Steps in the Oxidation of Indoleacetic Acid

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During the oxidation of indole-3-acetic acid (IAA) by an enzyme obtained from cultures of the fungus *Omphalia flavida* (*1*), the ultraviolet absorption spectrum was studied (*2*). At most wavelengths, the change in optical density is not proportional to disappearance of the substrate (IAA), as was shown by following the IAA simultaneously with the Salkowski color reaction (*3*). The spectrum continues to change after the IAA has been exhausted, indicating that the initial product of the reaction undergoes further transformation.

At the wavelengths 261 and 231 mμ however, the change in optical density is almost exactly proportional to substrate disappearance and practically ceases just when the IAA has been consumed. These must, therefore, be wavelengths at which products A and B of a reaction sequence,

$$\text{IAA} \longrightarrow A \longrightarrow B,$$

have identical molar extinction coefficients, ε. Thus the second reaction occasions no change in absorption, and the change in optical density at 261 mμ measures the conversion of IAA to $(A + B)$.

At 272 mμ, optical density remains nearly constant during the first few minutes of the reaction, then falls with increasing speed until the IAA is exhausted. It continues to fall without a break at that time and thereafter declines along a first-order path (Fig. 1). Hence, 272 mμ is a wavelength at which A and IAA have the same ε, so that change in optical density measures conversion of $(\text{IAA} + A)$ to B, which has a smaller ε. At other wavelengths the absorption changes throughout the reaction period, but the time-course curves show obvious breaks in slope at the time when absorption begins to fall at 272 mμ and when it ceases to rise at 261 mμ.

The rate of change of optical density at 261 mμ is strictly proportional to the enzyme concentration (Fig. 1) and is strongly inhibited by $10^{-3}M$ HCN, $1.1M$ HCHO, or by bringing the *p*H to 2.0 or

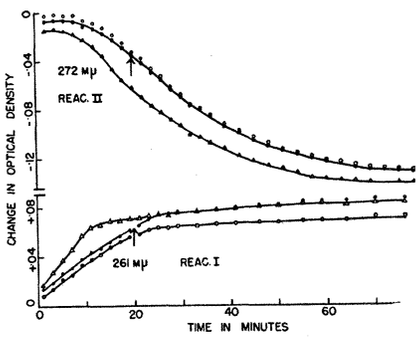

Fig. 1. Effect of enzyme concentration on reactions I and II (○, ●, 0.03 ml of enzyme; △, 0.06 ml of enzyme; ↑, 0.03 ml of enzyme added to ●). Change in optical density at 261 mμ and 272 mμ during the oxidation of 0.14 μmole of IAA in 3.1 ml of $0.005M$ citrate buffer at *p*H 3.7, containing the amounts of *Omphalia* enzyme preparation (dialyzed culture medium) shown, and 0.02 μmole of H_2O_2. The break in slope at 261 mμ occurs at the time the IAA is exhausted. The enzyme concentration is doubled at zero time in △ and just before the IAA is exhausted in ●. Optical densities are corrected for change caused by the addition; light path 1 cm.

7.0, as is the over-all oxidation. In contrast, the rate of change at 272 mμ is very little affected by these treatments (but accelerated at *p*H 2), provided that they are applied after the IAA has disappeared. The rate is also unaffected by varying the enzyme concentrations at that time (Fig. 1).

We deduce that the reaction IAA $\longrightarrow A$ is catalyzed by the enzyme, while $A \longrightarrow B$ is nonenzymatic. Removal of oxygen prevents the conversion of IAA to A but does not inhibit $A \longrightarrow B$, which thus appears to be nonoxidative. This second reaction is acid-catalyzed (Fig. 2), with velocity constant approximately

$$k_1 = (3.6 \times 10^{-2} + 35\ [\text{H}^+]M^{-1})\ \text{min.}^{-1}$$

at 27°C. Its first-order kinetics (Fig. 2) make it unlikely that two separate products of enzyme action from a scheme

