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Studies on the Oxidation of Indole-3-Acetic Acid by Peroxidase Enzymes. I. Colorimetric Determination of Indole-3-Acetic Acid Oxidation Products

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Summary. The method described here is based on a brief report by Harley-Mason and Archer. It involves the use of *p*-dimethylaminocinnamaldehyde (DMACA), a vinylogue of Ehrlich's reagent, as a color reagent for indoles. Colorimetric analyses of indoleacetic acid (IAA) oxidation reaction mixtures were made with the DMACA reagent as a solution rather than a spray. DMACA reagent will yield a wine-red color with IAA oxidation products in solution. Under similar conditions DMACA reacts with authentic IAA to yield only slight coloration at best. In comparison with other indoles, DMACA is more relative with IAA oxidation reaction products than either Salkowski or Ehrlich's reagents. Data discussed support a concept that the color produced with DMACA is due to the presence of tautomeric oxidation product(s) of IAA.

Colorimetric estimations of the oxidation of indole-3-acetic acid (IAA) by peroxidase enzymes have in the past been based entirely on the Salkowski reaction with unoxidized IAA in the oxidation reaction mixture. This reagent yields a pink color with IAA. The intensity of the color diminishes in proportion to the IAA oxidized. Whether the decrease in color intensity is entirely due to the decrease in IAA concentration, or contingent upon the formation of peroxides during the oxidation of IAA by peroxidase (9) is to be ascertained. Although the Salkowski assay may continue to be useful, particularly on chromatograms, its application in solution is limited to quantitative estimations only. In this communication we discuss a colorimetric method which lends itself to the chemical characterization of early oxidation products of IAA. The method described is based on a brief report by Harley-Mason and Archer (3) and involves the use of *p*-dimethylaminocinnamaldehyde (DMACA) a vinylogue of Ehrlich's reagent (10).

Materials and Methods

p-Dimethylaminocinnamaldehyde was obtained from the Aldrich Chemical Company[2] and was used as a 1 % solution dissolved in 2 N HCl. The solution was prepared fresh before each use. Solutions of DMACA change in color when exposed to air over a period of several days, but under refrigeration it will retain its sensitivity for at least 2 weeks.

Ehrlich's reagent was prepared by dissolving 2 gr. of *p*-dimethylaminobenzaldehyde in 2 N HCl and 80 % (v/v) ethanol. Salkowski reagent consisted of a mixture of 15 ml 0.5 M $FeCl_3$, 500 ml distilled water, and 300 ml of concentrated H_2SO_4 sp. gr. 1.84.

IAA oxidation mixture usually consisted of 0.2 mM IAA, 0.1 mM $MnCl_2$, 0.1 mM 2,4-dichlorophenol (DCP), 0.1 μM horseradish peroxidase (HRP) and 1 mM phosphate buffer (pH 6.1). The enzyme used was electrophoretically purified, crystalline horseradish peroxidase with a specific activity of 2900 units per milligram protein. All 3 color reagents were used as solutions and mixed with equal volumes of test solutions. Routinely, 2 ml of test solutions were mixed with 2 ml of reagent and stored for 70 minutes in the dark before measurements were made.

Results

Effect of Salkowski, Ehrlich and DMACA on Oxidation of IAA. The reaction of Ehrlich's re-

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[2] Mention of a vendor does not constitute the approval by the USDA to the exclusion of other vendors.

agent is chemically analogous to that of DMACA. Both aldehydes form condensation products with pyrrole derivatives and in the presence of acids, yield a quinoidal red-violet compound. Of the 2 reagents DMACA is more effective (10). Analogously, when reacted with indole and skatole (3) or with oxidized IAA (IAA ox), DMACA yields a greater increase in color than the Ehrlich's reagent (fig 1). Qualitatively, the Ehrlich's reagent reacts with IAA ox and yields a yellowish-brown product with maximum absorbance at 460 mμ. DMACA, on the other hand, condenses with IAA ox and yields a wine-red color absorbing maximally at 562 mμ. In comparison, solutions of Salkowski reagents gave only a slight coloration with IAA ox. Figure 1 shows the maximum absorbance obtained with the 3 reagents used. In this experiment 2 ml of test solutions were removed at 0, 1 and 2 hours after HRP was added, and assayed with equal volumes of reagents. The color intensity was measured 70 minutes after the reagents were added. Detailed colorimetric analysis during the course of the oxidation of IAA shows that the condensation reaction with the aldehyde commences about 30 seconds after the first changes are observed in the UV (251 mμ) figure 2. Subsequent studies indicate that the course of the condensation reaction coincides with the appearance of an absorption maximum at 260 mμ (Curve 3 in fig 3). The incipient change observed at 251 mμ (shown

