to speak as a shuttle, the *o*-benzoquinone, resulting from the oxidation of the catechol, oxidizing the hydroquinone and itself being reduced back to catechol. Adams and Nelson[3] found that the rate of oxygen uptake when hydroquinone was oxidized by tyrosinase, in the presence of a small amount of catechol, was proportional to the amount of enzyme used. They therefore proposed the use of this mixture for the determination of the enzyme's activity toward catechol. Subsequently Ludwig and Nelson[4] showed that the activity of their highly purified preparations of tyrosinase, when determined by the Adams and Nelson method, was proportional to the copper content of the enzyme.

It is well known that when catechol is oxidized by an excess of tyrosinase, two atoms of oxygen are consumed per molecule of catechol oxidized. Ludwig and Nelson, however, found that if less than a sufficient amount of their highly purified enzyme preparation was used, then the latter was inactivated before all of the catechol had been completely oxidized. The total amount of oxygen consumed in the inactivation of the enzyme amounted to 100 cu. mm. for each Adams and Nelson catecholase unit inactivated. Recently Parkinson and Nelson[5] have obtained highly purified tyrosinase preparations from the common mushroom, *Psalliota campestris*, which differ from those described by Keilin and Mann and by Ludwig and Nelson (obtained from the same source) in possessing a higher activity toward *p*-cresol. Furthermore, both the latter activity as well as the activity toward catechol were found to be proportional to the copper content of the enzyme. For convenience they designated their preparations as high cresolase preparations and preparations low in activity toward *p*-cresol but high in activity toward catechol, as for example those studied by Ludwig and Nelson, as high catecholase preparations of tyrosinase.

On determining the oxygen uptake corresponding to the inactivation of one Adams and Nelson unit of activity toward catechol, Parkinson and Nelson found, in the case of their high cresolase preparations, that it agreed with the enzyme's activity toward *p*-cresol but not with its activity toward catechol. This fact suggests that the same component of the enzyme responsible for the oxidation of *p*-cresol may be responsible for the oxidation of the hydroquinone.

Bordner and Nelson[6] have shown that tyrosinase cannot oxidize *p*-cresol without the simultaneous oxidation of some *o*-dihydric phenol, such as catechol. It is necessary, so to speak, to prime the enzyme by oxidizing catechol, in order that it can introduce the second hydroxyl group into the *p*-cresol. Gregg and Nelson[7] have shown the same holds true for the action of tyrosinase on phenol. Instead of the catechol acting as a shuttle in the oxidation of the hydroquinone, it primes the tyrosinase when it is oxidized so that the enzyme can oxidize the hydroquinone.

The observation made by Ludwig and Nelson that hydroquinone exerts a protective influence on the tyrosinase against inactivation just as *p*-cresol does is also in line with the view that the oxidation of these two substrates is brought about in the same manner. This protective influence of the hydroquinone is also shown upon comparing Curves I, III and V in Fig. 1. Curve V represents the oxidation of 4 mg. of catechol by a given amount of tyrosinase, insufficient to bring about complete oxidation of all the catechol present in the reaction mixture, so as to inactivate the en-

(1) G. Bertrand, *Compt. rend.*, **123**, 465 (1896).
(2) M. Graubard, *Enzymologia*, **5**, 332 (1939).
(3) M. H. Adams and J. M. Nelson, THIS JOURNAL, **60**, 2474 (1938).
(4) B. J. Ludwig and J. M. Nelson, *ibid.*, **61**, 2601 (1939).
(5) G. G. Parkinson and J. M. Nelson, *ibid.*, **62**, 1693 (1940).
(6) C. A. Bordner and J. M. Nelson, *ibid.*, **61**, 1507 (1939).
(7) D. C. Gregg and J. M. Nelson, *ibid.*, **62**, 2506 (1940).

zyme. Curve I shows that the same amount of enzyme was inactivated when a small amount of catechol (0.04 mg.) functioned as a shuttle in the presence of an excess of ascorbic acid. The latter is not oxidized by the enzyme and only serves to reduce the oxidized catechol back to catechol. These two experiments show that a given amount of the enzyme was inactivated when it had catalyzed the oxidation of a definite amount of catechol, irrespective of the concentration of the latter. When 2 mg. of hydroquinone was substituted in place of the 4 mg. of ascorbic acid in the above experiment (Curve I), then the rate of oxygen uptake was that represented by Curve III. It will be noticed that considerably more oxygen uptake occurred in the case of Curve III than in the case of the ascorbic acid experiment (Curve I). In other words, the enzyme was able to bring about more oxidation in the presence of the hydroquinone than it did in the presence of the ascorbic acid, indicating that besides the catechol, hydroquinone also was oxidized by the tyrosinase.

