CLXXVIII. THE OXIDATION OF CATECHOL AND 1:2:4-TRIHYDROXYBENZENE BY POLYPHENOL OXIDASE

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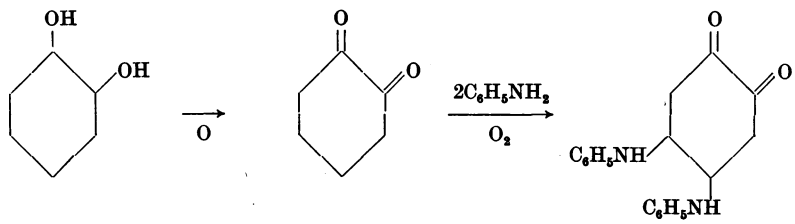
(*Received 21 July 1939*)

The enzyme polyphenol oxidase catalyses the oxidation of *o*-dihydric phenols in general. Oxidation of pyrogallol (1:2:3-trihydroxybenzene), for example, is accompanied by the absorption of 3 atoms of oxygen and the product (purpurogallin) crystallizes out during the reaction [Willstätter & Heiss, 1923]. Catechol, however, does not furnish any directly identifiable product on enzymic oxidation although manometric measurements have shown that 2 atoms of oxygen are absorbed in the reaction [Robinson & McCance, 1925; Pugh & Raper, 1927].

It was suggested by Szent-Györgyi [1925] that *o*-quinone might be the first product of oxidation and Pugh & Raper [1927] obtained experimental evidence in support of this view. They oxidized catechol enzymically in the presence of aniline when dianilo-*o*-quinone (I) separated as a scarlet solid. The following mechanism was therefore proposed:

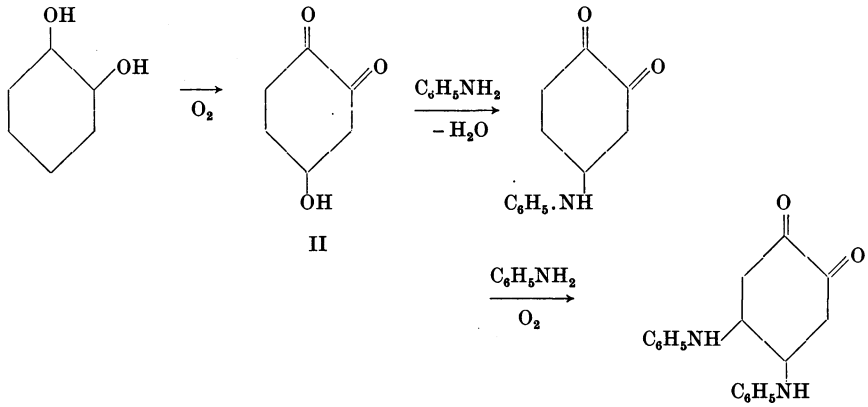


I

Subsequently Wagreich & Nelson [1936] found that the requisite 3 atoms of oxygen were absorbed in the reaction. These authors also found experimental evidence suggesting an alternative mechanism for the oxidation process, which possessed the additional advantage of accounting for the second atom of oxygen absorbed in the oxidation of catechol alone. The formation of *o*-quinone postulated by Pugh & Raper only required the absorption of 1 atom of oxygen prior to the spontaneous reaction of the quinone with aniline; the scheme suggested by Wagreich & Nelson necessitated the oxidation of each catechol molecule by 2 atoms of oxygen before reaction with aniline took place.

