tion. The cyclic AMP binding protein has been purified approximately 200-fold in this laboratory (Gill and Garren, 1970), and with the availability of higher specific activity of cyclic [^{3}H]AMP, the sensitivity of the assay can be markedly enhanced.

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Peroxidase-Catalyzed Formation of Indole-3-carbaldehyde and 4-Hydroxyquinoline from Indole-3-acetaldehyde*

Ren Yeh, Delbert Hemphill Jr., and Harold M. Sell†

ABSTRACT: Spectrophotometric studies on horseradish peroxidase (HRP) catalyzed oxidation of indole-3-acetic acid (IAA), indole-3-acetaldehyde (IAAld), and IAAld–$NaHSO_3$ have shown that oxidation of each compound led to different products and that the pH of the reaction mixture had a profound effect on the final oxidation product of IAAld. In the pH range 3.7–4.5, 3-methyleneoxindole was the major product of HRP-catalyzed oxidation of IAA, whereas oxidation of IAAld under the same conditions gave as high as 50% yield of indole-3-carbaldehyde (IAld). At pH near 7 HRP-catalyzed oxidation of IAAld plus equimolar $NaHSO_3$ gave a new product which was subsequently identified as 4-hydroxyquinoline (4-HQ) by the direct isolation of the oxidation product in crystalline form. However, at acidic pH IAld was the major end product in HRP-catalyzed oxidation of IAAld–$NaHSO_3$.

Kenten and Mann (1950) studied the oxidation of β-phenylethylamine by pea seedling extracts and found that the total oxygen uptake was in excess of that required for the oxidation of the amine to β-phenylacetaldehyde. Further studies on the pea seedling "aldehyde oxidase" activity by Kenten (1953) indicated that the proposed aldehyde oxidase was not a simple flavoprotein but a heme-containing peroxidase. The peroxidase-catalyzed oxidation of β-phenylacetaldehyde did not yield phenylacetic acid but benzaldehyde; hence Kenten suggested that the oxidation of indole-3-acetaldehyde (IAAld)[1] by plant peroxidase might cause the formation of indole-3-carbaldehyde (IAld). Evidence obtained in the present work supports Kenten's view that the oxidation of IAAld by peroxidase results in the formation of IAld as one of the major products.

The possibility that 4-hydroxyquinoline (4-HQ) can be produced as an oxidation product of IAA was not previously confirmed (Manning and Galston, 1955). However, our preliminary investigations indicate that 4-HQ was formed from horseradish peroxidase (HRP) oxidation of IAAld in

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[1] Abbreviations used are: IAA, indole-3-acetic acid; IAAld, indole-3-acetaldehyde; IAld, indole-3-carbaldehyde; 4-HQ, 4-hydroxyquinoline; and HRP, horseradish peroxidase.

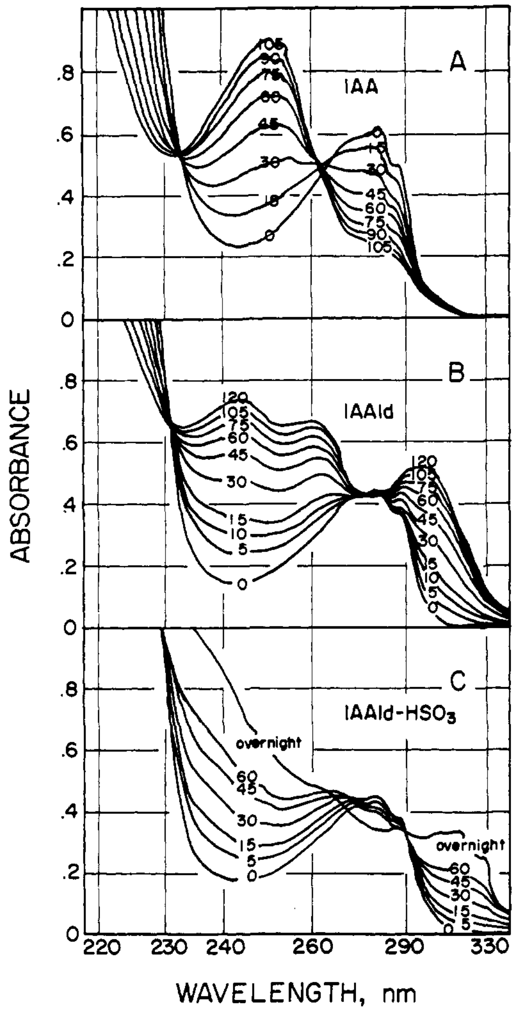

FIGURE 1: Changes in ultraviolet spectrum during enzymatic oxidation of IAA, IAAld, and IAAld-NaHSO$_3$. (A) Acetate buffer, pH 3.85; (B) acetate buffer, pH 4.58; (C) phosphate buffer, pH 7.80. Reaction mixtures contained: 8.4 μg (A and B) or 17.0 μg (C) of horseradish peroxidase, 0.32 μmole of either IAA, IAAld, or IAAld-NaHSO$_3$, 1.00 μmole of H_2O_2 in 0.050 M buffer solution. Total reaction mixture volume was 3.2 ml. Records were begun at 345 nm. Time elapsed at start of each recording is shown in figure; each record was complete in 1 min.