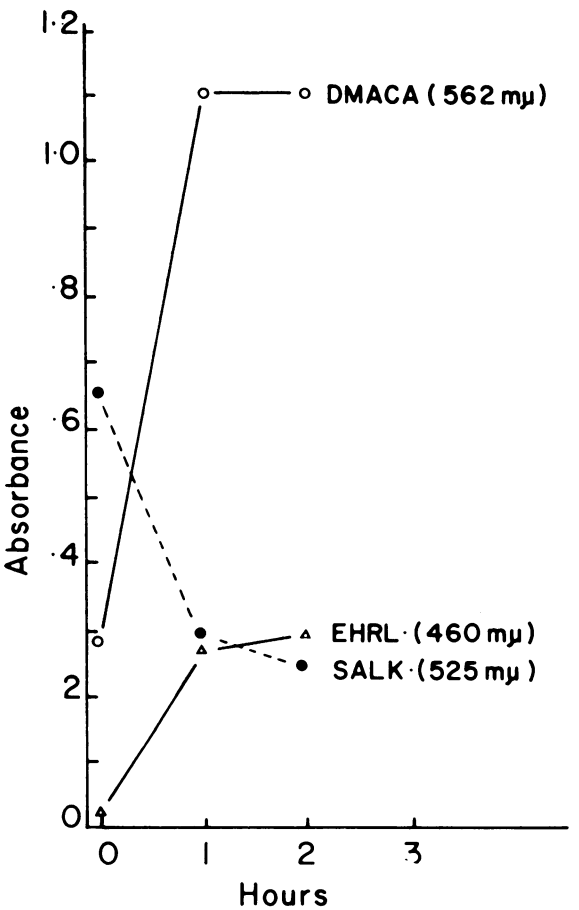

FIG. 1. Maximum absorbance of IAA oxidation reaction mixture reacted with DMACA, Ehrlich and Salkowski reagent. The reagents were added 0, 1 and 2 hours after HRP was added. The oxidation reaction mixture contained initially 0.6mM IAA, 1mM $MnCl_2$, 1mM DCP, and 0.1μM HRP. The color intensities were measured at the wave lengths indicated, 60 minutes after the reagents were added.

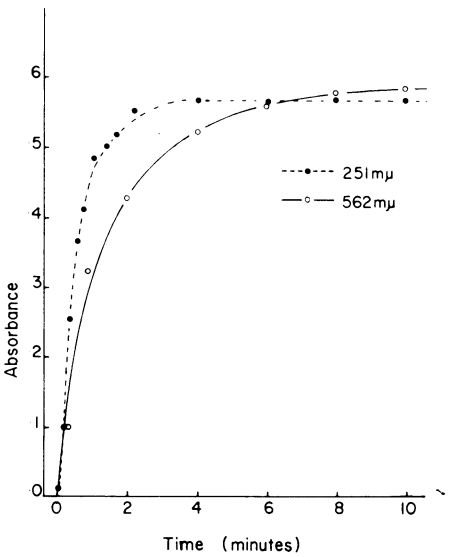

FIG. 2. Change of absorbance of an IAA oxidation reaction mixture as a function of absorbance at 251 mμ, and DMACA color complex measured at 562 mμ. The oxidation reaction mixtures contained 0.3mM IAA, cofactors DCP and $MnCl_2$ (0.1mM) and 0.1μM HRP.

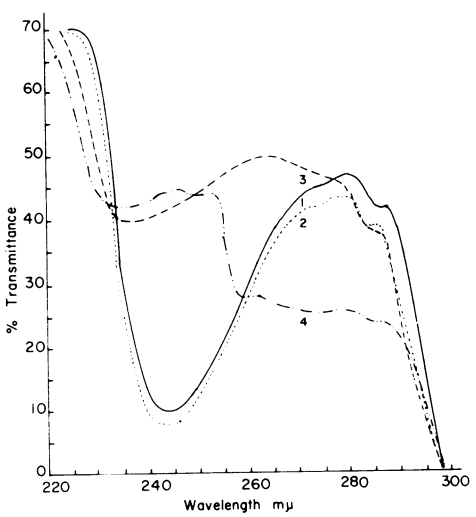

FIG. 3. UV Spectra of IAA oxidation reaction mixtures containing 0.2mM IAA, 0.1mM DCP, 0.1μM HRP. Curve 1) IAA plus cofactors in the absence of added HRP. Curve 2) IAA plus cofactors, 30 seconds after HRP was added. Curve 3) Same as in 2 but 5 minutes later. Curve 4) Same as in 2 but 12 hours later.

