THE OXIDATION OF INDOLE-3-ACETALDEHYDE AND ITS 1-METHYL DERIVATIVE BY HORSERADISH PEROXIDASE

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This is to certify that the

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THE OXIDATION OF INDOLE-3-ACETALDEHYDE AND ITS 1-METHYL DERIVATIVE BY HORSERADISH PEROXIDASE

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ABSTRACT

THE OXIDATION OF INDOLE-3-ACETALDEHYDE AND ITS 1-METHYL DERIVATIVE BY HORSERADISH PEROXIDASE

by Ren Homer Yeh

Indole-3-acetaldehyde exerts the same physiological effect on plant growth as does indole-3-acetic acid. Therefore, it is assumed that the former compound is oxidized enzymatically within the plant tissues to indole-3-acetic acid. Since this assumption has not been completely verified this investigation was undertaken to ascertain the alternative metabolic pathways of these two plant auxins.

Because peroxidase enzymes play a dual role as an "auxin oxidase" as well as an "aldehyde oxidase," horseradish peroxidase was used as a model enzyme system to study and compare the metabolism of indole-3-acetaldehyde and its 1-methyl derivative to that of indole-3-acetic acid.

In this study, a number of organic compounds including 1-methylindole-3-acetaldehyde (not synthesized previously) were prepared by various methods.

Our experimental results indicate that the peroxidase enzyme catalyzed the oxidation of indole-3-acetic acid to 3-methylene oxindole. However, oxidation of indole-3-acetaldehyde under the same conditions produced indole-3-

carboxaldehyde as one of the major products instead of 3-methylene oxindole.

The oxidation of the auxin derivatives including indole-3-acetic acid was found to be dependent on the conditions of the reaction. Changes in pH and the presence or absence of a cofactor altered the oxidative pathways of these compounds and gave rise to different oxidation products. For example, the presence of bisulfite ion at a pH range above neutrality stimulated the oxidation of indole-3-acetaldehyde to 4-hydroxyquinoline. The latter compound was isolated in crystalline form by use of silicic acid column chromatography. The same transformation of the 1-methyl derivative of indole-3-acetaldehyde to 1-methyl-4-hydroxyquinoline was not obtained, and apparently no single rule can be applied to the various patterns of peroxidase oxidation of indole auxins.

Thin-layer chromatographic techniques were used for identifying the oxidation products resulting from the oxidation of indole-3-acetaldehyde and 1-methylindole-3-carboxaldehyde by horseradish peroxidase.

Manganese and 2,4-dichlorophenol had no effect on the oxidation indole-3-acetaldehyde either at alkaline or acidic pH conditions, although these two cofactors are excellent stimulators of peroxidase oxidation of indole-3-acetic acid.

Indole-3-acetic acid at alkaline pH conditions inhibited strongly the formation of peroxide-peroxidase complexes of the horseradish peroxidase. This observation

supports the belief that at certain pH values, oxidation of auxin by peroxidase is controlled by auxin itself. Also these studies indicate a typical case of substrate inhibition of an enzyme reaction.

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LIST OF ABBREVIATIONS

4-HQ	4-Hydroxyquinoline
HRPO	Horseradish Peroxidase
IAA	Indole-3-acetic Acid
IAAld	Indole-3-acetaldehyde
IAld	Indole-3-carboxaldehyde
1-Methyl-IAAld	1-Methylindole-3-acetaldehyde
1-Methyl-IAld	1-Methylindole-3-carboxaldehyde
TLC	Thin-layer Chromatography
IAAld-$NaHSO_3$	Indole-3-acetaldehyde Sodium Bisulfite Adduct
1-Methyl-IAAld-$NaHSO_3$	1-Methylindole-3-acetaldehyde Sodium Bisulfite Adduct
IPyA	Indole-3-Pyruvic Acid
2,4-D	2,4-Dichlorophenol

Dedicated to My Wife

Ching-Chu

INTRODUCTION

INTRODUCTION

Literature Survey

In 1934 Kögl, Haagen-Smit and Erxleben (52) demonstrated that indole-3-acetic acid (IAA), which was isolated from urine, had a striking effect in promoting cell elongation in Avena coleoptile sections. It was not until 1946 that Haagen-Smit and Went (34) definitely showed the presence of IAA in Zea mays by isolating this compound in pure form. Most of their characterization of IAA was based on chromatographic analysis. IAA has been found to be widely distributed in the plant kingdom (8, 28, 88, 107). In addition to the wide occurrence of IAA in plants, the presence of other indolic compounds has been reported extensively (26, 27, 53, 60, 79, 117). All of these compounds, which are either the precursors of the active auxins or the end products of hormonal metabolism, constitute a pool of auxin growth regulators in plants.

The presence of indole-3-acetaldehyde (IAAld) in plant tissues was first reported by Larsen (57) two years before Kögl, Haagen-Smit and Erxleben's publication (52) on the identification of IAA. Evidence for the occurrence of this neutral auxin derivative was also confirmed by other investigators (29, 30, 55). Several investigators (55, 99, 108) suggest two metabolic pathways for the formation of

IAAld: one involves the decarboxylation of trytophan to tryptamine followed by oxidative deamination to indole-3-acetaldehyde; and the other involves an oxidative deamination to indole-3-pyruvic acid (IPyA) with subsequent decarboxylation to IAAld. Direct evidence (16) for the tryptamine pathway was provided by the finding that in watermelon tissue tryptophan could be transformed easily into tryptamine. Furthermore, the evidence that a purified amine oxidase obtained from pea seedlings (13) could convert tryptamine readily into IAAld lends support to this pathway. On the other hand, the presence of IPyA in plant tissues was not confirmed (40). However, it has been demonstrated that in aqueous solution IPyA can be decomposed readily to form IAAld (109). Observations that IAA can be formed easily from IPyA during chromatographic analysis (40, 88) give further support to the IPyA pathway.

The suggested biochemical pathways leading to the formation of IAAld are summarized in Figure 1.

It is apparent from experiments (87) that auxin-stimulated growth depends not only on the biosynthesis of auxins, but also on their metabolism. The presence in plants of a so-called auxin oxidase system (93, 94, 105), which catalyzes the oxidation of IAA and its derivatives, has been recognized extensively (10, 35, 76), but the chemistry of this "auxin oxidase" is controversial. Galston, Bonner, and Baker (22) postulate that "auxin oxidase" is actually a combination of enzymes: an oxygenase which is

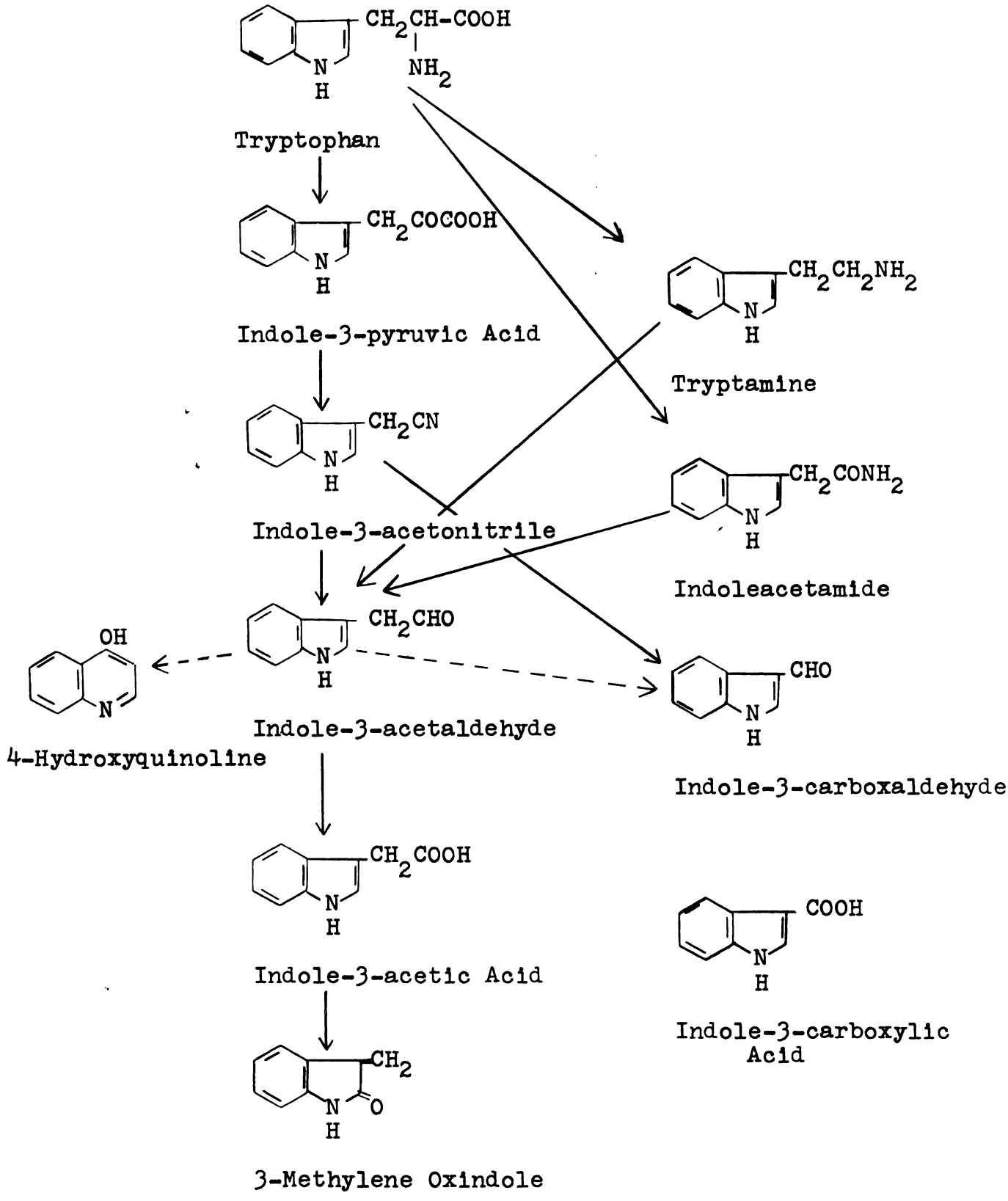


Figure 1. Pathways for the Metabolism of IAAld

a light-activated flavoprotein and a peroxidase which can use the hydrogen peroxide generated from the flavoprotein moiety to oxidize IAA. On the other hand, Kenten and Stutz (43, 91) could not identify the requirement for a light-activated flavoprotein factor in bean and lupine plants. They implied that an "auxin oxidase" is simply a peroxidase which can also act as oxidase in the presence of certain heat-stable cofactors such as monophenols and manganese ions. The belief that "auxin oxidase" is an oxidase-peroxidase is based on the following observations:

1. "Auxin oxidase" appears to be a hemoprotein since it can be inhibited by cyanide or by carbon monoxide. The inhibition by carbon monoxide is light-reversible (75).
2. "Auxin oxidase" is inhibited by catalase and stimulated by hydrogen peroxide (5, 43, 78).
3. Tissue extracts containing "auxin oxidase" can replace peroxidase for peroxidase substrates (75, 84, 91).
4. Highly purified horseradish peroxidase can also replace "auxin oxidase" to catalyze the oxidation of IAA (22, 42, 43, 105).
5. The seasonal changes and the activity of "IAA oxidase," manganese oxidase, and peroxidase are well correlated (70).
6. <u>In vitro</u> oxidation of IAA with purified horseradish peroxidase yields the same products as "IAA oxidase" (37).

7. The oxidation of IAA consumes one molecule of oxygen and evolves one molecule of carbon dioxide per molecule of IAA oxidized (94).

Obviously, a wealth of evidence suggests a close similarity between "IAA oxidase" and peroxidase.