Laccase differs from tyrosinase in that it does not possess the ability to oxidize monohydric phenols by inserting a hydroxyl group ortho to the one already present. When hydroquinone was oxidized by laccase, *p*-benzoquinone was formed and one atom of oxygen was consumed per molecule of the hydroquinone (Curve II). When, however, the same amount of hydroquinone was oxidized by means of tyrosinase in the presence of 0.04 mg. of catechol, considerably more than one atom of oxygen per molecule of the hydroquinone was taken up (Curve III). It is apparent, therefore, that the tyrosinase brought about a different type of oxidation than did the laccase, probably the introduction of a third hydroxyl group on the hydroquinone, as in the oxidation of monohydric phenols.

As further evidence that the activity of tyrosinase toward monohydric phenols (the introduction of a hydroxyl group) plays a part in the oxidation of hydroquinone is indicated by the experiment represented by Curve IV. This experiment differed from that belonging to Curve III in that a high cresolase preparation of tyrosinase was used instead of a high catecholase preparation as in the experiment corresponding to Curve III. Due to the relatively large activity toward monohydric phenols, as shown by its activity toward *p*-cresol, a much greater uptake of oxygen occurred.

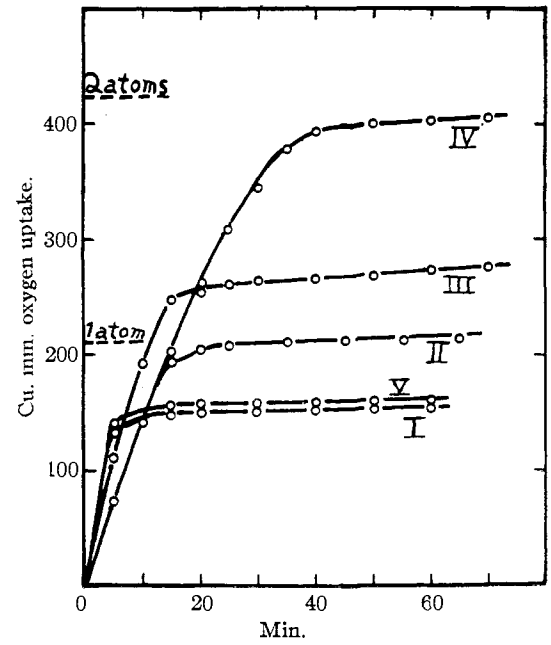

Fig. 1.—Showing the effect of hydroquinone on the oxidation of catechol. Reaction rates were followed by using Warburg respirometers, flask capacity 30 cc., temp. 25°. Curve I: Reaction mixture contained 2 cc. (0.2 *M*) citrate (0.4 *M*) phosphate buffer (*p*H 6.5), 1 cc. aqueous solution of gelatin containing 5 mg., 1 cc. aqueous solution of 5 mg. ascorbic acid and 0.1 mg. catechol, 1 cc. enzyme solution containing 2.8 catecholase and 0.32 cresolase units, (high catecholase prepn.) and sufficient water to make final volume 8 cc. Curve II: Reaction mixture contained buffer and gelatin solution as in Curve I; 1 cc. aqueous solution of 2 mg. hydroquinone and 0.04 mg. catechol was added from the side arm of the flask at zero time. The 1 cc. of enzyme solution contained 1.5 "hydroquinone" units of laccase from *Russula foetens* (Gregg and Miller, THIS JOURNAL, **62**, 1374 (1940)). Curve III: Reaction mixture same as Curve II except that the 1 cc. enzyme solution was a high catecholase tyrosinase preparation containing 2.8 catecholase and 0.32 cresolase units. Curve IV: Same as Curve II except the 1 cc. enzyme solution was a high cresolase tyrosinase preparation containing 2.8 catecholase and 1.5 cresolase units. Curve V: Same as Curve I except the substrate was 1 cc. of an aqueous solution of catechol (4 mg.) instead of the ascorbic acid–catechol mixture. The catecholase units used were those described by Gregg and Nelson, THIS JOURNAL, **62**, 2500 (1940). One of these units corresponds, in the case of high catecholase preparations, to 0.55 Adams and Nelson units. The cresolase units were those of Adams and Nelson.