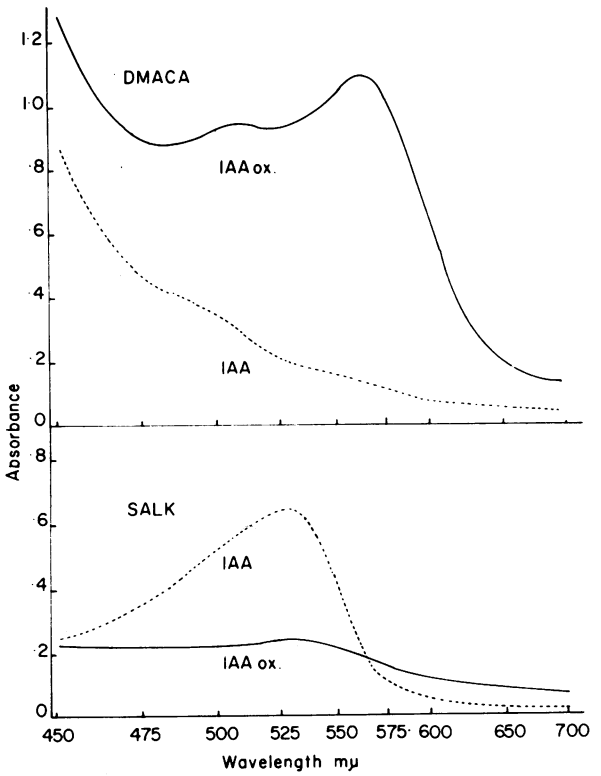

FIG. 4. Absorption spectra of oxidation reaction mixtures reacted with DMACA (top) and Salkowski reagent (bottom) at 0 and 7 minutes after the start of the enzymic oxidation of 0.4mM IAA.

in fig 2) is due to the 2 mμ shift of the indole spectrum (Curve 1 to Curve 2 in fig 3), observed during the first 30 seconds of the enzymatic reaction. Spectrum 2 in figure 3 resembles the spectrum of intermediate "A" conjectured by Ray (7) to be formed during the oxidation of IAA by the Omphalia enzyme. Changes in the UV absorption continue, however, at a rapid rate until the reaction mixture attains an absorption spectrum as shown in Curve 3 of figure 3. Subsequent changes are slow, giving rise to the non-indolic spectrum shown in Curve 4 figure 3, resembling that of an oxindole (5). Concomitant with the increase in absorbance at 240 to 250 mμ region of the spectrum, there is a decrease in the ability of the oxidation reaction mixture to react with DMACA. Spectral analysis of the colored complex formed with DMACA and Salkowski reagent reacted with authentic IAA (dotted line) is shown in figure 4. At the start of the enzymatic reaction, the oxidation reaction mixture contained 0.4 mM IAA, and was incubated for 7 minutes in the presence of HRP before the color reagents were added. The usual time course of the development of the color with DMACA is shown in figure 5. The oxidation reaction mixture contained initially 0.4 mM IAA, 0.1 mM DCP, 0.1 mM $MnCl_2$, 1 mM phosphate buffer (pH 6.1) and was incubated for 10 minutes in the presence of 0.1 μM HRP before DMACA was added. The color reaction was carried out both in the dark, Curve A, and in the light, Curve B. In the dark the reagent was somewhat more sensitive than in the light. Curve C shows the maximum absorbance obtained with IAA in the light and in the dark.

The transient color observed during the first 5 to 15 minutes after DMACA is added is characteristic of the color development and is probably due to an unstable intermediate product of IAA. After the initial change in color, the rate of decay amounts only to a drop of 0.02 OD units per hour.