the presence of bisulfite. That 4-HQ was formed in HRP-catalyzed oxidation of IAAld-NaHSO$_3$ was confirmed by direct isolation of this compound in crystalline form.

Materials and Methods

Enzyme and Oxidation Reactions. Electrophoretically purified HRP with an *RZ* value of 3.0 was purchased from the Worthington Chemical Corp. and was immediately stored at 0° on arrival (*RZ* calculated as A_{403}/A_{275}, $RZ = 3.04$ for pure HRP). Reaction mixtures contained the following: 3.0 ml of substrate (1.00×10^{-4} M IAA, IAAld, or IAAld-NaHSO$_3$) in 0.050 M buffer solution (acetate buffer for pH below 5.2 or phosphate buffer for pH above 5.2), 0.10 ml of enzyme solution containing 6 μg to 0.3 mg of protein, and 0.10 ml of 0.01 M H_2O_2. The total volume per cuvet was 3.2 ml. After reaction was initiated by the addition of enzyme solution, the rate of reaction was followed in a Beckman DK-2 spectrophotometer by monitoring the absorbance change at 244 nm or at 336 nm due to the disappearance of substrate or formation of reaction products, respectively. For thin-layer chromatographic studies and isolation of crystalline product, the same reaction mixture was used, but the amounts of the reaction components were proportionately increased. For isolation of 4-HQ the reaction mixture contained: 200 mg of an equimolar mixture of IAAld and NaHSO$_3$, 100 mg of HRP, and 2000 ml of 0.050 M phosphate buffer (pH 7.8). The reaction was initiated by adding dropwise 100 ml of 0.030% H_2O_2. Several batches of the same reaction mixture were run concurrently. A total of about 1 g of the bisulfite addition product of IAAld led to successful isolation of about 270 mg of crude product. The reaction was followed by monitoring the change in absorbance at 316 and 330 nm (typical absorption peaks for 4-HQ) in a Beckman DK-2. After the reaction was completed, the solvent was evaporated under vacuum to dryness, the residue was washed with isopropyl alcohol, and the crude material in isopropyl alcohol was further purified by fractionation through a silicic acid column.

Silicic Acid Column Chromatography. The silicic acid (Fisher Scientific Co., 200 mesh, chromatographic grade) was first washed in pure benzene and then twice in a mixture of 5% benzene in hexane. A slurry in 5% benzene-hexane was then transferred with continual stirring to a glass column, 2.0 cm in diameter, to a length of 20 cm. Samples of about 200 mg of enzyme reaction product in isopropyl alcohol were applied to the column which was first washed with 250 ml of a mixture of benzene-ethyl acetate (1:2, v/v) and then eluted with isopropyl alcohol. The eluate was collected in 2-ml fractions with an automatic fraction collector. Fractions having similar absorption patterns were combined for further purification.

Chemicals. IAAld was synthesized from tryptophan by the method of Gray (1959). Other chemicals were obtained from commercial sources.

Ultraviolet and Infrared Spectra. Ultraviolet spectra were recorded on a Beckman DK-2 spectrophotometer either in 95% ethanol or in aqueous solution. Infrared spectra were recorded with a Beckman IR-5 spectrophotometer. For liquid compounds such as free IAAld, the spectra were obtained with thin films on KBr cells; potassium bromide pellets were used for 4-HQ.

Results

Spectrophotometric Studies on the Oxidation of IAA and IAAld. The changes in ultraviolet spectra during the HRP-catalyzed oxidation of IAA and IAAld are shown in Figure 1A,B. Figure 1C shows the use of the sodium bisulfite addition product of IAAld as substrate instead of the free aldehyde. IAA, IAAld, and IAAld-HSO$_3$ each gave different products upon oxidation with HRP. The conversion of IAA at pH 3.85 into 3-methyleneoxidole was indicated by the appearance of the typical double peaks at 247 and 253 nm (Figure 1A) concomitant with the disappearance of indole absorption at 277 and 287 nm. These results are similar to observations reported previously (Hinman and Lang, 1965).