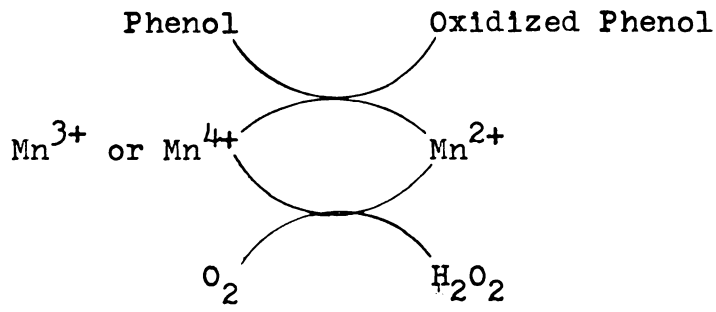
The purification of peroxidase from horseradish was first reported by Keilin and Mann (41) and crystallized by Theorell (97). It was the fourth hemoprotein to be crystallized, succeeding hemoglobin, myoglobin, and catalase. Other crystalline peroxidases from plant sources have been obtained from turnips, Japaneses radishes, wheat germ, and pineapples. Theorell and Akeson (96) have determined the amino acid and carbohydrate composition of horseradish peroxidase. Analyses indicate that the enzyme contains carbohydrate. Similarly, Japanese radish peroxidase also has a high content of carbohydrate residues: mannose (35), xylose (11), and arabinose (4) (69).

The heterogeneity of horseradish peroxidase has been studied extensively (95). The multiple components of horseradish peroxidase with identical spectra and activity are separable by salt precipitations, paper or starch electrophoresis, or chromatography. Theorell (98) reported the existence of a variable component, para-peroxidase or horseradish peroxidase I, which moves in a direction opposite to the major component, horseradish peroxidase II, during electrophoresis. In many aspects, horseradish peroxidase resembles methemoglobin in absorption spectrum and in complex formation with hydrogen peroxide.

As far as the kinetics of the enzyme reaction are concerned, the contribution of Chance (14, 15) should be mentioned. Mild treatment of horseradish peroxidase with hydrochloric acid cleaves the heme group of the enzyme, and the nondenaturated apoprotein with some of the original activity of the enzyme is obtained. This observation was reported earlier by Maehly (63). Recently Siegel and Galston (85) observed that the cleaved protein without the heme group did not oxidize IAA. However, IAA was oxidized in the presence of a monophenol cofactor and manganese ions. The cleavage of horseradish peroxidase was found to be reversible; the acid treated peroxidase could be restored to an active form by neutralization with alkali.

Numerous chemical compounds react with peroxidase to form stable compounds which can be detected spectroscopically (83). Many of these substances inhibit enzymatic activity. Cyanide, fluoride azide, hydroxylamine, and nitric oxide are several compounds known to be inhibitors of peroxidase activity. Some investigators (5, 23) suggest that these o-polyphenols act as inhibitors since they are capable of being oxidized rapidly by peroxidase with the subsequent result of a deficiency of hydrogen peroxide in the reaction mixutre. However, it is possible that the inhibiting effect of o-polyphenols is due to the enzyme catalyzed formation of the condensation products between IAA and the polyphenols (59). The stimulating effect of some naturally occurring monophenols may account for the

fact that these compounds are oxidizable in the presence of a peroxidase system (43). A cyclic mechanism was advanced by Kenten and Mann (45) to explain the oxidase character of the peroxidase. The suggested cyclic mechanism is illustrated by the following diagram:



In addition to the monophenols, maleic hydrazide (5, 43) was found to be a promoter in the oxidation of IAA, but its mode of action is unknown. Recently Klebanoff (49, 50, 51) reported that bisulfite or sulfite anions stimulated the oxidation of pyridine nucleotides by horseradish peroxidase and that thyroxine was a stimulator in the oxidation of adrenaline, uric acid, and ascorbic acid.

Some compounds act either as accelerators in certain reactions or as inhibitors in other reactions catalyzed by the same enzyme system. Thyroxine is an example of such a compound. It is a strong accelerator in the peroxidase oxidation of adrenaline, uric acid, and ascorbic acid but a strong inhibitor of peroxidase oxidation of iodide and pyrogallol. Among other compounds which are found to have a significant effect on peroxidase were ammonia, hydrazine, pyridine and imidazole (17).

Peroxidase catalyzes the reaction of hydrogen peroxide by electron donors other than hydrogen peroxide. A wide variety of chemical compounds have been found to be active as hydrogen donors in the oxidation and reduction systems. Therefore, the reaction involved in peroxidase-catalyzed reactions shows great versatility. Peroxidase can act as an oxidizing agent, halogenating agent, and hydroxylating agent as well. Peroxidase can also participate in polymerization, isomerization, and decarboxylation reactions. These observations on the versatility of peroxidase reactions have been reviewed by Mason and Saunders (65, 83).

Recently, IAAld was found to be an excellent substrate for rabbit muscle homogenates (81), liver homogenates (115), and pure horseradish peroxidase (116). Apparently, the peroxidase reactions are not only involved in the regulation of the hormonal level in plant tissues, but are closely related to the hormonal metabolism in mammalian tissues. The interconversion of steroid hormones in mammalian tissue depends greatly on the peroxidase system (47).

The mechanism of enzymatic degradation of auxins has for many years been a subject attractive to many investigators. The publications in this field are voluminous. The early report by Tang and Bonner (94) indicated that the oxidation of IAA consumed one molecule of oxygen and evolved one molecule of carbon dioxide per molecule of IAA. The proposed reaction product was indole-3-carboxaldehyde

(IAld). The results obtained by them demonstrated that the major feature of enzymatic degradation of IAA by "auxin oxidase" was decarboxylation of the side chain of the IAA molecule. However, later reports published by Manning and Galston (64) suggested that IAA could be oxidized in a manner analogous to the well-known kynurenine pathway of trypotophan metabolism in animal tissues, but they could neither identify the formation of indole-3-carboxaldehyde nor the production of formylkynurenine in the oxidation of IAA by pea homogenates.

The formation of a small quantity of indole-3-carboxaldehyde, among other products of IAA oxidation, later was detected by Racusen (73), Pilet (71), and Stutz (90) in reaction mixtures containing plant tissue homogenates. The formation of IAld from IAA oxidation catalyzed by Japanese radish peroxidase was reported recently (68). Furthermore, Stutz (90) discovered that the addition of cytochrome c and a cytochrome oxidase containing homogenate of rat liver to the lupine enzyme converted IAA to IAld in 50% yield. Moreover, Stutz (92) reported IAld could be formed almost quantitatively from indole-3-glycolic acid. Because of the ultraviolet and infrared spectra of the reaction product, Stowe, Ray, and Thimann (89) suggested that one of the IAA oxidation products might be 3-methyl oxindole. By studying the oxidation of IAA by a highly purified enzyme from lupine seedlings, Stutz (91) found at least five oxidation products of IAA.

Following the observation of Galston, Bonner, and Baker (22) that horseradish peroxidase itself could oxidize IAA, research has been conducted mainly on the oxidation of IAA in the presence of peroxidases alone. In the studies on the oxidation of IAA by Omphalia enzyme and horseradish peroxidase, Ray (77) observed the formation of an intermediate compound, and as a result he proposed a two-step reaction mechanism for the degradation of IAA by the enzyme system. This intermediate compound was regarded as the actual product of the enzymatic reaction. The compound in question was neutral and retained the indole structure, but was neither oxindole nor indole-3-carboxaldehyde. This intermediate compound was called compound A by Ray. The second step of the reaction, a course to the final products, was regarded as a nonenzymatic reaction.

Recently, Hinman and Lang (36) reported that the oxidation of IAA catalyzed by crystalline horseradish peroxidase yielded 3-methylene oxindole as a major product. They also showed that oxindole-3-carbinol was a precursor of 3-methylene oxindole. Consequently, oxindole-3-carbinol was regarded as the intermediate compound A in Ray's two-step reaction mechanism. Hinman and Lang (36) proposed a new mechanism for the degradation of IAA catalyzed by crystalline horseradish peroxidase. The mechanism involved, as a first intermediate, an indolenine hydroperoxide which is converted through an indolenine epoxide to oxindole-3-carbinol, which in turn undergoes a slow but spontaneous

transformation into 3-methylene oxindole. This suggested mechanism is of great interest and will be discussed further.

Recently, Latarjet and Caldas (58) made the interesting observation that certain bacteria can be made sterile by heavy exposure to ultraviolet light and then restored to activity by peroxidase. Kamerbeek (39) and Galston (20) reported, respectively, that there is remarkably greater peroxidase activity in dwarf varieties of beans or peas. These findings imply that the sui generis synthesis of peroxidase decides the height of certain variety of beans or peas . Mathan (66) reported that leaf shape or size of normal (La^+/La^+), Lanceolate (La^+/La^-), and several other leaf-shape mutants of tomato was determined by the content of peroxidase, catalase and phenoloxidase.

Another theory advanced by Galston and Dalberg (21) suggested that the ageing process in plants is closely correlated to the activity of peroxidase in plant tissues. This assumption was based on the observation that the IAA destroying activity in pea stem or root sections increased longitudinally from the apex (minimum activity) toward the point where growth ceased and cell maturation occurred. Siegel (84) noted that the lignification of vascular tissue involved the action of peroxidase.

Apparently, other than the oxidation and reduction processes, the biologically significant aspects of the peroxidase catalyzed oxidation of auxins may be exhibited in the areas of growth, ageing, morphogenesis, and protection

or adaptability of plants to the specific conditions they may encounter. The observations of Saunders et al. (83) indicate that the versatility of peroxidases may be related to the biosynthesis of certain complex organic compounds in plants.

Recently, Galston and co-workers (19), and Key and Ingle (46) mentioned that growth processes controlled by auxin were closely related to the synthesis of RNA, especially messenger RNA, since auxin could enhance the synthesis of RNA in excised plant tissue. Such processes involved the combination of IAA to the RNA fraction, but the combination could only be shown in the presence of peroxidase.

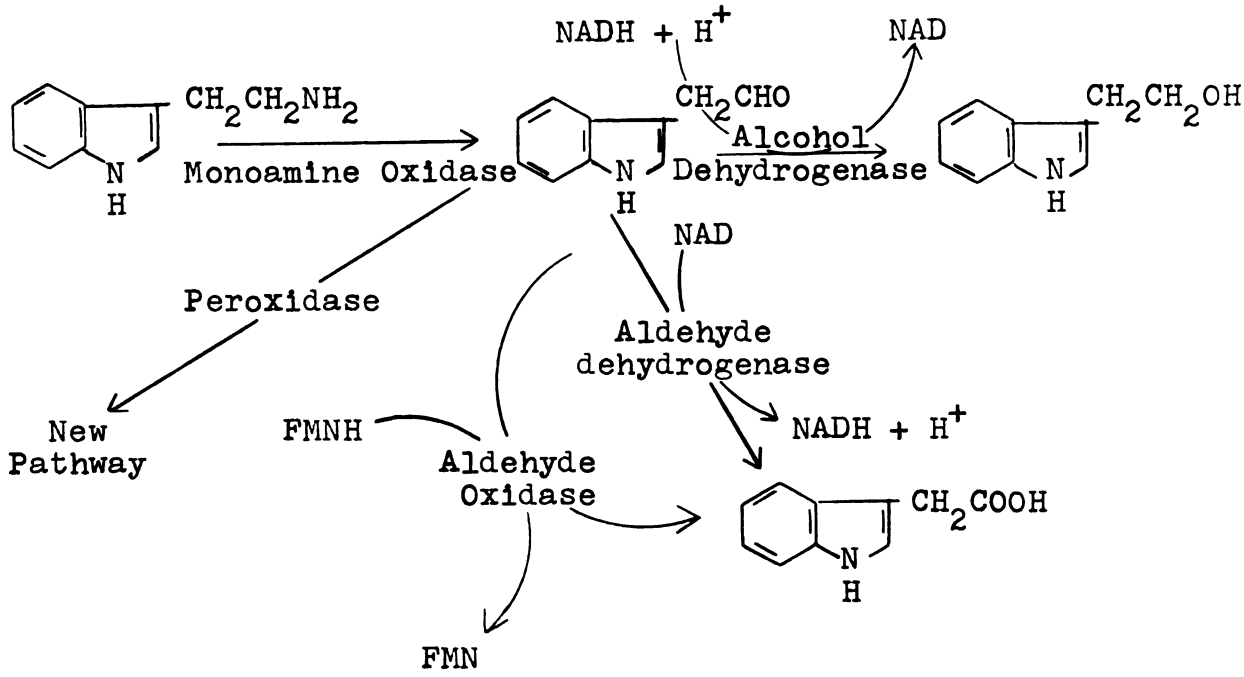
Statement of Problem

Several investigators (6, 29, 55) propose that IAld is an immediate precursor of IAA. Indeed, the presence of an enzyme system which will catalyze readily the oxidation of IAAld to IAA has been reported (29, 55). However, a satisfactory stoichiometric relationship between IAAld disappearance and IAA formation has never been obtained. Therefore, some workers (6, 44) suggest that in plant tissues IAAld and its analogs may be metabolized in a manner other than by oxidation or dismutation to the corresponding acids (56). Larsen (56) studied the metabolism of IAAld and naphthalene-1-acetaldehyde by Avena coleoptile sap, and found that at least one-half of the aldehydes added to the coleoptile sap disappeared so rapidly that they did not give

rise to the formation of IAA. Consequently, he suggested that the conversion of IAAld to IAA might involve a dismutation reaction in which equivalent amounts of alcohol and acid are formed.

