VI. MECHANISM OF THE OXIDATION OF CATECHOL BY TYROSINASE*

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The accompanying diagram illustrates a mechanism proposed for the enzymic oxidation of catechol (1–5). In the present study the oxidations of catechol and hydroxyhydroquinone at varying concentrations of substrate, enzyme, and hydrogen ion have been followed spectrophotometrically. Step 1, first proposed by Raper on chemical grounds (6), has been confirmed. Further results indicate that the catechol melanin obtained under these conditions is not a polymer of hydroxy-p-quinone.

EXPERIMENTAL

Tyrosinase was prepared from Psalliota campestris by the procedure of Ludwig and Nelson (7). The preparations contained between 83 and 107 chronometric catecholase units per mg. of dry weight, determined by the procedure of Miller et al. (8). Catechol melted at 103–104° (corrected). o-Benzoin (9, 10) consisted of bright red needles which melted and decomposed between 60–70°. Hydroxyhydroquinone (11) melted at 139–140°. Hydroxy-p-quinone prepared from it (12) crystallized from ether in yellow prisms which melted at 122–125°. 2,4,5,2',4',5'-Hexahydroxydiphenyl was prepared from vanillin according to the procedure of Erdtman (13); the granular gray powder possessed the properties previously described (13–15). It was further identified by preparation of the corresponding hexaacetate, which melted at 172.5–174.5°. A dilute ethereal solution shaken with silver oxide and dry sodium sulfate first turned deep red-purple, then lemon yellow. Yellow crystals melting and decomposing at 180° were deposited from the concentrated filtrate. The small amounts available forestalled further identification, but the method of preparation and the color sequence observed during the reaction (cf. 4,4'-dimethoxydi-p-quinone of Erdtman) indicate that this substance was 4,4'-dihydroxydi-p-quinone.

Buffers were prepared by adding 0.1 N NaOH to 50 ml. of 0.1 M KH₂PO₄

* Some of the results reported in this paper were the subject of a preliminary note (J. Am. Chem. Soc., 67, 1233 (1945)). For Paper V in this series see Mason and Wright (J. Biol. Chem., 180, 235 (1949).

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and diluting the mixture to 100 ml. All spectrophotometric experiments were conducted at temperatures of 25–28° with a Beckman ultraviolet spectrophotometer and matched quartz cells having a light path of 1.0 cm. The constant procedure of adding 0.1 ml. of enzyme, appropriately diluted, to 3.0 ml. of substrate-buffer mixture in the spectrophotometer cell was utilized. The spectrophotometric conventions and experimental details have been previously described (16, 17).

**Enzymic Oxidation of Catechol**—The spectrophotometric course of the oxidation of catechol in the presence of tyrosinase at pH 5.4 is depicted in Fig. 1. Although the concentration of catechol is varied between $7.54 \times 10^{-5}$ and $2.27 \times 10^{-4}$ mole per ml. and the concentration of tyrosinase
between 5.3 and 21.2 catecholase units per 3.1 ml., the observations are qualitatively alike. The absorption maximum at 276 m\(\mu\), characteristic of catechol, is replaced rapidly by principal absorption at 390 m\(\mu\), which will presently be shown to be characteristic of \(o\)-benzoquinone. General
absorption displaying a weak maximum at 255 to 260 mμ then develops over a period of 60 to 90 minutes. The results obtained with the same range of enzyme and substrate concentrations at pH 8.4 are depicted in

Fig. 2. Spectrophotometric course of the enzymic oxidation of catechol at pH 8.4. The designations have the same significance as those in Fig. 1.

Fig. 2. Intermediate spectra are more transient; the maximum absorption of catechol at this pH, 277.5 mμ, is directly replaced by general absorption displaying weak maxima or inflections at 260 to 270 mμ.