HRP oxidation of IAAld at pH 4.58 is seen in Figure 1B. Obviously, there are significant differences in the spectroscopic changes in Figure 1A,B. Oxidation of IAAld caused immediate changes in the spectra at 244, 260, and 300 nm,

TABLE I: Thin-Layer Chromatographic R_F Values of Authentic IAld and the Product of IAAld Oxidation in Four Solvent Systems.[a]

Solvent System	IAld	Isolated IAld
n-Propyl alcohol–hexane (1:4, v/v)	0.76	0.79
Chloroform–acetic acid (95:5, v/v)	0.80	0.82
Benzene–acetic acid–water (4:1:1, v/v)	0.69	0.70
n-Butyl alcohol–hexane (18% *n*-butyl alcohol)	0.62	0.62

[a] The thin-layer plates were Eastman Type K 301, R2 silica gel without fluorescent indicator. Spots were developed by spraying with 2,4-dinitrophenylhydrazine in ethanol.

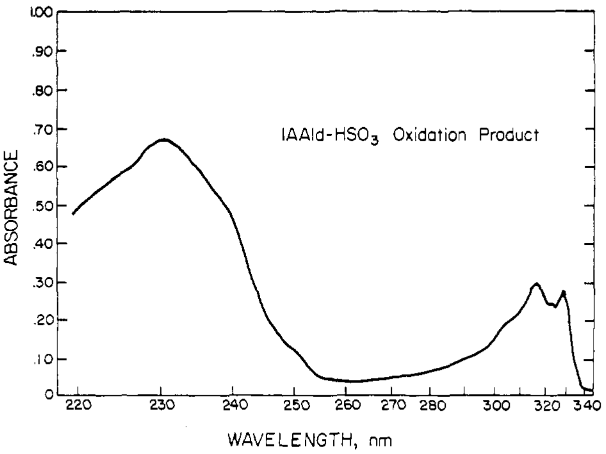

FIGURE 2: Ultraviolet spectrum of IAAld–$NaHSO_3$ oxidation product. The spectrum was obtained with a 0.20×10^{-4} M aqueous solution of the isolated product.

but not at 253 and 247 nm (characteristic peaks for 3-methyleneoxindole). At equilibrium the spectrum showed no similarity to that of 3-methyleneoxindole. As seen below, subsequent thin-layer chromatographic analysis indicated that these spectra represented formation of IAld.

The spectroscopic changes in HRP oxidation of IAAld–HSO_3 at pH 7.8 are shown in Figure 1C. Neither the peaks at 247 and 253 nor those at 244, 260, and 300 nm were observed; but appearance of a double peak at 316 and 330 nm was always observed. This peak was not due to the absorption of bisulfite itself, but was due to the formation of a new compound. In contrast, in the pH range 3.7–4.4, the changes in the spectrum during HRP oxidation of IAAld plus $NaHSO_3$ showed no significant difference from those observed for free IAAld at the same pH.

Thin-Layer Chromatographic Evidence for the Formation of IAld from HRP-Catalyzed Oxidation of IAAld. The results of thin-layer chromatography of the IAAld oxidation product in four solvent systems are shown in Table I. It is evident that the R_F values of the isolated product corresponded very well to the R_F for authentic IAld. Further evidence for the formation of IAld was provided by ultraviolet spectra and 2,4-dinitrophenylhydrazone formation.

The yield of IAld obtained from peroxidation of 1.0×10^{-4} mole of IAAld at pH 4.5 was approximately 50%. The yield was calculated from the spectral changes at 289 nm when the reaction reached equilibrium.

Isolation and Identification of 4-Hydroxyquinoline as an Oxidation Product of IAAld-HSO_3. In order to isolate the new oxidation product having maxima at 316 and 330 nm, fractions were collected from the silicic acid column described above. The new compound remained at the top of the column during the benzene–ethyl acetate wash and was isolated in a fairly purified form after elution with isopropyl alcohol. It was crystallized in an acetone–ethyl acetate mixture and finally recrystallized in pure acetone several times until a constant melting point was attained.

In addition to maxima at 316 and 330 nm the ultraviolet spectrum of the unknown compound (see Figure 2) showed a broad peak at 234 nm. This corresponded with the ultraviolet spectrum of authentic 4-HQ. The infrared spectrum of the unknown compound was also identical with the spectrum of authentic 4-HQ as is seen in Figure 3A,B.

4-Hydroxyquinoline, purchased from the Aldrich Chemical Co., was a trihydrate having a melting point of 200–202°. After drying over phosphorus pentoxide, the commercial 4-HQ melted at 207–208° (uncor) as did the compound isolated from the HRP reaction mixture. A mixture of both compounds showed no depression of the melting point. *Anal.* Calcd for C_9H_7NO: C, 74.47; H, 4.82; N, 9.65; O, 11.06. Found: C, 74.47; H, 4.90; N, 9.68; O, 10.95. Thus all available data indicated that the new oxidation product was 4-HQ. The mole ratio of 4-HQ formed to substrate oxidized was approximately 0.2.