However, Kaper and Veldstra (40), who studied the metabolism of tryptophan by Agrobacterium tumefaciens, did not find the 1:1 ratio between IAA and tryptophol formation reported by Larsen (56). The ratio of IAA to tryptophol depended on the experimental conditions used. Ashby (6) also studied the metabolism of naphthalene-1-acetaldehyde by Artemisia-seedling root sap and found less than 1 mole of acid per 2 moles of aldehyde. These facts do not support Larsen's (56) dismutation theory for the explanation of the extra loss of the aldehydes in plant tissues.

Apparently, no single enzyme called aldehyde mutase is present in both plant and animal tissues. The purified aldehyde mutase from beef liver was found by Racker (72) to be actually a mixture of two separable enzymes, an alcohol dehydrogenase and aldehyde dehydrogenase. The enzymes, which will catalyze the transformation of IAAld and its analogs to the corresponding acids or alcohols, are illustrated in the following scheme:



The lack of a stoichiometric relation between aldehyde disappearance and acid formation suggests that there are at least two metabolic pathways which are involved in the metabolism of IAAld or its analogs. One catalyzes the transformation of IAAld to IAA and the other catalyzes the direct metabolism of the active auxin precursors. These facts indicate a balance between these two enzymatic systems according to the stage of development of the living organisms.

Kenten and Mann (45) 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. They suggested that a so-called aldehyde oxidase was present in the extracts. Further studies on this pea-seedling "aldehyde oxidase" by Kenten (44) indicated that the rate of oxygen

uptake required for the oxidation of β-phenylacetaldehyde can be inhibited to the extent of 85% when a high concentration of catalase is added. But for a simple flavoprotein, the inhibition of catalase would, at most, reduce the reaction rate to half of the total oxygen uptake. From this and other evidence, Kenten (44) concluded that the "aldehyde oxidase" catalyzed reaction is very similar to the oxidase-peroxidase reaction, and that the oxidase-peroxidase-catalyzed oxidation of β-phenylacetaldehyde does not yield phenylacetic acid but phenylcarboxaldehyde. Some observations (44) suggest that the wide distribution of oxidase-peroxidase systems in nature may be responsible for the rapid disappearance of IAAld and its analogs in various plant tissues.

Recently, Bartholini et al. (7) noted the presence of enzyme systems in platelets and erythrocytes which can readily catalyze the transformation of IAAld to its corresponding alcohol and acid. However, a lack of stoichiometric relationship between aldehyde disappearance and acid formation was also observed in these experiments. Udenfriend et al. (103) reported that only 30% of the serotonin metabolized by rat liver homogenate could be recovered as 5-hydroxyindole-3-acetic acid, whereas Wu (111) could not detect any IAA when rat liver mitochondria was employed as a catalyst for tryptamine oxidation. Similar results (115) were observed when IAAld was directly used as substrate for the oxidation studies by liver homogenates. Evidence has

accumulated to support the existence of an alternative pathway other than IAA formation for the metabolism of the hormonal substances such as IAAld and its analogs in both plant and animal tissues.

Moreover, several investigators (3, 4, 9, 12, 32, 54) have studied the effect of IAAld on various auxin tests. Results of these tests indicate that IAAld becomes active by being oxidized enzymatically into IAA. Moreover, another noteworthy observation is that in all of these tests, the relative activity of IAAld is not always the same. According to Larsen (54), the differences found in the relative activity of IAAld in different tests may be due to test objects with different properties in absorbing IAAld or to conversion of IAAld into IAA. Indirectly, this finding also suggests the existence of an alternative pathway for the metabolism of IAAld.

Because of the dual role of horseradish peroxidase to act both as an "IAA oxidase" and an "aldehyde oxidase," the present work on this enzyme was taken as a model to study this new metabolic pathway of IAAld. Attempts were made to identify the oxidation products of IAAld catalyzed by this crystalline enzyme. Studies were made also on 1-methyl-IAAld to give further information concerning the mechanism of horseradish peroxidase-catalyzed oxidation of indole aldehydes.

MATERIALS AND METHODS

MATERIALS AND METHODS

Synthesis of Compounds

A number of different methods were selected for the preparation of indole derivatives for this thesis. Detailed procedure for synthesis of the compounds is outlined below:

Indole-3-acetaldehyde.--Indole-3-acetaldehyde was synthesized from tryptophan by treatment with dilute sodium hypochlorite solution by the method of Gray (32).

Three grams of DL-tryptophan were dissolved in 100 ml of a 10% sodium hydroxide solution which was then adjusted to a pH of 8.5 with 4 N hydrochloric acid. The solution was poured at once into a 4,000 ml Erlenmeyer flask containing 1,200 ml of distilled water and 700 to 800 ml of benzene; then 200 ml of a 0.52% sodium hypochlorite solution (1 part Clorox with 9 parts water), was added. Immediately after the addition of the hypochlorite solution, the flask and contents were shaken with a swirling motion in hot water bath. The temperature was raised to 50° over a period of 20 minutes and then held between 50 and 51° for an additional 15 minutes. During this operation, the benzene layer became yellow to orange in color indicating a successful synthesis.

While still warm, the benzene layer was separated from the water phase and concentrated to a volume of 65 ml

under vacuum while maintaining the temperature below 50°. A crystalline product was obtained by shaking the resulting benzene solution with 50 ml of saturated sodium bisulfite solution. The crystals were collected on a Büchner funnel and washed several times with 95% ethanol and finally with absolute ethanol and ether. The product was recrystallized by dissolving IAAld-$NaHSO_3$ in a minimum amount of water and then diluting with absolute ethanol to make a final concentration of 90% ethanol. The yield was 1.9 to 2.0 g of pure IAAld-$NaHSO_3$.

To obtain the free aldehyde, 200 mg of IAAld-$NaHSO_3$ was dissolved in 8 ml of distilled water and a few drops of concentrated Na_2CO_3 was added. The aldehyde was then extracted with peroxide-free ether. After evaporation of the ether solution to dryness under vacuum, 82 μg IAAld was attained.

1-Methylindole-3-acetaldehyde.--The title compound was made by the same procedure described by Gray (32) for indole-3-acetaldehyde and was also obtained in the form of bisulfite salt. Since the bisulfite salt of 1-methyl-IAAld is very soluble in 95% ethanol and cannot be recrystallized by the method outlined for IAAld-$NaHSO_3$, it was recrystallized by the following procedure. First, the salt was dissolved in a minimum amount of water to make a saturated solution, and then it was filtered. A small amount of isopropanol was added to the clear filtrate to induce cloudiness. Then the mixture was set in a refrigerator overnight

or longer for crystallization to occur. One and one-half grams of white needles of 1-methyl-IAAld-$NaHSO_3$ was obtained from 4 g of 1-methyltryptophan. The free 1-methyl-IAAld was obtained in the same manner as described for indole-3-acetaldehyde (32).

1-Methyltryptophan.--1-Methyltryptophan was obtained from the sodium salt of tryptophan with methiodide in liquid ammonia according to a process outlined by Yamada et al. (112).

Metallic Na (3.2 g, 0.14 atomic mass units) was added in small pieces to a mixture of about 450 ml of liquid ammonia containing 0.2 g of ferric nitrate monohydrate while the mixture was being stirred. After the sodium metal was completely dissolved, 12.3 g of DL-tryptophan which was suspended in an appropriate amount of anhydrous ether, was added to the mixture while it was being stirred. After 30 minutes 11.4 g of methyl iodide was added dropwise over a 15 minute period and stirring was continued until all ammonia was completely evaporated. The residue was then slowly transferred to a mixture containing 350 ml of ethanol and 350 ml of H_2O. This mixture was then evaporated under vacuum to dryness. The residue was added to 350 ml H_2O, which was heated to dissolve the mass and then filtered. The warm solution was adjusted to pH 5.0 with glacial acetic acid and diluted with 350 ml of 95% ethanol. The mixture was then placed in a refrigerator overnight. The resulting white precipitate was collected on a Büchner funnel and washed successively with

40 ml of H_2O, 40 ml of 50% aqueous ethanol, 40 ml absolute ethanol, and finally with 40 ml of dry ether. This precipitate, which was recrystallized from 50% aqueous ethanol, consisted of colorless needles of melting point 269° (lit., mp 269°) (112) and gave a positive ninhydrin test.

1-Methylindole-3-carboxaldehyde.--The title compound was synthesized from 1-methylindole by reaction of dimethylformamide on indole in the presence of phosphorus oxychloride by a method described by Tyson and Shaw (102).

To a flask protected from atmospheric moisture, fitted with a mechanical stirrer, and containing 1.18 mole of dimethylformamide cooled to -5°, 2.0 mole of phosphorus oxychloride was added. During this procedure the mixture was stirred constantly to prevent the temperature from rising above 10°. After phosphorus oxychloride was added, 1.0 mole of 1-methylindole was added in small quantities while a temperature between 23 and 27° was maintained. Stirring was continued at 25° for 0.5 hour. Then 4.0 g of finely divided calcium carbonate was added to the mixture and the temperature was gradually raised from 30 to 35°. From this point on the temperature was controlled by cooling the flask with ice from time to time until the temperature reached 55 to 60° in 0.5 hour. The slurry was cooled externally to 10° with ice and then 20.0 ml of 30% solution of sodium acetate was poured into the flask as the mixture was stirred. Then the mixture was diluted with water to a volume of 1,000 ml. After 1.18 moles of sodium hydroxide was added and the mixture was

refluxed for 3 hours, dimethylamine formed copiously. The mixture was diluted with water so that its volume reached 3.5 liters. Then it was heated to reflux and filtered. After the resulting filtrate was treated as described above, it yielded colorless needles of melting point 69° (lit. mp 69°) (82).

1-Methyl-4-hydroxyquinoline.--1-Methyl-4-hydroxyquinoline was made by using the method reported by Simpson and Wright (86) for the synthesis of 6-nitro-1-methyl-4-quinoline.