$$\text{IAA} \begin{cases} \nearrow A_1 \longrightarrow B_1 \\ \searrow A_2 \longrightarrow B_2 \end{cases}$$

are present. From the kinetics it is also improbable that a dismutation, dimerization, or polymerization of A is occurring. However, it is possible that A decomposes by two independent pathways:

$$A \begin{cases} \nearrow B_1 \\ \searrow B_2 \end{cases}$$

Yet the absorption at 261 mμ remained constant and the final absorption spectrum was the same when the temperature was varied from 5° to 37°C and when the *p*H was varied from 2 to 6.5 during the second reaction: both factors vary the rate of this reaction enormously and might well affect differentially two such independent decompositions. Although the oxidation product is known (by chromatography) to consist of more than one substance, it is not certain at present whether this is the result of the second reaction or of changes during the working-up procedure.

The absorption spectrum of A can be estimated in two ways: (i) from the initial rate of change of optical density (when the second reaction is very slow), or (ii) from the absorption at the time the IAA has just disappeared (by subtracting absorption caused by B, which is calculated from change in optical density at 272 mμ). Figure 3 shows an estimate obtained by the first method.

The substance(s) B (ultraviolet spectrum shown in Fig. 3) is believed to be an oxindole derivative (*4*), and the

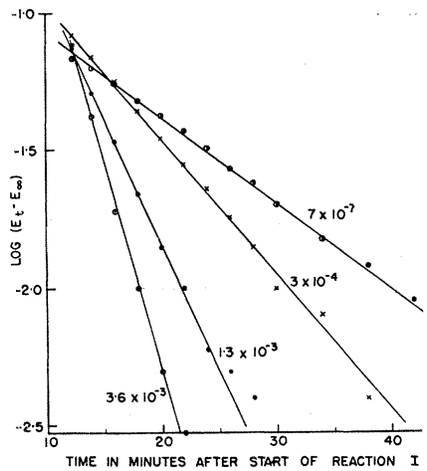

Fig. 2. H^+ catalysis of reaction II. First-order decline in optical density at 272 mμ after the disappearance of IAA, as a function of $[H^+]$. Ordinate shows log of difference between optical density at time t and final optical density. The unbuffered solution (3.1 ml) contained 0.135 μmole of IAA, 0.02 μmole of H_2O_2, and enzyme; it was adjusted to *p*H 3.5 with $0.01M$ HCl. Just after the IAA had disappeared, 12 min after the start of the reaction, the $[H^+]$ was altered by addition of HCl (lower two curves) or sodium phosphate buffer (upper curve). Final $[H^+]$ in moles per liter is given for each curve; light path 1 cm.

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stoichiometry is fulfilled by 3-methyldioxindole (III). The spectrum of the intermediate *A* is not that of indole-3-aldehyde, 5- or 7-hydroxyindoleacetic acid (*5*), or any acetophenone derivative for which we have information. It is rather similar to that of 11-hydroperoxytetrahydrocarbazolenine (*6*); this compound was considered by Witkop and Patrick (*7*) to be a model for the intermediate in the biological conversion of tryptophan to formylkynurenine (*8*). The hydroperoxide (*7*) opened by an acid-catalyzed rearrangement to give a cyclic lactam of δ-*o*-aminobenzoylvaleric acid, analogous to formylkynurenine. A similar hydroperoxide (II) could be formed by the (enzymatic) addition of O_2 to, and loss of CO_2 from, the isoindole form (Ib) of IAA, but this would probably not open between the 2- and 3-carbons, because the C-H bond at C-2