The experimental evidence for this hypothesis was based on the observation that when catechol was oxidized at *p*H 6·0 until 2 atoms of oxygen were absorbed, introduction of aniline and fresh enzyme was followed by the gradual absorption of a further atom of oxygen and the formation of dianilo-*o*-quinone. It was

concluded that catechol is probably oxidized to 4-hydroxy-1:2-quinone (II) which then reacts with aniline in the following manner:



In their investigation, Wagreich & Nelson believed that when exactly 2 atoms of oxygen had been absorbed the absence of *o*-quinone could be justifiably assumed. Since, however, oxidation of catechol proceeds to about 2·2 atoms this assumption is by no means certain. Furthermore these authors merely stated that *the same aniloquinone is formed* without giving any information on the important question of how much was produced. The object of the present investigation was to find out more definitely whether *o*-quinone was the enzymic oxidation product of catechol which produced dianiloquinone or whether hydroxyquinone was really the direct product of oxidation reacting with aniline to form dianiloquinone. The experimental procedure adopted consisted of a quantitative study of aniloquinone formation during oxidation of catechol and an examination of the enzymic and autoxidation of 1:2:4-trihydroxybenzene alone and in the presence of aniline.

Experimental

Polyphenol oxidase

The enzyme used by Wagreich & Nelson [1936] was a comparatively crude preparation from mushrooms. The enzyme used in the present work was prepared from the same source, but considerably purified according to the method of Keilin & Mann [1938]. The preparation had a dry weight of 1·8 mg./ml. and Q_{O_2}, 140,000 estimated at 20°.

Catechol oxidations

These were carried out in the usual manner in Barcroft respirometers. The reaction flask received 1 ml. of catechol solution (2·28 mg./ml.), 2 ml. of phosphate buffer (Sørensen, *M*/15, *p*H 5·9 or 7·1) and a dangling tube containing 0·3 ml. of enzyme concentrate. Addition of aniline to the reacting mixture at any stage of the oxidation was effected by rapidly detaching the reaction flask and fixing a second dangling tube in position containing aniline (30–40 mg.), whilst 0·3 ml. of enzyme solution was added to the reaction fluid. After replacing the apparatus in the thermostat and dislodging the tube, shaking was continued for 4 hr. and a final reading taken next morning. The manipulation could be accomplished without seriously affecting the equilibrium of the system.

Detection and estimation of dianilo-o-quinone

The formation of aniloquinone was easily observed qualitatively by the intense red colour which preceded the separation of the insoluble pigment. Extraction with ether provided a means of detecting traces of this compound when no visible reaction occurred, preliminary experiment having shown that no ether-soluble pigment was produced during oxidation of catechol alone. In quantitative determinations the aniloquinone produced subsequent to aniline addition was estimated colorimetrically after 20 hr. by extraction with ether and adjusting the volume of the extract to 50 ml. The results were expressed as percentages of the quantity of aniloquinone formed when oxidation was carried out entirely in the presence of aniline (Table II).

Preparation of 1:2:4-trihydroxybenzene

The following improved method gave an excellent yield of almost colourless product.

(*a*) *1:2:4-Triacetoxybenzene.* *p*-Quinone (15 g.) was mixed with acetic anhydride (45 ml.) and conc. H_2SO_4 (1 ml.) added dropwise (10 min.), the temperature being maintained below 40° by cooling. The pale brown product (20–25 g.) was precipitated by pouring into water. One crystallization from alcohol gave the white crystalline triacetate, M.P. 97–98°.

(*b*) *Hydrolysis of 1:2:4-triacetoxybenzene.* The acetate (10 g.) was mixed with cold, absolute alcohol (30 ml.) and conc. HCl (1 ml.) added. The mixture was heated for 1·5 hr. in an inert gas stream on a water bath at 80°. The liquid remained colourless or became very pale yellow (if refluxed in air, intense red solutions were obtained). The solvent and acid were removed *in vacuo* leaving a yellow syrup which solidified on treatment with chloroform and scratching. The 1:2:4-trihydroxybenzene (4–5 g.) so obtained, M.P. 135–136°, remained quite stable if kept dry.

Oxidations of 1:2:4-trihydroxybenzene

In respirometers, the reaction flask contained phosphate buffer (3·0 ml.) with or without aniline for autoxidations, buffer and enzyme (0·3 ml.) or buffer, enzyme and aniline for enzymic oxidations, the weighed trihydric phenol (4 mg.) being suspended in a dangling tube. For large scale oxidations a quantitative apparatus was constructed having a reaction flask of 1·5 l. capacity connected to a burette system capable of measuring gaseous absorption up to 500 ml. (for general details of this type of apparatus see Jackson, 1938).