Methyl sulfate (2.2 ml) was added to a solution of 1.5 g of 4-hydroxyquinoline in 48 ml of aqueous potassium hydroxide (1.9%) at 50°. At the end of the reaction, excess alkali was added, and the 1-methyl-4-hydroxyquinoline (2.1 g) extracted with chloroform. The product was recrystallized from benzene, and gave colorless prisms of melting point 150 to 152° (lit. 150-152°) (1). The chloride of 1-methyl-4-hydroxyquinoline was obtained by dissolving the 1-methyl-4-hydroxyquinoline in diluted hydrochloric acid solution. After the solution was evaporated to dryness, the residue was recrystallized from isopropanol. The crystals melted at 164 to 166° (lit. 164-166°) (67).

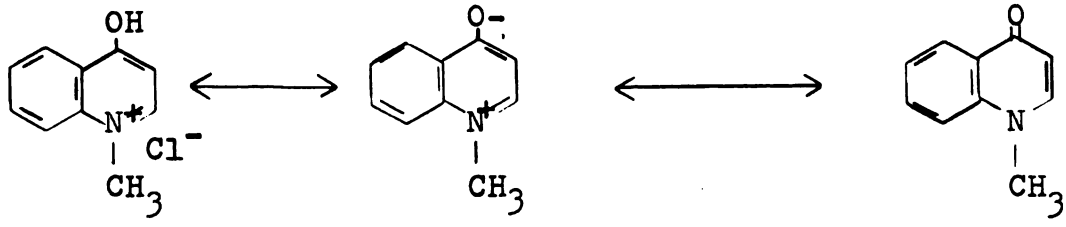
Identification of Compounds

1-Methylindole-3-carboxaldehyde.--The synthesized aldehyde gave positive tests with 2,4-dinitrophenylhydrazine

and xanthydrol, but responded poorly to Schiff's and Ehrlich's color reaction. Its crystals were colorless needles of 69° (lit. 69°) (82). λ_{max}^{KBr} 1780, 1720, 1650, 1560, 1470, 1400, 1380, 1375, 1327, 1260, 1190, 1125, 1075, 1032, 1012, 787, 749, and 730 cm^{-1}. The NH stretching band could not be shown. λ_{max}^{EtOH} 262 mμ.

The infrared spectrum of 1-methylindole-3-carboxaldehyde was very similar to indole-3-carboxaldehyde. The two infrared spectra are shown in Figures 2 (A) and 2 (B) respectively, for comparison.

1-Methyl-4-hydroxyquinoline.--According to Allison et al. (1), 1-methyl-4-hydroxyquinoline has the following reasonance structures:

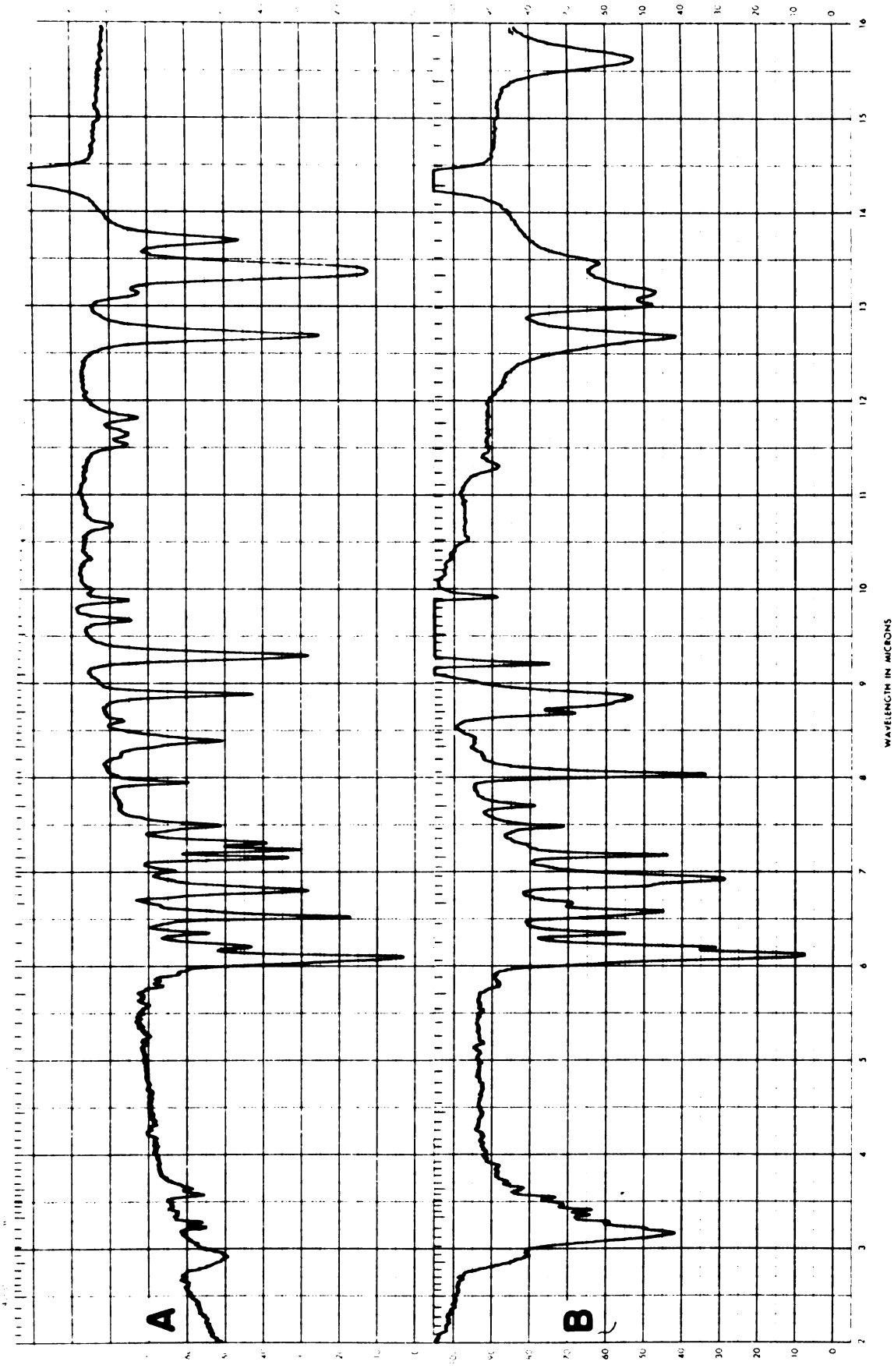


I II III

The quinolinone, structure II, shows no ketonic properties and is unaffected by methiodide in boiling methanol, indicating that the ionic "aromatic" form II makes the major contribution to the reasonance structure.

The free 1-methyl-4-quinolinone crystals were colorless needles of melting point 150 to 152° (lit. 150-152°) (1). The chloride melted at 164 to 166° (lit. 164-166°) (68) λ_{max}^{EtOH} 313, 329 mμ.

Figure 2. Infrared absorption spectra for 1-methylindole-3-carboxaldehyde (A) and indole-3-carboxaldehyde (B) in potassium bromide pellet. The spectra were determined in a Beckman IR-5 spectrophotometer.


A
B
WAVELENGTH IN MICRONS

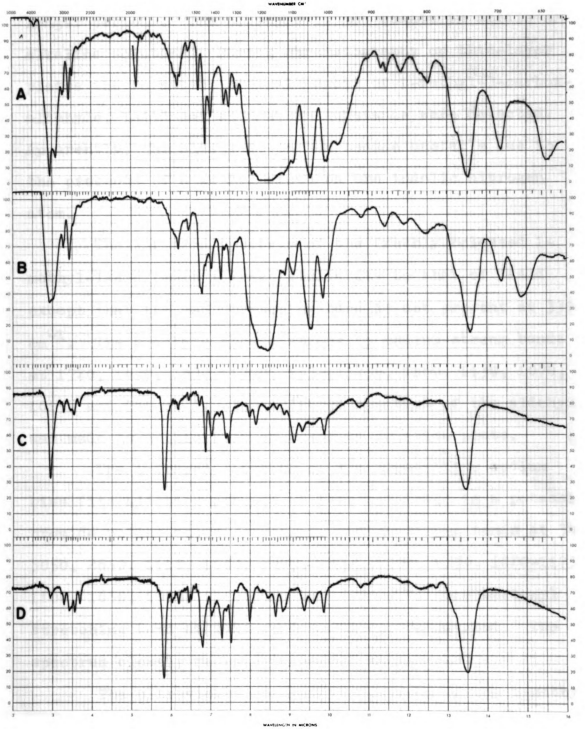
Indole-3-acetaldehyde.--The title aldehyde was identical to the one reported by Gray (32). It was a colorless syrupy liquid and was positive to Schiff's and 2,4-dinitrophenylhydrazine reactions for aldehydes and to Salkowski, Ehrlich, and xanthydrol color reactions for the indole ring.

The infrared spectrum of the freshly released indole-3-acetaldehyde was the same as that reported by Brown (12) and Gray (32). It showed strong absorption bands at 3400, 1710, 747 cm^{-1} and medium strong bands at 1465, 1430, 1350, and 1100 cm^{-1}. The infrared spectrum of the crystalline sodium bisulfite addition product of IAAld in a KBr pellet showed no carbonyl stretching band at 1710 cm^{-1}. Strong to medium-strong absorption bands were noticed at 3400, 3200, 2930, 1620, 1490, 1460, 1345, 1300, 1242, 1050, 1005, 880, 870, 845, 800, 740, 700, and 645 cm^{-1}. The ultraviolet absorption spectrum of the free IAAld in ethanol had absorption maxima at wavelengths of 273, 281, and 289 mμ The infrared spectra of IAAld-$NaHSO_3$ and free IAAld are given in Figures 3 (A) and 3 (C), respectively.

1-Methylindole-3-acetaldehyde.--The free 1-methyl-indole-3-acetaldehyde was a colorless syrupy liquid. It gave positive tests to Schiff's and 2,4-dinitrophenylhydrazine reactions for aldehydes; and to Salkowski, Ehrlich and xanthydrol reactions for the indole ring.

The bisulfite salt of 1-methyl-IAAld was as stable as that of IAAld-$NaHSO_3$. The aldehyde was stable enough for

Figure 3. Infrared absorption spectra of indole-3-acetaldehyde sodium bisulfite salt (A), 1-methylindole-3-acetaldehyde sodium bisulfite salt (B), indole-3-acetaldehyde (C) and 1-methylindole-3-acetaldehyde (D).


A
B
C
D

chemical analysis, but it is highly recommended that the aldehyde be stored under an atmosphere of nitrogen for longer periods. The aldehyde is not stable in acidic or alkaline solution. During chromatography on thin-layer plates the formation of small spots of 1-methyl-3-carboxaldehyde, IAAld and an unknown substance were detected. Apparently, the aldehydic carbon became unstable due to the presence of a methyl group on the indolic nitrogen atom.

The infrared spectrum of the freshly released 1-methyl-IAAld bears a great similarity to that of IAAld except that the N-H stretching band is not present at 3390 cm^{-1}. The aldehyde showed strong to medium-strong bands at 1720, 1470, 1430, 1370, 1335, 1250, 1160, 1130, 1070, 1015, and 740 cm^{-1}. The infrared spectrum of the aldehyde was made as a thin film on a sodium chloride plate.

The infrared spectrum of the crystalline sodium bisulfite addition product of 1-methyl-IAAld in a potassium bromide pellet showed strong to medium-strong bands at 3450, 3050, 2850, 1610, 1550, 1470, 1420, 1370, 1330, 1220-1175, 1100, 1050, 1015, 735, 695, and 670 cm^{-1}. The carbonyl stretching band at 1720 cm^{-1} could not be located. The spectrum closely resembles that of IAAld-$NaHSO_3$.

The ultraviolet absorption spectrum of the 1-methyl-IAAld in ethanol showed absorption maximum at wavelength of 288 mμ. The bisulfite addition product in water gave a similar ultraviolet absorption curve with a single maximum

at the same wavelength.

The infrared spectra of the aldehyde and its bisulfite salt are shown in Figures 3 (D) and 3 (B), respectively.