The reason why the Adams and Nelson method, based on the use of a mixture of 5 mg. of hydroquinone and 0.1 mg. of catechol, could be used as a measure of the catecholase activity of high catecholase preparations is due to the fact that the oxidation of the hydroquinone, under these

conditions, is proportional to the copper content of these preparations. Since it has been shown by Keilin and Mann, and confirmed in these laboratories, that the activity toward catechol, in the case of these high catecholase preparations, is also proportional to the copper content, it follows that the Adams and Nelson values would also be proportional to the catecholase activity.

Summary

1. Evidence is offered indicating that the action of tyrosinase on hydroquinone resembles the action of the enzyme on monohydric phenols.

2. When hydroquinone is oxidized by tyrosinase, in the presence of a small amount of catechol, the reaction and the product formed are different from those occurring when hydroquinone is oxidized by means of laccase.

3. A greater total oxygen uptake occurs in the oxidation of hydroquinone by tyrosinase when the ratio of the activity toward monohydric phenols over the activity toward *o*-dihydric phenols is large.

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Aldehyde–Resorcinol Condensations[1]

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A crystalline condensation product of acetaldehyde with resorcinol has been reported by several investigators[3,4,5] but the statements relative to the constitution of this compound are conflicting. Thus the conventional alkylidene-diphenol[3] as well as the acetal[4] structure has been suggested. It is the purpose of the present investigation to contribute to the elucidation of the structure of not only this disputed compound, but also of its derivatives and homologs and to compare the products with the recently established structures of certain crystalline ketone–resorcinol condensation products[6] which involved multiple alkylation of the resorcinol.

Quantitative elementary analysis of the crystalline, water-free acetates and propionates of the acetaldehyde–, propionaldehyde– and iso-valeraldehyde–resorcinol condensation products indicated an equimolar ratio of the reactants, the same as encountered in the aldehyde–phenol and cresol condensations.[7] However, molecular weight determinations indicated that the ratio of reactants is 4:4. The compounds are insoluble in water, yet seem to possess eight free phenolic hydroxyl groups as indicated by the formation of crystalline octa-acetates, octa-propionates and octa-methyl ethers. The original condensation products, as well as their methyl ethers, retain various amounts of water of crystallization very tenaciously, which may explain the difference in the analytical results obtained by the previous investigators. The condensation products do not appear to undergo hydrolysis upon refluxing with alcoholic sulfuric acid (absence of acetal linkages) nor with hydriodic acid (absence of ether linkages). Thus carbon to carbon linkages throughout are strongly indicated.

In the condensation of resorcinol with ketones, two alkyl radicals always enter the resorcinol molecule. Undoubtedly a similar behavior must be anticipated when an aldehyde is used instead of a ketone in such condensations. On the other hand, equimolar condensation of aldehydes with phenols as previously observed also has to be considered. Of the few structural formulas which would satisfy the above enumerated physical and chemical behavior, as well as that of analogous compounds, open chain as well as ring structures have to be taken into consideration. An open chain structure would imply polymerization and there would be no reason why polymerization should stop at exactly the tetrameric stage, regardless of the aldehyde used, or of the reaction conditions employed. Also the crystalline state and the high melting points of the compounds do not favor such a structure.

(1) This communication is part of a paper entitled: "Syntheses of New Poiycyclics" which was presented before the Division of Organic Chemistry at the Detroit meeting of the American Chemical Society, September, 1940.

(2) Abstracted from the thesis submitted by Heinz J. Vogel to the faculty of the Graduate School of New York University in partial fulfillment of the requirements for the degree of Master of Science.

(3) Michael and Comey, *Am. Chem. J.*, **5**, 349 (1883).

(4) Moehlau and Koch, *Ber.*, **27**, 2887 (1894).

(5) Causse, *Ann. chim.*, [vii] **1**, 90 (1894).

(6) J. B. Niederl and V. Niederl, THIS JOURNAL, **61**, 348 (1939).

(7) J. B. Niederl and co-workers, *ibid.*, **59**, 1113 (1937).