Isolation of 5-anilo-4-hydroxy-1:2-quinone

(*a*) *By enzymic oxidation of 1:2:4-trihydroxybenzene.* The reaction flask received phosphate buffer *p*H 5·9 (*M*/30, 700 ml.) and aniline (10 ml.), the *p*H being adjusted to 5·9 by addition of dilute HCl. Enzyme concentrate (50 ml.) was now added and 1:2:4-trihydroxybenzene (1·26 g.) suspended in a weighed tube in the reaction flask, which was then connected to the measuring system and the entire apparatus filled with oxygen. After shaking to temperature equilibrium with the surroundings, the trihydroxybenzene was added to the reaction fluid when oxidation proceeded rapidly, 2 atomic equivalents of oxygen being absorbed in 10 min. Equilibrium was reached after 1 hr. (260 ml. absorbed; required for 2 atoms at 20°, 240 ml.), when the separated solid was filtered off. Yield, 1·5 g. of brownish-maroon powder, M.P. 150° (decomp.), not sharply.

Considerable difficulty was experienced in the purification of this anilo-compound owing to contamination with brown material. The most satisfactory procedure consisted in shaking the crude powder with cold acetone-ether mixture (2 : 3, 150 ml.) which removed a considerable amount of brown resin. The residue was boiled with acetone (50 ml.) and filtered. A deep violet solid remained which was suspended by a thimble in a reflux apparatus containing acetone so that a hot extraction was carried out. The intense red extract yielded an iridescent, violet solid (500 mg.), M.P. 208° (decomp.), which was dissolved by a similar process in benzene (80 ml.). On cooling, a violet-black microcrystalline solid separated. This was finally purified by repeated crystallization from acetic or propionic acid, from which the hydroxymonoaniloquinone separated in masses of glistening, purple micro-needles, M.P. 210° (decomp.). (Found: C, 67·1; H, 4·5; N, 6·5%. $C_{12}H_9O_3N$ requires C, 67·0; H, 4·2; N, 6·5%.)

The hydroxyaniloquinone was easily removed from ethereal solution by dilute sodium carbonate, in which it formed a yellow solution turning violet on acidification. It dissolved readily in aniline in the cold, followed almost immediately by separation of a light brown solid consisting of yellow micro-needles. On heating the mixture, these dissolved, forming a deep red solution turning brown again on cooling due to separation of the solid, which could be filtered off and washed with light petroleum (200 mg. of hydroxyaniloquinone with 7·5 ml. of aniline gave 100 mg. of product). This substance proved to be an unstable addition compound of aniline and hydroxyaniloquinone since, on heating to 100° or boiling with a little benzene followed by dilution with light petroleum, the aniloquinone was recovered quantitatively. (100 mg. of aniline compound gave 70 mg. of hydroxyaniloquinone; $C_6H_5NH_2.C_{12}H_9O_3N$ (100 mg.) requires $C_{12}H_9O_3N$ (70 mg.).)

(*b*) *From autoxidation of 1:2:4-trihydroxybenzene in the presence of aniline.* The procedure was identical with that described under (*a*) except that no enzyme was used. An oxygen absorption of nearly 4 atomic equivalents took place during 1 hr. (460 ml. found; 480 ml. required) and the solid which had separated from the deep red solution was filtered off. Yield, 2 g. of red powder, decomposing above 200°. Purification was effected by boiling with methyl alcohol (50 ml.) and refluxing the undissolved material with hot acetone as described in (*a*). The filtrate gave a deep violet solid (500 mg.), M.P. 210° (decomp.), giving no depression in melting point with hydroxyaniloquinone from (*a*) and behaving in identical manner with aniline, alkali and acid. The methyl alcohol filtrate from above gave a further quantity of red solid (300 mg.) on cooling, which deposited as purple needles of the aniloquinone from acetic acid. A similar colour change from red to purple took place on heating.