Analysis of $C_{11}H_{12}O_4NSNa$:

Calcd. C, 47.65; H, 4.36; N, 5.05%

Found. C, 46.04; H, 4.86; N, 4.93%

1-Methyltryptophan.--The synthesized 1-methyltryptophan gave a positive ninhydrin reaction. It crystallized as colorless prisms with a melting point of 269°, which is in agreement with that reported by Yamada et al. (112).

Methods for Determination of Substrate Concentrations

The concentrations of IAA and IAAld were determined by xanthydrol reaction with slight modifications of the method described by Weissbach et al. (106). Before each determination a fresh solution of 0.1% xanthydrol in glacial acetic acid was prepared. The following procedure was used. To 1.0 ml of the sample in water was added 0.8 ml of 12 N hydrochloric acid and 2.0 ml of the xanthydrol reagent. Five minutes later 1.0 ml of acetone was added. After each addition of reagent, the solution was mixed well. The intensity of the pink color, which developed, was measured within 5 to 10 minutes at 520 mμ in a spectrophotometer. The standards and blanks were treated in the same manner. The xanthydrol reaction is very sensitive to compounds having

an indole ring. As little as 8 μg of IAA could be detected, and the optical density was found to be proportional to IAA concentration over the entire range from 8 to 200 μg. The calibration curves for IAA and IAAld were determined as previously described. The data are shown in Figure 4.

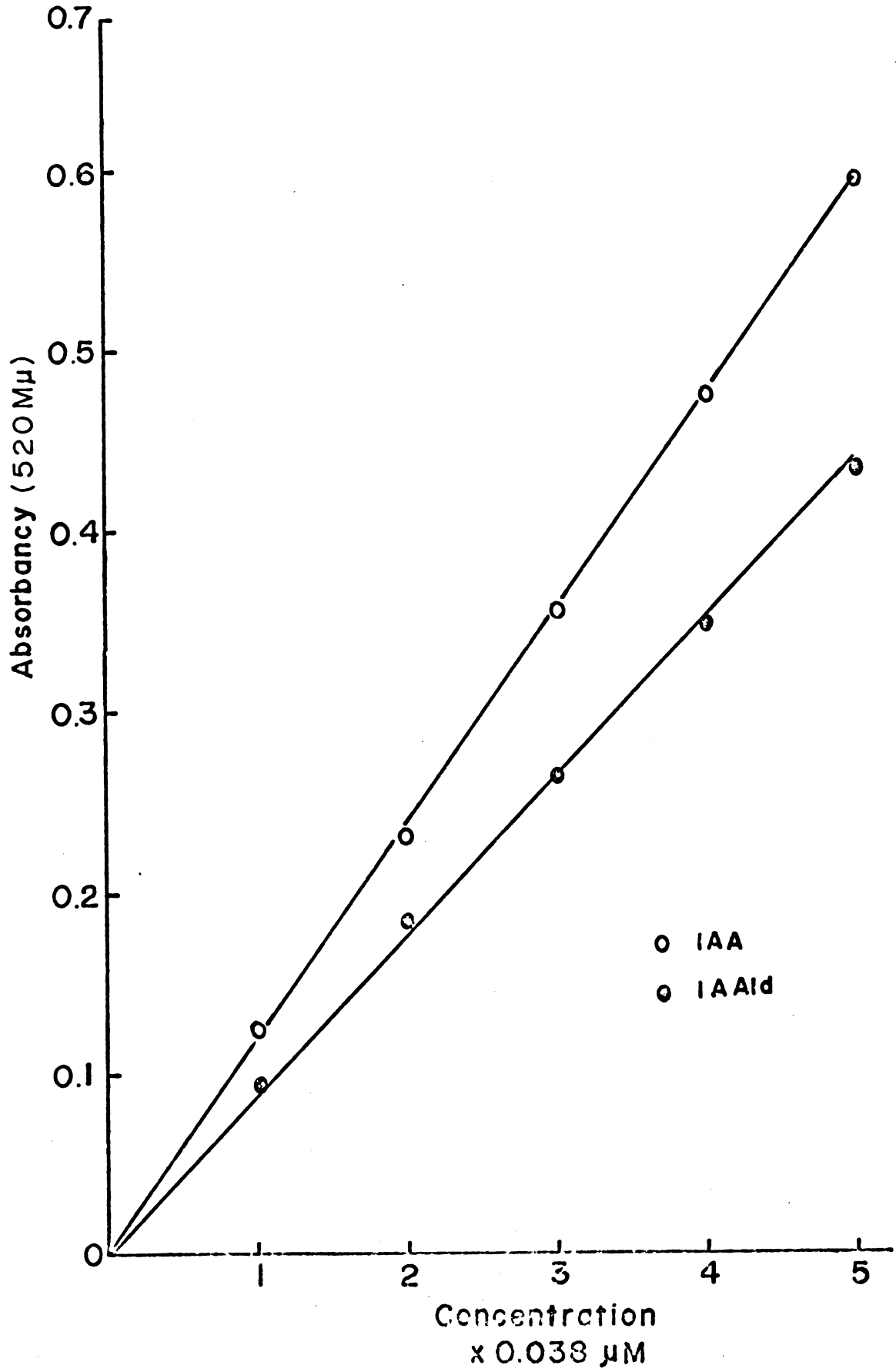
Enzyme

The crystalline horseradish peroxidase (RZ = 3), which was procured from Worthington Chemical Company, was stored at 0° immediately after arrival. Enzyme with RZ less than 3 was also employed, but had no effect on the results reported in this thesis.

Enzyme Reactions

For the enzymatic studies the reaction mixture contained the following: 3 ml of substrate (1×10^{-4} M of IAA or IAAld) in 0.05 M of buffer solutions (acetate buffer for pH below 5.2 or phosphate buffer for pH above 5.6), 0.1 ml of enzyme solution (usually contained 6 μg to 0.3 mg enzyme protein), and 0.1 ml of hydrogen peroxide (1×10^{-2} M). Other cofactors (manganese, 2,4-dichlorophenol, and bisulfite), if used, were added to the substrate solutions. Total reaction mixture per cuvette was 3.2 ml. After reaction was started by the addition of hydrogen peroxide, the rate of the reaction was followed in a Beckman DK-2 Ratio Recording Spectrophotometer to determine the change of the absorbancy which was due either to the oxidation of the substrate or to the formation of the reaction products.

Figure 4. Calibration curves for estimating IAA and IAAld by xanthydrol reaction.


0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
Absorbancy (520 Mμ)
1
2
3
4
5
Concentration
x 0.038 μM
IAA
IAAld

Incubation Conditions for Biosynthesis of a New Oxidation Product

4-Hydroxyquinoline.--The new oxidation product, 4-hydroxyquinoline, was isolated from a reaction mixture consisting of 2,000 ml of 200 mg of IAAld-$NaHSO_3$, 40 mg of peroxidase, and 0.05 M of phosphate buffer solution at pH 7.8. The reaction was started by addition of 160 ml of 2×10^{-3} M hydrogen peroxide and the rate of reaction was determined by the xanthydrol reaction and also by the changes of the optical density at 316 and 330 mμ (a typical absorption maximum of 4-hydroxyquinoline) in the spectrophotometer. After changes in the structure of the substrate were completed, the same amount of IAAld-$NaHSO_3$ was added together with 20 mg of peroxidase. This procedure was repeated in the same way until finally 800 mg of IAAld-$NaHSO_3$ and 100 mg of peroxidase were added. In a similar manner 160 ml of 2×10^{-3} M of hydrogen peroxide was added dropwise to the reaction mixture until a total of 650 ml of the hydrogen peroxide solution was added. After the reaction was completed, the solvent was evaporated under vacuum to dryness. The residue was extracted with isopropanol and the crude material (267.9 mg) in the isopropanol extract was further purified by fractionation through a silicic acid column.

Silicic Acid Column Chromatography

The silicic acid (Fisher Scientific Company, 200 mesh, chromatographic grade) was suspended with stirring in a

sufficient quantity of pure benzene to make a slurry. The supernatant was decanted and the silicic acid was washed twice in the same manner with a mixture of 5% benzene in hexane. After the final wash, the silicic acid was resuspended in 5% benzene in hexane. This suspension was stirred continually while it was transferred to a glass column, 2.0 cm in diameter, to give a silicic acid column having a length of 20 cm.

Samples were applied in amounts of about 200 mg. After the sample, which was obtained from the enzyme reaction mixture, was applied to the top of the adsorbent in the column, the adsorbent and sample were first developed with a solvent system consisting of hexane and ethyl acetate (50:100) and then with isopropanol. About 250 ml of the hexane-ethyl acetate mixture was used for the first development. Samples were collected with an automatic fractionation collector and fractions having similar absorption patterns were combined for further purification.

Thin-Layer Chromatography

For routine work the thin-layer plates were prepared by the method of Randerath (74). For the results formally recorded in this thesis, Silica Gel F_{254} (from Brinkmann Instruments, Inc.) and Eastman Chromatogram Sheet (Type K 301, R2 Silica Gel without fluorescent indicator) were used. The following solvent systems were employed: (1) n-propanol-hexane (1:4); (2) chloroform-acetic acid (95:5); (3) benzene-

acetic acid-water (4:1:1); and (4) n-butanol-hexane (18% n-BuOH).

IAld and 1-methyl-IAld were identified by spraying the adsorbent on the plates with 2,4-dinitrophenylhydrazine reagent, which was prepared according to Randerath (74). Since both IAld and 1-methyl-IAld responded poorly to Erhlich's reagent, the latter reagent was not employed for identification of these two compounds. For identification of 4-hydroxyquinoline or 1-methyl-4-hydroxyquinoline ultraviolet light was used as detecting agent with plates made of fluorescence background.

Ultraviolet and Infrared Spectra

Ultraviolet spectra were determined in a Beckmann DK-2 Ratio Recording Spectrophotometer either in 95% ethanol solution or in water solution. Infrared spectra were made in a Beckmann IR-5 Infrared Spectrophotometer. For liquid compounds such as IAAld and 1-methyl-IAAld, the spectra were obtained as a thin film and for other crystalline compounds as potassium bromide pellets.

RESULTS

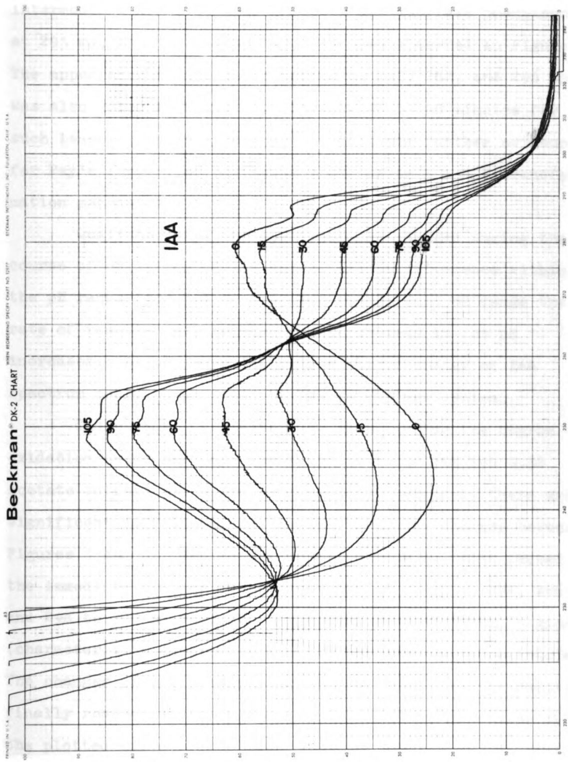
RESULTS

Spectrophotometric Studies on the Oxidation of IAA, IAAld and IAAld-$NaHSO_3$

The changes in ultraviolet spectra during the peroxidase reactions were followed by using the Beckman DK-2 Ratio Recording Spectrophotometer. The incubation conditions were the same as those described under the section on methods. The series of ultraviolet spectra of IAA, IAAld, and IAAld-$NaHSO_3$ are shown, respectively, in Figures 5, 6, and 7.

