OXIDATION OF METADIHYDROXYPHENOLS BY ENZYMES OF
PIRICALIRIA ORYZAE

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Piricularia oryzae possesses an enzyme that catalyzes the oxidation of 1,3-dihydroxyphenols (Neufeld, Latterell, and Weintraub, Arch. Biochem. Biophys. 76:317, 1958). This communication presents findings regarding the mechanism of oxidation involved with this metapolyphenoloxidase, and argues against the hypothesis that 2,2'-dihydroxy-4,4'-diphenooquinone or related compounds are formed. These have been reported as intermediates or as end products of the nonenzymatic oxidation of resorcinol (Heinrich, Setz. phys. med. soc. Erlangen 71:199, 1939; Valyashko and Shecherbak, J. Gen. Chem. (U. S. S. R.) 8:1641, 1938), or they might be postulated from simple stoichiometric considerations when this compound is used as a substrate.

Enzymes were prepared and oxidation was measured as described by Neufeld et al. (Arch. Biochem. Biophys. 76: 317, 1958). When oxygen uptake ceased, the reaction mixture was acidified and lyophilized, and the residue was extracted with methanol and chromatographed (n-butanol-pyridine-water, 3:2:1.5). Spots were visualized with ultraviolet light and their absorption spectra were determined. The resorcinol reaction gave fluorescent spots with the following $R_F$ values: 0.19, 0.27, 0.62, 0.91; orcinol gave spots 0.10, 0.40, 0.56, 0.89. The material of spot $R_F$ 0.27 was a hygroscopic glass, carbonized without melting, and exhibited absorption maxima at 250, 256, 262, and 425 m$\mu$. Reducing agents caused the 425 m$\mu$ peak to shift to 386 m$\mu$. In the infrared spectrum, strong quinoid, hydroxyl, and ether bands were observed. Spectra of the remaining resorcinol- and orcinol-derived spots also exhibited the ultraviolet band triad, and materials eluted from these spots were reducible.

Two possible precursor phenols were incubated with the enzyme. 2,4,2',4'-Tetrahydroxybibiphenyl and 2,9-dihydroxydiphenylene oxide, which theoretically could yield 2,2'-dihydroxy-4,4'-diphenooquinone or (when followed by loss of a molecule of water), could yield the corresponding 2,7-dioxodiphenylene oxide. Slow polymerization was observed with little oxygen uptake. When the tetrahydroxy compound was oxidized with silver oxide or with oxygen in alkaline solution containing cupric ion, different polymers were obtained in either system that were not identical with the products from the corresponding enzyme-catalyzed reaction. The diphenylene oxide failed to oxidize in alkali, but gave the expected 2,7-dioxodiphenylene oxide...
with silver oxide. Resorcinol, when oxidized in alkaline solution, yielded a product identical with the substance from spot \( R_F 0.27 \), and the expected diphenoquinone.

Another phenol, 2, 4, 5-tribromoresorcinol, polymerized rapidly without oxygen consumption and without liberation of bromide ion when subjected to the action of the enzyme.

Oxidation mechanisms for phenolases such as those proposed by Nelson and Dawson (J. Am. Chem. Soc. 61:245, 1938), where a phenol is oxidized directly by oxygen, cannot explain the lack of oxygen utilization in the tribromoresorcinol oxidation. Also not applicable are mechanisms postulating reduction of the cupric ion moiety in such enzymes concomitant with the formation of a quinone and followed by subsequent regeneration of the cupric state via oxidation with elemental oxygen (Kubowitz, Biochem. Z. 292:221, 1937). The rapidity of the reaction, and the polymeric nature of the products with this as well as with other phenols, indicates the formation of an intermediate similar to a free radical. Failure to utilize oxygen in these cases suggests the creation of, conditions such that a chain-sustaining polymerization occurs without further participation of enzyme or oxygen.

In those reactions where oxygen was consumed, the diversity of products could arise by utilization of primary oxidation or coupling products as substrates for accompanying phenoloxidases, as Neufeld et al. have shown their enzyme preparation to be heterogeneous.

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NEW INDOLE TEST REQUIRING ONLY SIMPLE REAGENTS
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Although Kovacs' method is now commonly used in testing for indole production by Enterobacteriaceae, it requires the use of \( p \)-dimethylaminobenzaldehyde. This compound is not always easy to obtain in some areas of the world and it is unstable in solution, necessitating storage in the cold.

To develop an indole test which can be carried out in any laboratory with common reagents, the combined action of formaldehyde, sulfuric acid, and potassium dichromate was utilized as follows.

Two separate solutions are necessary:

Solution I. Formalin (about 37% formaldehyde), 1 part; concentrated sulfuric acid, 2 parts. Mix cautiously in a pyrex Erlenmeyer flask, pouring the sulfuric acid very slowly into the formaldehyde. This mixture keeps indefinitely.

Solution II. Potassium dichromate, 0.5% in distilled water.

Use. For each milliliter of 24- to 48-hr bacterial broth culture to be tested, add two drops (about 0.1 ml) of solution I. Invert the stoppered tube to mix the contents completely. Then add, for each milliliter of culture, one drop of the solution II and mix. A distinctly pink hue appears immediately whenever indole is present, in contrast to an accentuated yellow color seen in the absence of indole.

This reaction has been checked by comparison with Kovacs' reaction in more than two thousand (2,234) tests on different strains of Enterobacteriaceae; without exception both tests were coincident. It appears that this test could be submitted advantageously as a method for demonstrating indole production when the reagents for the Kovacs' method, especially \( p \)-dimethylaminobenzaldehyde, are not available.