Discussion of results

The theory proposed by Wagreich & Nelson, of the formation of hydroxyquinone rather than *o*-quinone during the enzymic oxidation of catechol, depends upon two experimental observations. These are, that when catechol is oxidized at *p*H 6·0 until 2 atoms of oxygen are absorbed, subsequent addition of aniline is followed by the formation of dianilo-*o*-quinone with the absorption of a further atom of oxygen.

The results in Table I show that during oxidation of catechol at *p*H 7·1, addition of aniline after an absorption of 1·71 atoms of oxygen gives only a trace of aniloquinone and none at all after preliminary oxidation of catechol to 1·79 atoms. At *p*H 5·9, however, after 2 atoms of oxygen have been absorbed,

Table I. *Catechol oxidations at* p*H 7·1*

Oxygen absorption at aniline addition (atoms)	Time min.	Total oxygen absorption (20 hr.) atoms	Dianiloquinone
—	—	2·1	—
1·71	34	2·37	+ve
1·79	38	2·20	−ve
1·83	40	2·31	−ve

Table II. *Catechol oxidations at* p*H 5·9*

Aniline added after min.	Oxygen uptake at aniline addition atoms	Total oxygen absorption 20 hr. atoms	% aniloquinone	Oxygen uptake % of 3 atoms
—	—	2·18	—	—
0	0	2·96	100	100
0	0	3·06	100	100
1	—	—	95	—
3	—	—	79	—
8	1·18	2·76	59	91
28	1·52	3·00	53	100
35	1·71	2·83	51	94
56	1·78	2·79	51	93
97	1·98	2·79	30	93
97	1·99	2·76	27	91
198	2·20	2·83	15	94
198	2·20	2·95	15	98

a deep red colour appears with aniline and further oxygen is absorbed to a final value of 2·8 atoms after 20 hr. Yet only a few flecks of aniloquinone can be seen in the brown reaction fluid. When oxidation is first completed (2·2 atoms) before addition of aniline, no colour develops with the amine and only a small quantity of pigment is subsequently extracted by ether. If dianiloquinone is formed from 4-hydroxy-1:2-quinone and aniline, as Wagreich & Nelson suggest, it follows that comparable quantities of the anilo-compound should be formed when aniline is added at the commencement or at the 2 atom stage of the oxidation of catechol; otherwise the total absorption of 3 atoms of oxygen in the latter case can be of no significance. Quantitative estimation of the aniloquinone produced by addition of aniline at various stages of the oxidation (Table II) shows that the maximum quantity is formed when aniline is present from the commencement of the oxidation, the amount decreasing rapidly at first as addition of aniline is delayed and later diminishing much more slowly. After 2 atoms of oxygen are absorbed (97 min. required, compared with 90 min. in Wagreich & Nelson's experiment) only 30 % of aniloquinone is formed; when 200 min. have elapsed before adding aniline the figure is reduced to 15 %. In the preparation of the standard aniloquinone solutions, it is noticeable that one extraction with ether completely removes the red solid leaving the aqueous phase clear and colourless; as introduction of aniline into the oxidizing catechol is gradually delayed, subsequent extraction with ether leaves the aqueous liquid more and more brown in colour.

These observations suggest that catechol is first oxidized to *o*-quinone which is unstable and in the absence of aniline rapidly oxidizes to brown material. The composite graph (Fig. 1) shows the oxygen absorption occurring after additions of aniline to oxidizing catechol in experiments in which the aniloquinone was later estimated. The diminution in the rapid uptake following the additions corresponds to the observed fall in the amount of aniloquinone produced.

Furthermore, in all these experiments the total oxygen absorption after 20 hr. is about 3 atoms (Table II) irrespective of the amount of aniloquinone formed. It follows, therefore, that the uptake of an additional atom of oxygen by oxidizing catechol when aniline is introduced at the 2 atom stage, cannot safely be used in formulating the mechanism of aniloquinone formation.