For the three different substrates used, it can be clearly seen that the breakdown of IAA, IAAld, and IAAld-$NaHSO_3$ by the pure horseradish peroxidase followed different patterns of oxidative pathways. The conversion of IAA to 3-methylene oxindole is clearly noted by the appearance of the typical intense double peaks at λ_{max} 247 and 253 mμ (Figure 5) parallel with the disappearance of the indole absorption at 277 and 285 mμ. These changes in spectrum, which were observed when IAA was used as substrate under the present conditions, are very similar to the observations previously reported by Hinman *et al.* (36, 37) and Ray (77). The experiments shown in Figure 5 were carried out at pH 3.9 acetate buffer solution without addition of any cofactor such as manganese, 2,4-dichlorophenol, and sulfite or bisulfite anion. It is clearly observed that at 265 mμ,

Figure 5. Changes in ultraviolet absorption spectrum during enzymatic oxidation of IAA. 0.1 ml enzyme (8.4 µg/cuvette), 3 ml of 1.07×10^{-4} M IAA in 0.05 M acetate buffer solution, pH 3.90, and 0.1 ml of 1×10^{-2} M H_2O_2 solution. Total volume of the reaction mixture per cuvette was 3.2 ml. Records were begun at 345 mµ. Curves were taken at 0, 15, 30, 45, 60, 75, 90, and 105 min.; each record was completed in 1 min. Final concentration of IAA was 1×10^{-4} M and that of H_2O_2 was 3×10^{-4} M.


Beckman® DK-2 CHART
IAA
0
15
30
45
60
75
90
105

absorbancy increases at first and then decreases; this behavior was shown by Ray to be evidence for the formation of an intermediate A. A similar increase and decrease phenomenon at 295 mμ, as reported by Ray, was also observed in Figure 5. The appearance of isosbetic points at 233, 262, and 296 mμ was also noted in Figure 5. It took 45 to 60 minutes for such isosbetic points to appear. This was further evidence for Ray's support of his sequential IAA to A and B transformation mechanism.

Qualitatively, the spectroscopic changes during the course of oxidation of IAA showed little difference within the pH range from 3.7 to 4.5, but within this pH range the rate of reaction was reduced quantitatively as the pH increased. At pH above 6.1 only a slight change of IAA spectrum could be produced by the peroxidase system.

The changes of spectrum of IAAld during the course of oxidation under the same condition used for IAA (pH 4.58 acetate buffer) are shown in Figure 6. Obviously there are significant differences in the spectroscopic changes between Figures 5 and 6. The action of peroxidase on IAAld caused the immediate changes of the spectrum of IAAld at 244, 246, and 298 to 300 mμ regions, but not at 253 and 247 mμ regions (characteristic absorption maximum for 3-methylene oxindole). The changes of the spectrum during the course of the reaction finally reached a steady state as is shown by the shape of the plotted curve. This absorption behavior shows no similarity with that of the typical absorption of the 3-methylene-

Figure 6. Changes in ultraviolet absorption spectrum during enzymatic oxidation of IAAld. 0.1 ml enzyme (8.5 µg/cuvette), 3 ml of 1.07×10^{-4} M IAAld solution in 0.05 M acetate buffer solution, pH 4.58, and 0.1 ml of 1×10^{-2} M H_2O_2 solution. Final concentration of IAAld of the reaction mixture was 1×10^{-4} M and that of H_2O_2 was 3×10^{-4} M. Total volume of the reaction mixture per cuvette was 3.2 ml. Records were begun at 345 mµ. Curves were taken at 0, 15, 30, 45, 60, 75, 105 and 120 min.; each record was completed in 1 min.

Beckman DK-2 CHART
IAAd
120
105
75
60
45
30
15
10
5
0
180
135
90
75
60
45
30
15
10
5
0

oxindole. The typical absorption spectrum produced was found later to represent the absorption of IAld (Figure 22).

After careful study of the spectroscopic changes of IAAld during the course of oxidation at pH between 3.7 to 4.4, it was noted that at certain wavelengths (ca. 274 and 280 mμ regions) the optical density first decreased and then increased. These observations might be used as an interpretation for the possible formation of an intermediate substance during the course of oxidative breakdown of the IAAld. However, because of the lack of substantial evidence, a conclusion could not be drawn on the basis of this simple observation.

Catalytic breakdown of IAAld-$NaHSO_3$ by the peroxidase system gave a completely different picture as compared to that observed for IAA and IAAld. At pH between 3.7 and 4.4, the oxidation of IAAld-$NaHSO_3$ followed the same pattern as that observed for free IAAld. The reaction rate decreased as pH was increased from 3.7 to 6.1. Apparently, the increase of the hydroxide ions in the reaction mixture gradually stopped the activity of the enzymatic system. However, when pH was increased above 6.1, the activity of the enzymatic system toward the substrate (IAAld-$NaHSO_3$) was started again by some unknown mechanism. The rate of reaction increased profoundly as the pH was increased above 6.1. This observation is of considerable interest for the study of enzymatic oxidation of the indole aldehyde.

The spectroscopic changes of IAAld-$NaHSO_3$ oxidized at pH between 6 to 8 is shown in Figure 7. Large differences in the changing of the spectra can be seen clearly as compared to those described for IAA and IAAld. Neither the peaks at 247 and 253 mμ nor those at 244, 246, and 300 mμ could be observed. Instead, as a result of oxidative breakdown of IAAld-$NaHSO_3$, an appearance of a double peak at 316 and 330 mμ regions was always observed. The appearance of these peaks was not due to the absorption of bisulfite present in the substrate molecule because apparently the bisulfite compound itself represents an individual entity in the formation of a new compound.

Effect of pH on the Oxidation of IAA, IAAld, and IAAld-$NaHSO_3$

The effect of pH on the oxidation of these substances is illustrated in Figure 8. The rate of the enzymatic reaction was followed at 244 mμ since the changes of optical density at this wavelength represent the overall reaction. The oxidation of IAA, IAAld, and IAAld-$NaHSO_3$ was found to be dependent on the concentration of hydrogen ions. The data in Figure 8 indicate that all three of these substrates were oxidized rapidly at a pH range between 3.6 and 5.2, a normal optimal pH for horseradish peroxidase. The reaction rate decreased as the pH was increased from 5.6 to 7.8. As the pH was increased above 6.2, a differential behavior of the enzymatic system toward the oxidation of IAAld-$NaHSO_3$

Figure 7. Changes in ultraviolet absorption spectrum during enzymatic oxidation of IAAld-$NaHSO_3$ at pH 7.68. 0.1 ml enzyme (17.0 µg/cuvette), 3 ml of 1.07 x 10^{-4} M IAAld-$NaHSO_3$ solution in 0.05 M phosphate buffer solution, pH 7.68, and 0.1 ml of 1 x 10^{-2} M H_2O_2 solution. Total volume of the reaction mixture per cuvette was 3.2 ml. Final concentration of IAAld-$NaHSO_3$ of the reaction mixture was 1 x 10^{-4} M and that of H_2O_2 was 3 x 10^{-4} M. The records were begun at 345 mµ. Curves were taken at 0, 5, 15, 30, 45, and 60 min.; the last curve was taken 8 hours after reaction started.

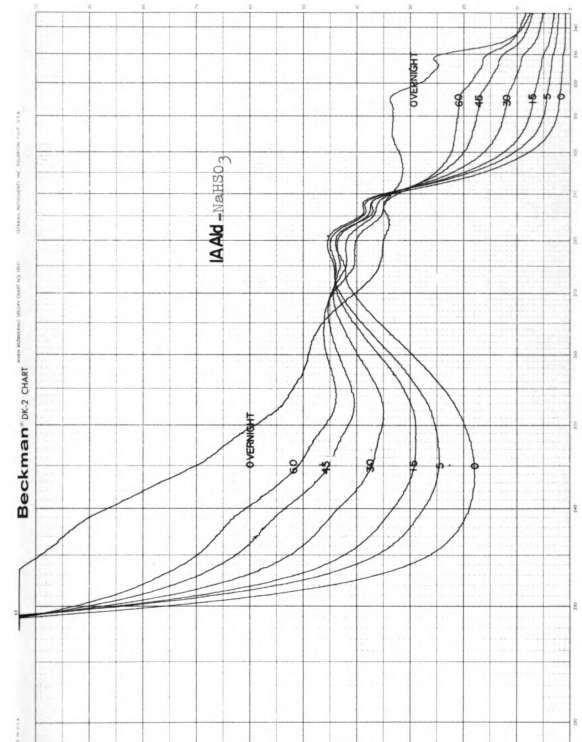
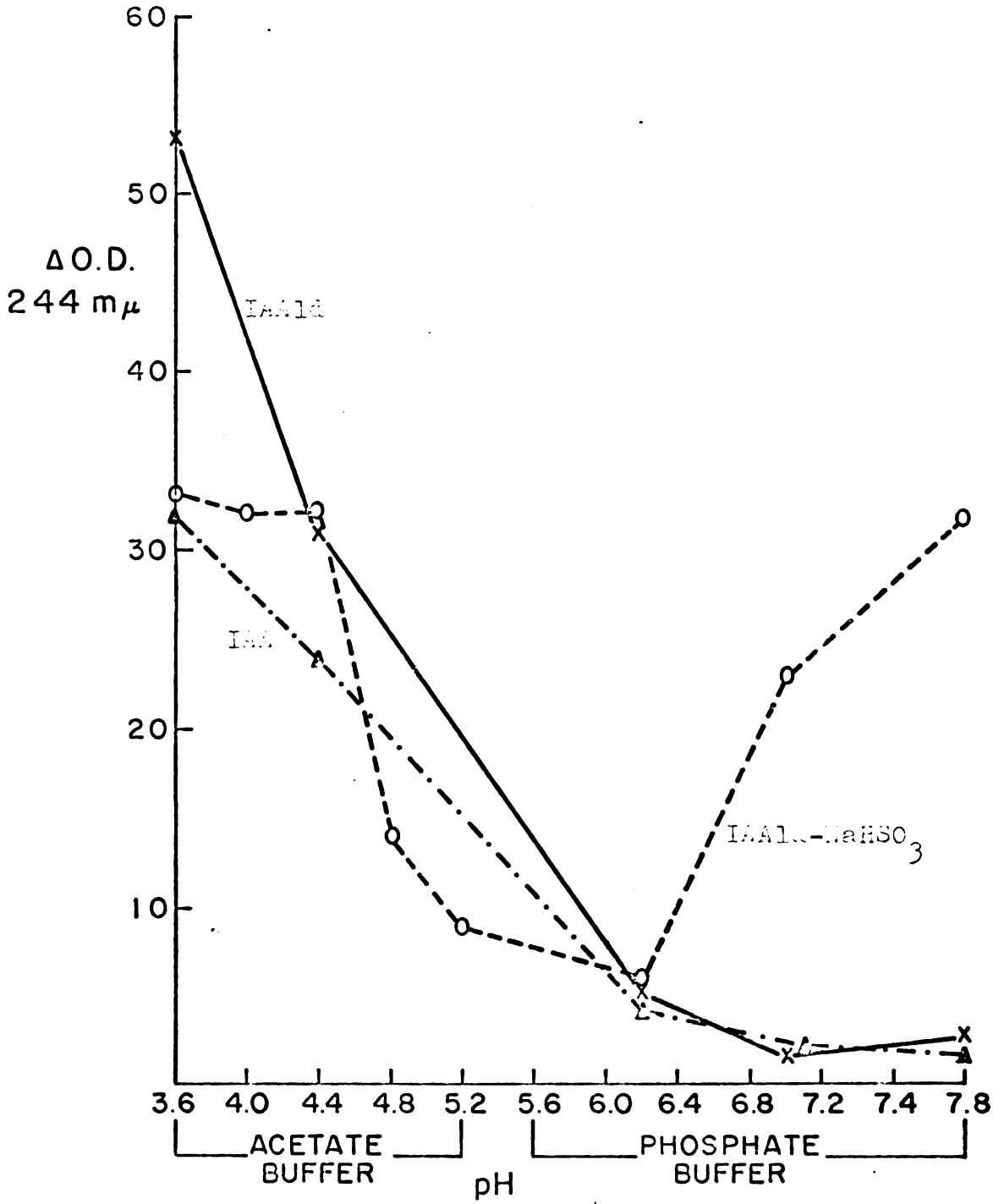