Discussion

As a result of the stimulatory effect of bisulfite at slightly alkaline pH, a new oxidation product having absorption peaks at 316 and 330 nm was detected. By the use of silicic acid

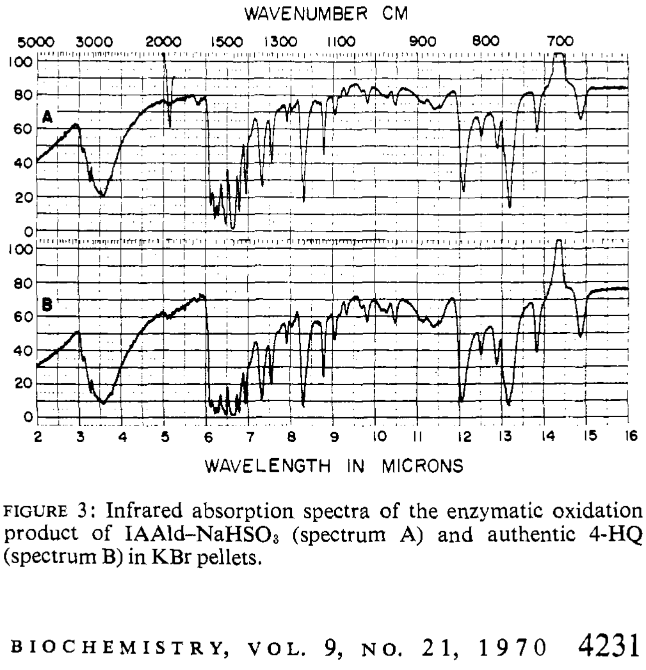

FIGURE 3: Infrared absorption spectra of the enzymatic oxidation product of IAAld–$NaHSO_3$ (spectrum A) and authentic 4-HQ (spectrum B) in KBr pellets.

column chromatography this compound was isolated and identified as 4-HQ. Since HRP is normally rather inactive in the oxidation of IAAld at pH above 6, the question was raised as to whether sodium bisulfite is one of the reactants in the transformation of IAAld to 4-HQ. Thus far no evidence is available to show the involvement of a sulfonate intermediate in the reaction. However, unpublished data from this laboratory point out that in the pH range 5–6, the presence of 4-HQ can be detected either chromatographically or spectrophotometrically as a result of HRP oxidation of IAAld as well as IAAld–HSO_3 (Yeh, 1967). These data indicate that the formation of 4-HQ may not involve a sulfonate intermediate. Observations by several investigators (Yamazaki *et al.*, 1960; Fridovich and Handler, 1961) suggest that the stimulatory effect of bisulfite on peroxidase oxidation of IAAld and IAA occurs because bisulfite can be oxidized through a free-radical chain reaction which is initiated by the peroxide–peroxidase system.

Although the formation of a small amount of IAld from peroxidase oxidation of IAA has been reported (Racusen, 1955; Ray, 1956; Pilet, 1960; Morita *et al.*, 1962), the major oxidation product was not IAld but 3-methyleneoxindole (Hinman and Lang, 1965). However, the present work shows that as high as 50% yield of IAld can be obtained from peroxidase oxidation of IAAld. These results suggest that IAA and IAAld are degraded in different ways by the same peroxidase system and that IAA is not an intermediate in the course of HRP-catalyzed oxidation of IAAld. The results with IAAld–$NaHSO_3$ also suggest at least two possible pathways for IAAld oxidation.

van Tamelen and Haarstad (1961) reported the synthesis of 4-acetylquinoline from 2-methyltryptophan. By using 2-methylindole-3-acetaldehyde as one of the model intermediates, they suggested a sequence of reactions to explain the reaction pathway. The oxidation of IAAld to 4-HQ may follow a reaction mechanism similar to that suggested by van Tamelen and Haarstad with loss of the carbon atom at the 2-position. However, an alternative explanation may also be suggested if the carbon atom removed in the oxidation reaction is not the second carbon of the indole ring but rather is the first carbon of the side chain (as is the case when IAld and 3-methyleneoxindole are formed). Hinman and Lang (1965) suggested an "epoxide mechanism" to explain HRP-catalyzed oxidation of IAA to 3-methyleneoxindole and IAld. They proposed that the aldehydic oxygen in IAld is derived from H_2O_2, while the oxygen at the 2 position of 3-methyleneoxindole is derived from water. Whether a similar mechanism exists for the transformation of IAAld to IAld and 4-HQ is unknown.

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