Figure 6 shows that the color intensity obtained

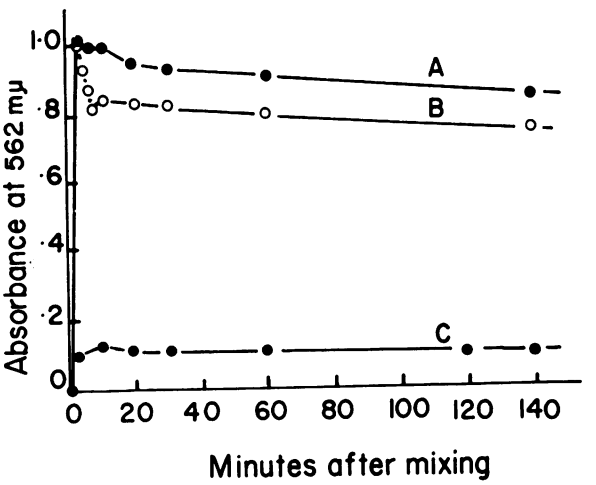

FIG. 5. Time course of the development of DMACA color in the presence of 0.4mM IAA plus cofactors and HRP oxidized for 10 minutes A) in the dark, B) in the light, C) for IAA plus cofactors but in the absence of HRP.

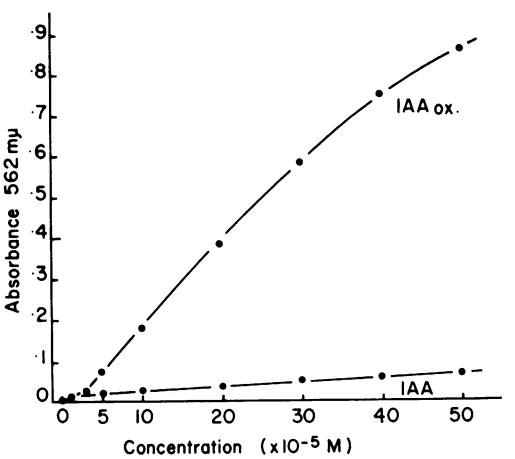

FIG. 6. IAA concentration response curve. Concentrations indicated represent final concentrations of IAA. IAA ox oxidation reaction mixture containing IAA, 0.1mM DCP, 0.1mM $MnCl_2$, and 0.1μM HRP. DMACA was added 10 minutes after HRP was added. IAA) same oxidation reaction mixture as in IAAox but in the absence of added HRP.

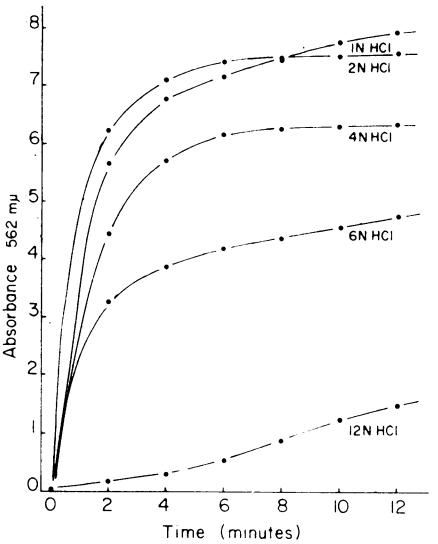

FIG. 7. Time course of oxidation of 0.4mM IAA as assayed by a 1% solution of DMACA dissolved in various concentrations of HCl.

with DMACA is linear with respect to concentration of IAA. Concentrations indicated on the abscissa represent IAA concentration at the start of the enzymatic oxidation. Under the conditions used, IAA ox from initially 50 μM IAA may be detected. Each determination was made against a DMACA blank. Figure 7 shows that the sensitivity of DMACA is greatly affected by the concentration of HCl used with the reagent. Although, 12 N HCl is recommended when DMACA is used as a spray, 12 N HCl reduces the sensitivity when DMACA is used as a solution. As shown in figure 7, a 1% DMACA solution dissolved in 1 or 2 N HCl gave the most intense color with IAA ox.

Reactivity with Other Indole Derivatives and with Peroxides. Inasmuch as DMACA reacts with many indole derivatives when used as a spray, it reacts only with a few indoles when used as a solution. Results in table I show that when DMACA was used as a spray on chromatograms, 13 of the 15 indoles tested yielded colored products. Indole-3-glyoxylic acid and 2-oxindole did not react. IAA oxidation reaction mixtures resolved into 4 DMACA positive compounds but none gave the red color observed when DMACA is added directly to the IAA reaction mixture. As a solution, DMACA reacted only with indole, skatole and the oxidation mixture of IAA. These results suggest that the color reaction observed in solution rests on another kind of condensation than that of the color reaction of indoles on paper.