Beckman DK-2 CHART
IAAd – $NaHSO_3$
OVERNIGHT
60
45
30
15
5
0
OVERNIGHT
60
45
30
15
5
0

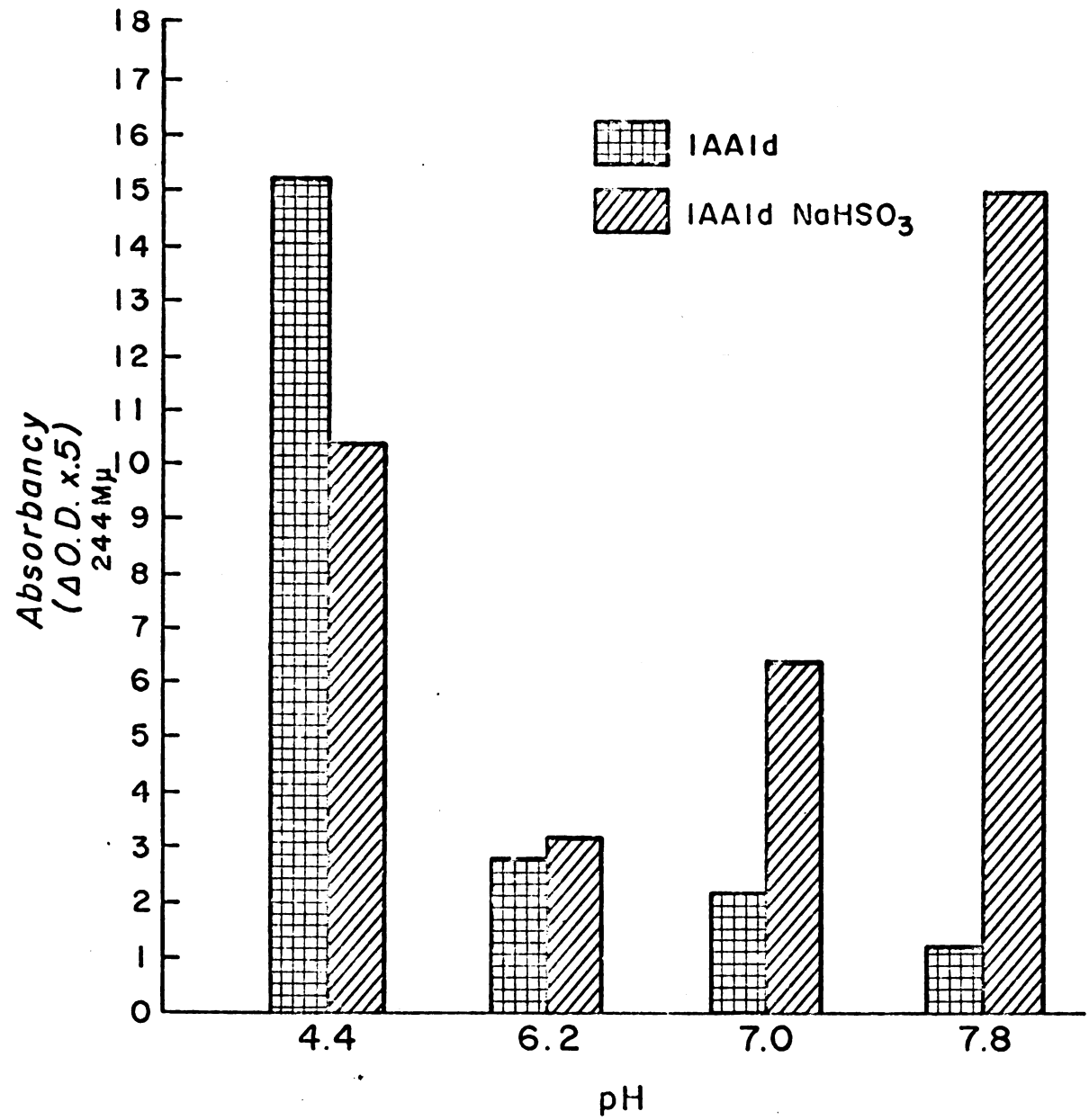
Figure 8. Effect of pH on the oxidation of IAA, IAAld, IAAld-$NaHSO_3$. The rate of the enzymatic reaction was followed by the change of absorbance at 244 mμ per hour. Reaction mixture contained 1 x 10^{-4} M substrates, IAA or IAAld or IAAld-$NaHSO_3$ in 0.05 M buffer solution, 6 μg of enzyme (RZ = 2.0) and 1 x 10^{-4} M H_2O_2. For pH values ranging from 3.6 to 5.2 and from 5.6 to 7.8 acetate and phosphate buffers were used, respectively.


60
50
40
30
20
10
ΔO.D.
244 mμ
IAAld
IAA
IAAld-NaHSO3
3.6
4.0
4.4
4.8
5.2
5.6
6.0
6.4
6.8
7.2
7.4
7.8
ACETATE BUFFER
PHOSPHATE BUFFER
pH

was clearly seen; the oxidation of IAA and IAAld at a pH above 6.2 decreased continually as the pH was increased, whereas the oxidation of IAAld-$NaHSO_3$ increased profoundly as the pH was raised above 6.2. Apparently, the competition between the hydroxide ions and sodium bisulfite may account for the differential behavior of the enzymatic system toward these three different substrates. It is possible that the increase of the concentration of hydroxide ions inactivated the enzymatic system, whereas the presence of sodium bisulfite prevented the inhibitory effect of the hydroxide ions and restored the activity of the enzymatic system at the elevated pH values. Hochster and Quastel (38) believe that the inactivation of the enzyme system toward oxidation of IAA at high pH values may be due to the formation of manganese carbonate at the higher pH values which lowers the effective manganese concentration below optimum. However, since there were no manganese ions present in the reaction mixtures, the lowering of the effective manganese concentration does not account for the reduced activity of the reaction system at the elevated pH values.

The effect of pH on the spectroscopic changes of IAA, IAAld, and IAAld-$NaHSO_3$ has been described previously and, therefore, will not be elucidated further. However, one should recall that the presence of sodium bisulfite had a profound effect on the enzymatic system, whereas at the lower pH conditions sodium bisulfite exhibited, to some extent, an inhibitory effect on the activity of the enzyme. At higher pH values the enzymatic action was enhanced (Figure 9).

Figure 9. Effect of sodium bisulfite on the oxidation of IAAld at different pH values. Data were taken from Figure 8.


18
17
16
15
14
13
12
11
10
9
8
7
6
5
4
3
2
1
0
Absorbancy
(Δ O.D. x.5)
244 Mμ
IAAld
IAAld NaHSO3
4.4
6.2
7.0
7.8
pH

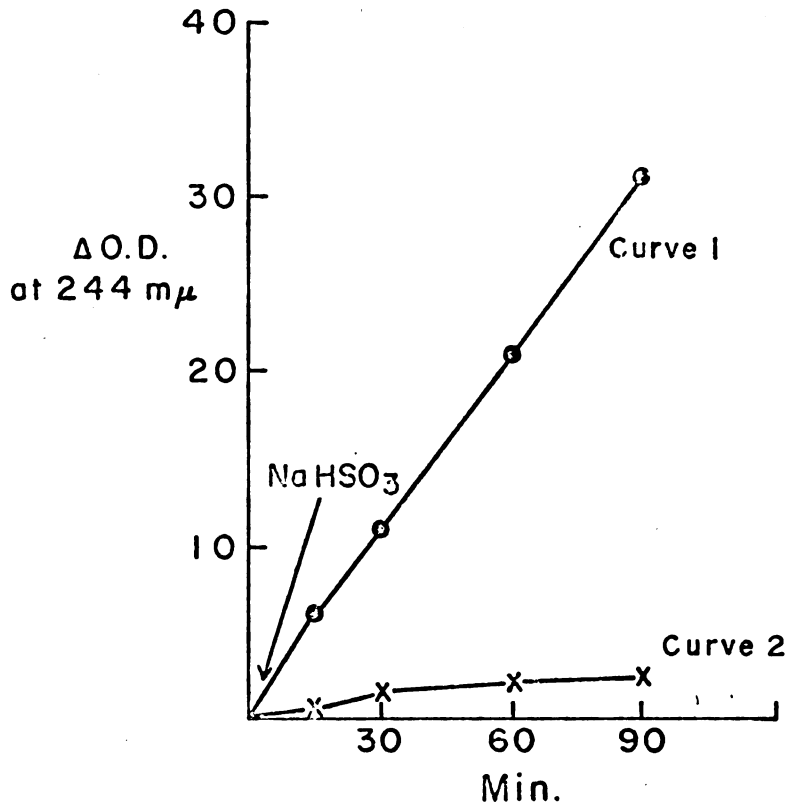
Effect of HSO_3^- or SO_3^{-2} Ions on the Oxidation of IAA and IAAld

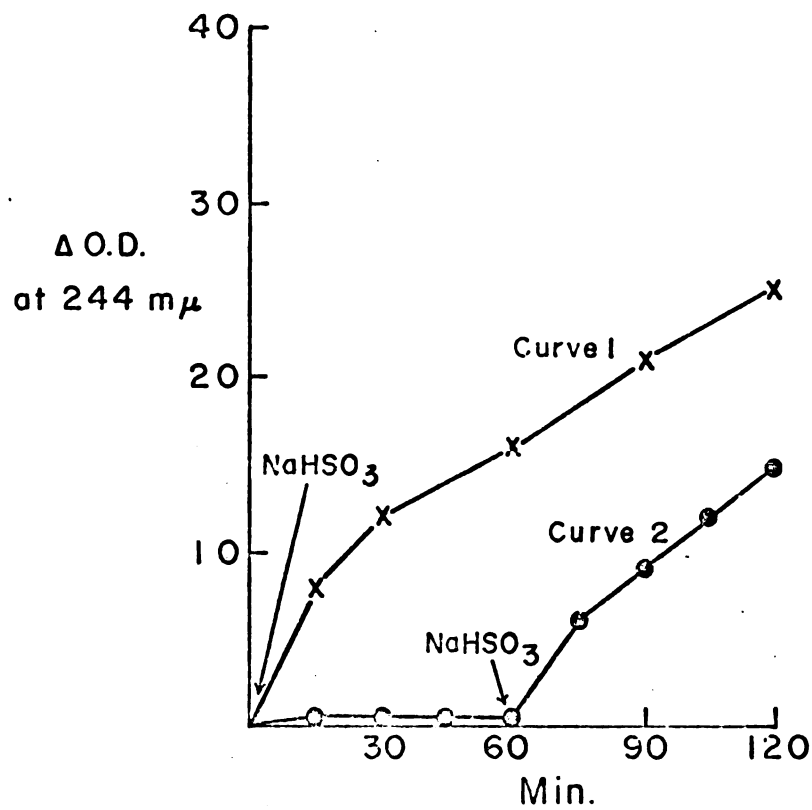
The effect on sodium bisulfite on the oxidation of IAA has been reported by Ray (77). He found that sodium bisulfite prevented further changes of optical density of IAA oxidized by the peroxidase system. Similar results were also obtained by Tonhazy and Pelczar (100) from *P. versicolor* enzyme preparation. But it is noteworthy to add that in Ray's experiments the pH of the reaction mixtures was 3.7, whereas the optimal pH for *P. versicolor* was 4.5. These results correspond well with those observed in the present work (Figure 9). It has been noted previously that at pH between 3.7 and 4.4 the effect of bisulfite ions on the enzymatic system was inhibitory rather than stimulatory in contrast to that observed at high pH values.