**Enzymic Oxidation of Hydroxyhydroquinone**—The spectrophotometric course of the oxidation of hydroxyhydroquinone in the presence of tyrosinase
at pH 5.4 is depicted in Fig. 3. The concentrations of enzyme and substrate are the same as in the previous experiments with catechol. In each, the initially observed maximum at 288 m\(\mu\), characteristic of hydroxyhydroquinone, is rapidly replaced by two new maxima at 260 and 480 to
485 m. These will presently be shown to constitute a portion of the absorption spectrum of hydroxy-p-quinone. Under the conditions of the experiment they are metastable and are replaced in 60 minutes by a band, the position of which is related to the amount of enzyme in solution: 320 m. in the presence of 21.2 units and 340 m. in the presence of 5.3 units. Increases in the concentrations of hydroxyhydroquinone and enzyme produce expected increases in the rate of appearance and intensities of the several bands serially observed.

At pH 8.4 the rate of autooxidative transformation of hydroxyhydroquinone is too rapid to be observably affected by the presence of enzyme
Immediately after solution of the phenol, absorption maxima at 260 and 480 μm are observed. These are replaced more slowly by a single maximum at 320 μm.

**Enzymic Oxidation of Mixtures of Catechol and Hydroxyhydroquinone**—Mixtures of catechol and hydroxyhydroquinone, the combined concentrations of which equaled \(2.27 \times 10^{-4}\) mole per ml., were oxidized in the presence of 21.2 units of enzyme at pH 5.4. The absorption spectra which developed immediately after addition of the oxidase contained bands at both 395 and 490 μm, the relative intensities of which were proportional to the mole fractions of catechol and hydroxyhydroquinone, respectively. General absorption then appeared. When the concentration of hydroxyhydroquinone was 50 per cent or more of the total sub-

<table>
<thead>
<tr>
<th>Compound</th>
<th>pH of buffer solvent</th>
<th>Maximum μm</th>
<th>Log E</th>
<th>Maximum μm</th>
<th>Log E</th>
</tr>
</thead>
<tbody>
<tr>
<td>Catechol</td>
<td>5.4</td>
<td>276</td>
<td>3.362</td>
<td>5.4</td>
<td>3.410</td>
</tr>
<tr>
<td>o-Benzquinone (enzymic)</td>
<td>5.4</td>
<td>390</td>
<td>3.202</td>
<td>5.4</td>
<td>3.124</td>
</tr>
<tr>
<td>Hydroxyhydroquinone</td>
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<td>3.441</td>
<td>5.4</td>
<td>3.441</td>
</tr>
<tr>
<td>Hydroxy-p-quinone (enzymic)</td>
<td>5.4</td>
<td>260</td>
<td>4.690</td>
<td>480-485</td>
<td>3.312</td>
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<tr>
<td>2,4,5,2',4',5'-Hexahydroxydiphenyl</td>
<td>5.4</td>
<td>292</td>
<td>4.019</td>
<td>4.400</td>
<td>3.324</td>
</tr>
<tr>
<td>4,4'-Dihydroxydip-quinone</td>
<td>5.4</td>
<td>275</td>
<td>4.260</td>
<td>5.4</td>
<td>4.260</td>
</tr>
</tbody>
</table>

strate concentration, an inflection or maximum simultaneously appeared in the region of 340 μm.

**Absorption Spectra of Hydroxy-p-quinone and o-Benzquinone**—The absorption spectrum of hydroxy-p-quinone at pH 5.4 is depicted in Fig. 4, B. Unstable maxima are observed at 260 and 480 to 485 μm; over a period of 60 minutes these diminish in intensity and a new band at 340 μm appears. o-Benzquinone displays an initial maximum at 390 μm; this rapidly disappears and general absorption in which a well defined maximum at 270 μm is apparent develops (Fig. 4, C). The absorption maxima and molecular extinction coefficients of catechol, hydroxyhydroquinone, the corresponding quinones, and related substances relevant to the following discussion and observed in the present study are listed in Table I.
DISCUSSION

The isolation of 4,5-dianilino-o-benzoquinone as a derivative from the products of the oxidation of catechol in the presence of tyrosinase (2, 6) indicated that o-benzoquinone is formed under these conditions. The present study provides confirmation, since the absorption spectrum observed immediately after addition of tyrosinase to catechol solutions and that of o-benzoquinone itself are identical at pH 5.4 (Table I). o-Benzoquinone must therefore be formed more rapidly than it is consumed in subsequent reactions at this pH. At pH 8.4, however, the quinone must be utilized as rapidly as it forms, for only general absorption can be observed in the region of 390 mμ after addition of enzyme to catechol solutions (Fig. 2).