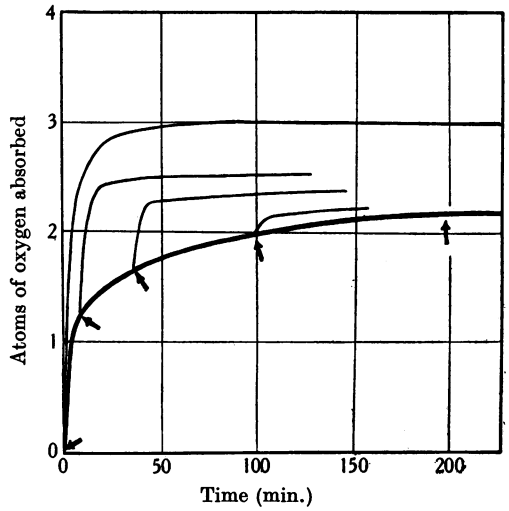
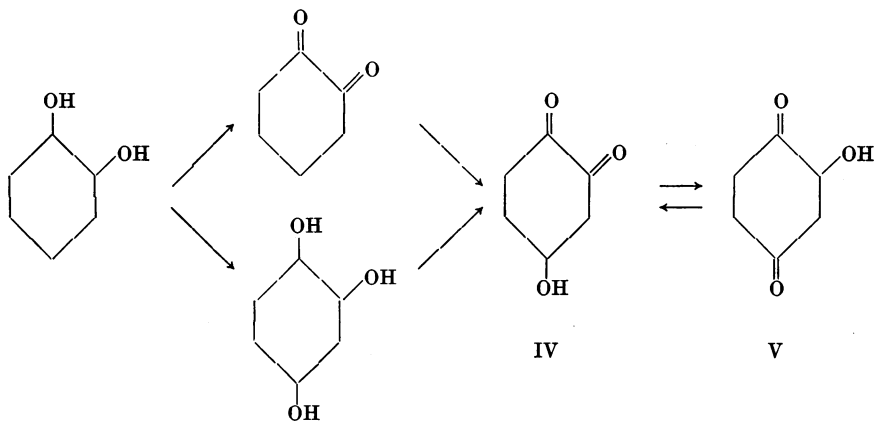


Fig. 1. Composite curve showing the oxygen absorption following introduction of aniline at various stages during the enzymic oxidation of catechol. The points indicated show the time of addition of aniline in different experiments.

The oxidation of catechol to 4-hydroxy-1:2-quinone could hardly take place directly as Wagreich & Nelson suggest. Either *o*-quinone or 1:2:4-trihydroxybenzene would be expected as an intermediate product.



Oxidation of 1:2:4-trihydroxybenzene could proceed either to 4-hydroxy-1:2-quinone (IV) or 2-hydroxy-1:4-quinone (V), which however are tautomeric forms. Although the trihydric phenol autoxidizes quite readily in aqueous solution at *p*H 5·9, polyphenol oxidase exerts a strong catalytic action on the

reaction (curves *a* and *b*, Fig. 2); the enzymic oxidation probably proceeds via the 1:2-quinone. One atom of oxygen is rapidly absorbed with the development of an intense red colour which rapidly turns brown in a few minutes with further slight absorption of oxygen indicating the unstable nature of the quinone. In the presence of aniline and enzyme, 2 atoms of oxygen are rapidly absorbed (curve *c*) but no trace of dianilo-*o*-quinone is formed, the reaction fluid being