To account for the reactivity of the IAA oxidation reaction mixture with DMACA, subsequent studies led to the testing of the possibility that the color formed is due to a reaction with peroxides, known products of the oxidation of IAA (5), and demonstrated to be responsible for the decrease in intensity of the Salkowski color (9). Various organic peroxides and H_2O_2 were tested but none reacted with DMACA to yield a colored product. Peroxides included in this test were 8-*p*-methyl hydroperoxide,

Table I. *Color Reaction with Indoles*

All indoles were tested at 1mM. For the color reactions in solution, the test solutions were reacted with equal volume of reagent. The colors were estimated about 70 minutes after initial mixing.

Compounds	Color in solution with Salkowski	Color in solution with DMACA	DMACA as a spray
1. Indole-3-acetic acid	Pink	None	Purple
2. Indole-3-acetic acid oxidation mixture	Slight pink	Red	Purple**
3. Indole	Yellow	Red*	Green
4. Skatol	Yellow	Red	Purple, violet†
5. Indole-3-butyric acid	Yellow	None	?
6. 2,3-Dimethyl indole	Blue	None	Purple
7. Indole-3-propionic acid	Yellow	None	Purple***
8. Indole-3-carboxaldehyde	Pale peach	None	Purple, Bluish green***
9. Indole-3-acetaldehyde	Brown	None	Grayish blue***
10. Indole-3-glycolic acid	Purple	None	Purple
11. Indole-3-glyoxylic acid	Purple	None	None; weak brown***
12. 5-Hydroxyindole-3-acetic acid	Pink	None	Purple
13. Indole-3-acetamide	Pink	None	Violet***
14. Indole-3-acetylaspartic acid	Purple	None	Violet***
15. Tryptophane	None	None	Purple
16. 2-Oxindole	None	None	None

* Green at 0.1mM and red at 1.0μM.

** Paper chromatography in Isopropanol: Ammonium hydroxide water 10:1:1 resolved the IAA oxidation mixture into 4 products all reacting with DMACA to give a purple color. (Unpublished)

*** From Wightman (11)

† Harley-Mason and Archer (3)

tert-butyl hydroperoxide, benzoyl peroxide, and lauroyl peroxide. The contingency exists, therefore, that the color with DMACA is not due to the presence of peroxides.

Discussion

In contrast to the Salkowski method, DMACA yields a wine-red color with an early oxidation product(s) of IAA and does not react with authentic IAA. In general, the reaction of indoles with the vinylogue of Ehrlich's reagent parallels that with other pyrroles. The reaction is based on the fact that pyrroles react in their tautomeric forms (pyrrolenine) with aldehydes to form a quinoidal red-violet compound. For the reaction to proceed, the pyrrole derivatives must have an intact CH group in the 2 or 3 position relative to the cyclic NH group (2). Any substitution on the pyrrole ring which tends to decrease the tautomeric characteristic will reduce condensation with aldehydes (10). Accordingly, indoles are expected to react analogously to pyrrole. The electrophilic substitution of indole differs, however, from that of pyrrole in favoring the 3-position and if this is blocked, the position 2 is attacked (1). Condensation of aldehydes with indoles is best explained by assuming that the indoles react in the form of their 1,3-tautomers (6). The presence of electron attracting groups like $-COOH$, $-COR$, and $-CN$ (4), which tends to withdraw electrons from the indole nucleus reduces 1,3-tautomerization and conceivably restrict condensation at the 2 or 3 position of the indole ring. The presence of alkyl groups, $-NR$ or $-OR$ groups, which tend to donate electrons (4) when attached to the pyrrole portion of the indole ring, would be expected to favor 1,3-tautomerization and thus condensation with DMACA. Results in table I are in keeping with this concept. Indolic acids, aldehydes and the amide do not undergo the condensation reaction while the 2 or 3 methyl indole derivatives do. Oxindole does not react because a 1,3-tautomerization is prevented in favor of the more likely 1,2-tautomerization (6). The condensation of IAA ox with DMACA might, therefore, be explained by the possibility that IAA ox reacts as an indolenine derivative conjectured to be an early oxidation product of IAA, by Ray and Thimann (8), and Hinman and Lang (5).

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