The final stages of the enzymic oxidation of catechol are characterized by inflections or maxima in the region of 255 to 270 mμ. These are generally more pronounced in the presence of low initial enzyme concentrations. Solutions of pure o-benzoquinone develop similar maxima upon standing (Fig. 4, C) and catechol itself can readily be isolated from such solutions. For this reason it is probable that these peaks do not characterize the polymeric product.

The enzymic oxidation of hydroxyhydroquinone follows a completely different spectrophotometric course. In the first phase the spectrum of this polyphenol is replaced by absorption maxima at 260 and 480 to 485 mμ (pH 5.4) (Fig. 3). Similar changes take place autoxidatively at pH 8.4. This spectrum is identical to that of hydroxy-p-quinone (Fig. 4, B; Table I).

The instability of this compound in aqueous solution does not, as in the case of o-benzoquinone, lead directly to general absorption but to new specific absorption in the region of 320 to 340 mμ (Figs. 3, 4, A, and 4, B). The appearance of this absorption band 60 minutes after the solution or enzymic formation of hydroxy-p-quinone is an additional characteristic of the compound at pH 5.4. Furthermore, the 320 to 340 mμ band develops perceptibly when the concentration of hydroxy-p-quinone is 50 per cent or more of the total in a mixture with o-benzoquinone formed by the enzymic oxidation of the corresponding phenols at pH 5.4. Since neither this band nor those characterizing hydroxy-p-quinone itself appear any time during the enzymic oxidation of catechol at pH 5.4 to 8.4, it is probable that the mechanism postulated by Nelson and coworkers does not obtain under these conditions.

The hypothesis that hydroxy-p-quinone is formed during the enzymic oxidation of catechol developed from the observation that, after the enzymic consumption of 2 atoms of oxygen per molecule of catechol, addition of aniline results in the formation of 4,5-dianilino-o-quinone (2).
However, it is known that hydroxy-\(p\)-quinone does not give rise to 4,5-dianilino-\(o\)-quinone (18). Accordingly, this basis for the hypothesis is without foundation. An alternative explanation for the isolation of 4,5-dianilino-\(o\)-quinone by Wagreich and Nelson lies in the observation that the complete enzymic oxidation of catechol requires 2.5 to 3.1 atoms of oxygen per molecule of catechol (19). The consumption of only 2.0 atoms per molecule may permit unchanged \(o\)-benzoquinone to remain. This would form the derivative obtained.

The kinetics of the disappearance of \(o\)-benzoquinone from dilute aqueous solutions has also been interpreted as indicative of a simple hydration reaction between the quinone and water (3). The analytical procedure employed for following the concentration of \(o\)-benzoquinone in this work did not, however, discriminate between \(o\)-benzoquinone initially present and quinones subsequently formed.

Under the conditions of the experiment, hydroxy-\(p\)-quinone did not condense to polyhydroxydiphenyls of the type described by Erdtman (13), since these substances do not possess absorption maxima in the 320 to 340 \(\text{m} \mu\) region (Table I). While the products of the enzymic oxidation of hydroxyhydroquinone are readily dialyzable and do not precipitate upon the addition of strong acid, catechol melanins prepared under the same conditions are retained by a cellulose membrane and are almost completely precipitated from solution at \(\text{pH} \ 2\). This evidence together with failure to detect intermediate substances spectroscopically suggests that \(o\)-benzoquinone polymerizes directly to phenolic polyphenyls susceptible to further oxidation.

**SUMMARY**

Comparison of the spectrophotometric course of the enzymic oxidations of catechol and hydroxyhydroquinone under varying conditions of substrate, enzyme, and hydrogen ion concentrations indicates that the catechol melanins obtained under these conditions are not polymers of hydroxy-\(p\)-quinone.

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