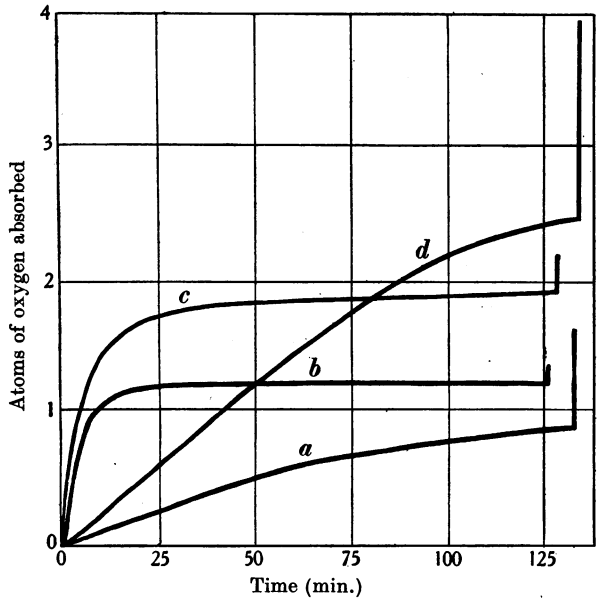
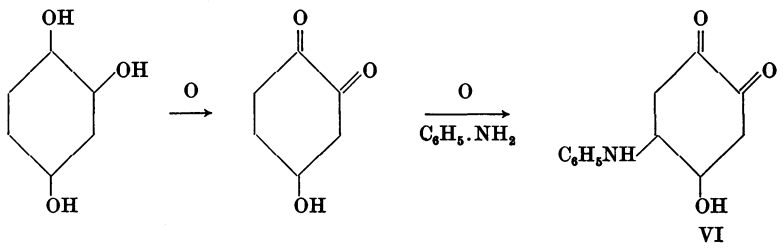


Fig. 2. The enzymic and autoxidation of 1:2:4-trihydroxybenzene alone (curves *b* and *a* respectively), and in the presence of aniline (*c* and *d* respectively). The vertical part of each curve represents O_2 uptake up to 20 hr.

opalescent violet-brown in appearance. The colour is completely removed by ether forming a violet-red solution. The pigment, isolated from large-scale oxidations, crystallizes in purple micro-needles giving analytical figures agreeing with the expected hydroxymonoaniloquinone (VI). Autoxidation of the trihydric phenol in the presence of aniline also gives no dianilo-*o*-quinone and 3·9 atoms of oxygen are absorbed (curve *d*). The reaction fluid is similar in appearance and behaviour to that from the enzymic oxidation and the only product which can be isolated is a considerable proportion of the compound (VI). In view of the oxygen uptake this is quite unexpected and is as yet inexplicable.

The enzymic oxidation of 1:2:4-trihydroxybenzene can therefore be represented in the following manner:



VI

Thus hydroxyquinone cannot accumulate during the enzymic oxidation of catechol owing to its instability, or take any part in the formation of dianilo-*o*-quinone since it reacts with aniline to form a stable hydroxyaniloquinone. There is no evidence, therefore, of an alternative mechanism for the enzymic oxidation of catechol—*o*-quinone must be the initial product. Subsequent oxidation probably consists in the rapid decomposition of the unstable quinone.

Summary

1. The oxidation of catechol by polyphenol oxidase in the presence of aniline has been investigated.

2. The amount of dianilo-*o*-quinone formed diminishes as introduction of aniline into the oxidizing catechol is delayed; the total oxygen absorbed, however, remains approximately constant at 3 atoms per molecule of catechol.

3. Oxidation of 1:2:4-trihydroxybenzene at *p*H 5·9 is catalysed by polyphenol oxidase. In the presence of aniline, 2 atoms of oxygen per molecule of trihydric phenol are absorbed; autoxidation under the same conditions requires approximately 4 atoms of oxygen. The only product which can be isolated in both cases is a hydroxymonoaniloquinone, no dianilo-*o*-quinone being formed.

4. These results do not support the suggestion that the enzymic oxidation of catechol proceeds to hydroxyquinone, or that the formation of dianilo-*o*-quinone takes place by interaction of aniline with hydroxyquinone or any higher oxidation product of catechol than *o*-quinone.

The author wishes to thank Prof. H. S. Raper for his interest and advice during the progress of the investigation.

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