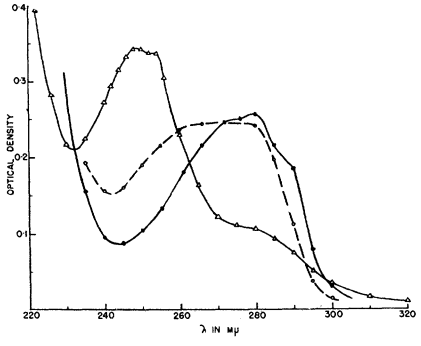
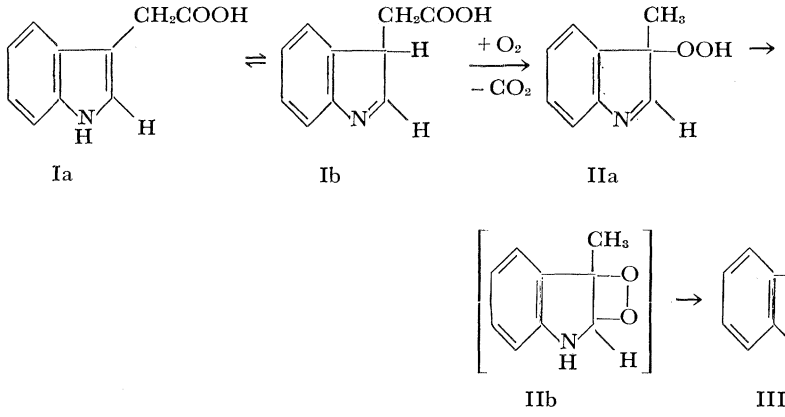


Fig. 3. Ultraviolet absorption spectra of IAA (●), the final product *B* (△) after enzymatic oxidation, and the intermediate *A* (broken line). The latter shows the spectrum that would have resulted if the changes in optical density during the first 4 min of the reaction, at each wavelength, had continued proportionate to the disappearance of IAA until this was exhausted. Three milliliters of $4.5 \times 10^{-5}M$ IAA in $0.005M$ citrate buffer at *p*H 3.7, containing enzyme and 0.01 μmole of H_2O_2, against blank containing enzyme and buffer; light path 1 cm.



would more readily lose electrons. Instead, 3-methyldioxindole (III) would be formed. Side reactions involving formation of indoxyl derivatives and/or dimerization might be anticipated from the reactions of 11-hydroxytetrahydrocarbazolenine (*9*). These are speculations only, but they are interesting in view of the possible relationship of our observations to the persistent problem of identifying the intermediate between tryptophan and formylkynurenine (*10*).

The reaction sequence described here is strikingly similar to that which has been found for the oxidation of uric acid by uricase (*11*) or by peroxidase (*12*). Uric acid is converted enzymatically to an intermediate, which decomposes by nonenzymatic reactions into the final products (allantoin, alloxan, or other substances), and thus ultraviolet absorption continues to change for some time after the uric acid has disappeared. In this case there is other than spectroscopic evidence for the existence of an intermediate (*13*).

It is of interest to mention that the oxidation of IAA by horseradish peroxidase in the presence of H_2O_2 appears to follow kinetics similar to those described here. Furthermore, the spectrum of the final product is almost identical with that of *B* (Fig. 3). This suggests that the reactions studied here may occur generally in the destruction of IAA by higher plants.

References and Notes

1. L. Sequeira and T. Steeves, *Plant Physiol,* 29, 11 (1954).
2. This work was carried out during the tenure of a National Science Foundation fellowship by one of us (P.M.R.). Support is also acknowledged from the Committee on Growth of the National Research Council, acting for the American Cancer Society, and from the National Science Foundation.
3. S. A. Gordon and R. P. Weber, *Plant Physiol.* 26, 192 (1951).
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5. These last two compounds are indeed decomposed under the same conditions but at so slow a rate that they are in any case excluded from being intermediates.
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The scientist should be a man willing to listen to every suggestion, but determined to judge for himself. He should not be biased by appearances; have no favorite hypothesis; be of no school; in doctrine have no master. He should not be a respecter of persons, but of things. Truth should be his primary object. If to these qualities be added industry, he may indeed hope to walk within the veil of the temple of nature.—MICHAEL FARADAY.