The effect of bisulfite ions on the activity of enzyme system toward IAA and IAAld is shown in Figures 10 and 11, respectively. Curve 1, Figure 10 shows the effect of sodium bisulfite on the oxidation of IAAld and curve 2 shows the activity in the absence of bisulfite anions. In curve 1, Figure 11, the experiment was made by the addition of sodium bisulfite to the reaction mixture containing IAA at the beginning of the reaction, whereas in curve 2 the sodium bisulfite was added 60 minutes after incubation was started. These results clearly indicate that the oxidative degradation of both

Figure 10. Effect of $NaHSO_3$ on the oxidation of IAAld. The incubation condition was similar to that mentioned for IAA in Figure 11. Curve 1 was obtained in the presence of 0.1 ml of 3×10^{-3} M $NaHSO_3$ (final concentration was 0.9×10^{-4} M); curve 2 was obtained in the absence of $NaHSO_3$.

Figure 11. Effect of $NaHSO_3$ on the oxidation of IAA. Reaction mixture contained 3 ml of 1×10^{-4} M IAA solution in a 0.05 M phosphate buffer, pH 7.8, 0.3 mg of enzyme (RZ = 1.5) in 0.1 ml water solution, and 0.1 ml of 1×10^{-2} M H_2O_2. Total volume was 3.2 ml per cuvette. Curve 1 was obtained by addition of 0.1 ml of 3×10^{-3} M $NaHSO_3$ to the reaction mixture at the beginning of the incubation. Curve 2 was obtained when 0.1 ml of the $NaHSO_3$ solution was added 60 min. after incubation. Reaction rate was followed at 244 mμ.


40
30
20
10
Δ O.D.
at 244 mμ
Curve 1
NaHSO3
Curve 2
30
60
90
Min.

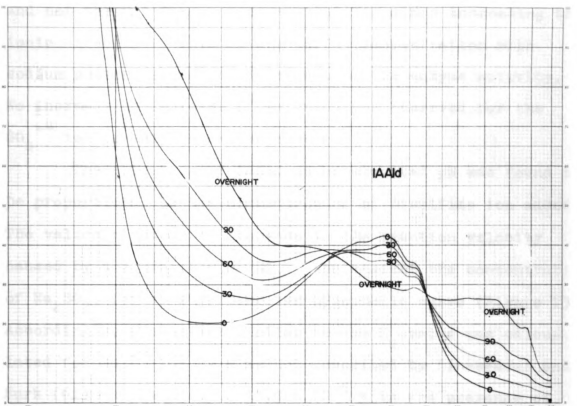

40
30
20
10
Δ O.D.
at 244 mμ
Curve 1
NaHSO3
Curve 2
NaHSO3
30
60
90
120
Min.

IAAld and IAA at the high pH values requires the presence of bisulfite ions. Although in the absence of bisulfite ions there was little observable change in the spectrum, the addition of a small amount (1×10^{-4} M) of sodium bisulfite solution to the reaction mixture enhanced the oxidative degradation of both IAAld and IAA immediately. The complete reaction system required only the presence of an active enzyme, hydrogen peroxide and sodium bisulfite. The addition of manganese ions (Mn^{2+}) and 2,4-dichlorophenol had no effect on the optical density of the substrates at pH 7.8.

The spectroscopic change of IAAld during the course of oxidation by the addition of sodium bisulfite to the reaction mixture was identical to that observed for the oxidation of IAAld-$NaHSO_3$ at the same pH conditions (Figure 12, A). It is noteworthy that the oxidation of IAA at the same alkaline pH value, in the presence of bisulfite ions gave the same pattern of spectroscopic changes as that observed for the oxidation of IAAld-$NaHSO_3$ under similar conditions (Figure 12, B). Apparently, the presence of bisulfite ions during the oxidation of both IAA and IAAld by the enzyme system results in the same oxidation product along with the formation of the typical double peak in 316 and 330 mμ regions. A comparison of the spectroscopic changes during the oxidation of IAA and IAAld at pH 7.8 in the presence of bisulfite ions are illustrated in Figure 12.

Figure 12, A. Spectroscopic changes during the oxidation of IAAld at pH 7.8 in the presence of $NaHSO_3$. Enzyme (0.3 mg per cuvette RZ = 1.5), 3 ml of 1 x 10^{-4} M of IAAld in 0.05 M phosphate buffer solution, pH 7.8, and 0.1 ml of 1 x 10^{-2} M H_2O_2. Reaction mixture contained 0.9 x 10^{-4} M of $NaHSO_3$ (0.1 ml of 3 x 10^{-3} M $NaHSO_3$ per cuvette of 3.2 ml).

Figure 12, B. Spectroscopic changes during the oxidation of IAA at pH 7.8 in the presence of $NaHSO_3$. The incubation condition was similar to that used in A, except the reaction mixture contained 1.35 x 10^{-4} M of $NaHSO_3$ (0.15 ml of 3 x 10^{-3} M $NaHSO_3$ per cuvette of 3.2 ml).


IAAId
OVERNIGHT
120
60
30
0
0
30
60
120
OVERNIGHT
OVERNIGHT
30
60
120
0

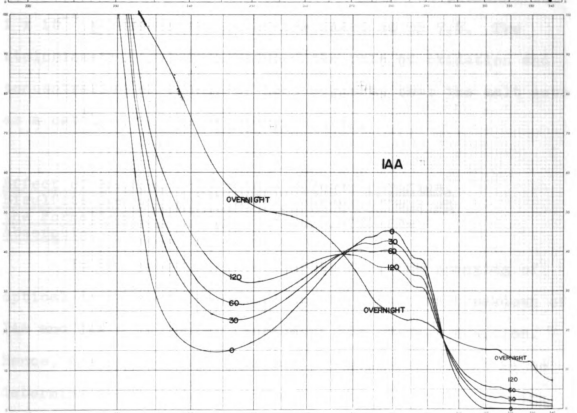
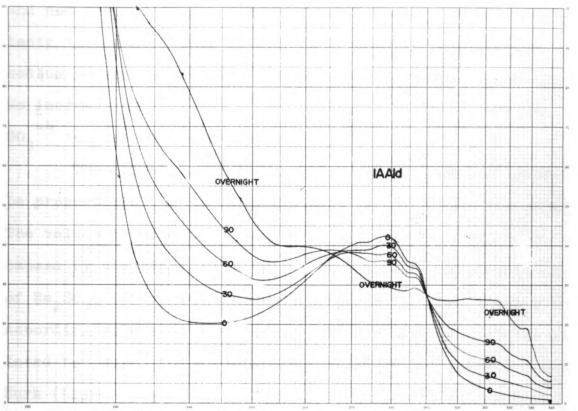
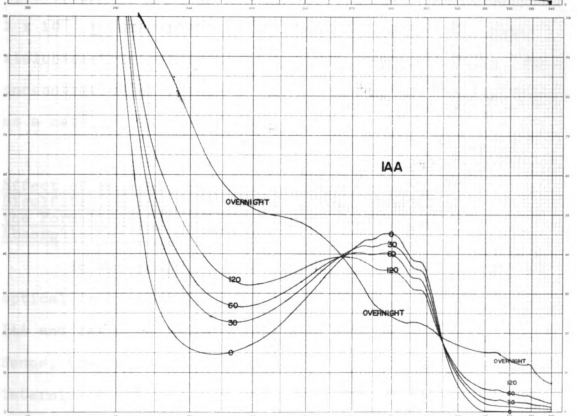

IAA
OVERNIGHT
0
30
60
120
120
60
30
0
OVERNIGHT
OVERNIGHT
120
60
30
0

Figure 12, A. Spectroscopic changes during the oxidation of IAAld at pH 7.8 in the presence of $NaHSO_3$. Enzyme (0.3 mg per cuvette RZ = 1.5), 3 ml of 1×10^{-4} M of IAAld in 0.05 M phosphate buffer solution, pH 7.8, and 0.1 ml of 1×10^{-2} M H_2O_2. Reaction mixture contained 0.9×10^{-4} M of $NaHSO_3$ (0.1 ml of 3×10^{-3} M $NaHSO_3$ per cuvette of 3.2 ml).

Figure 12, B. Spectroscopic changes during the oxidation of IAA at pH 7.8 in the presence of $NaHSO_3$. The incubation condition was similar to that used in A, except the reaction mixture contained 1.35×10^{-4} M of $NaHSO_3$ (0.15 ml of 3×10^{-3} M $NaHSO_3$ per cuvette of 3.2 ml).


IAAId
OVERNIGHT
90
60
30
0
0
30
60
90
OVERNIGHT
OVERNIGHT
90
60
30
0


IAA
OVERNIGHT
120
60
30
0
0
30
60
120
OVERNIGHT
OVERNIGHT
120
60
30
0

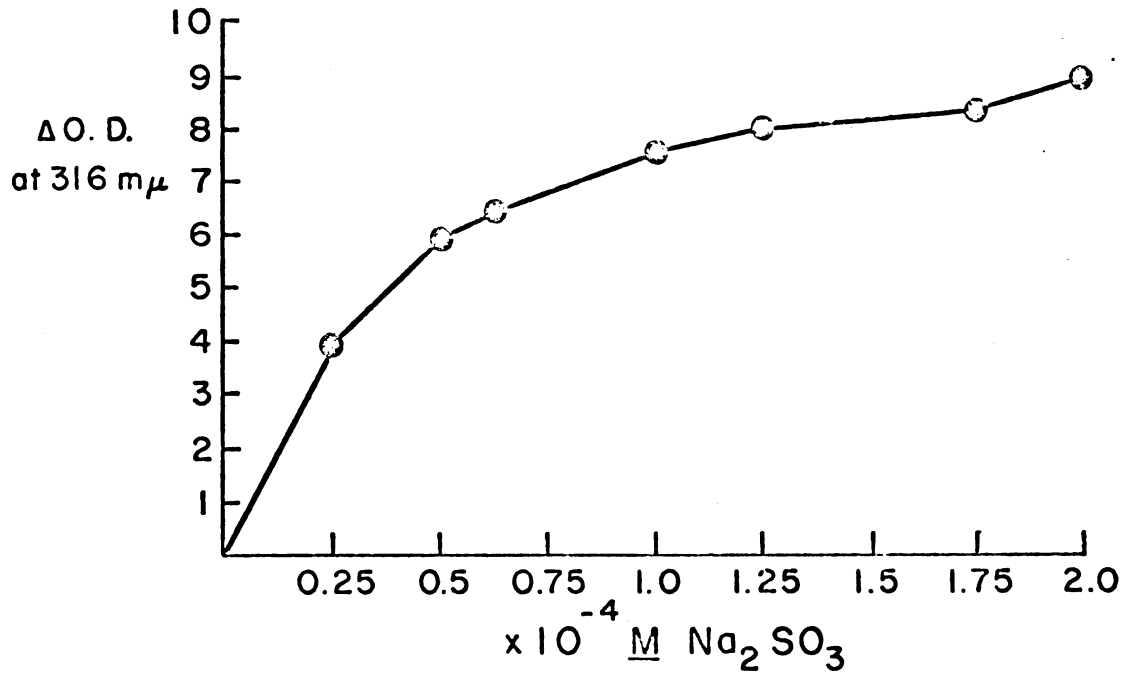
The results indicate that the SO_3^{-2} or HSO_3^{-2} ions and not the Na^+ ions acted as catalysts since increasing the ionic strength by raising the buffer concentration with sodium phosphate had little effect on the enzyme activity. No increase or decrease of activity was observed for the SO_4^{-4} ion toward the enzyme system.

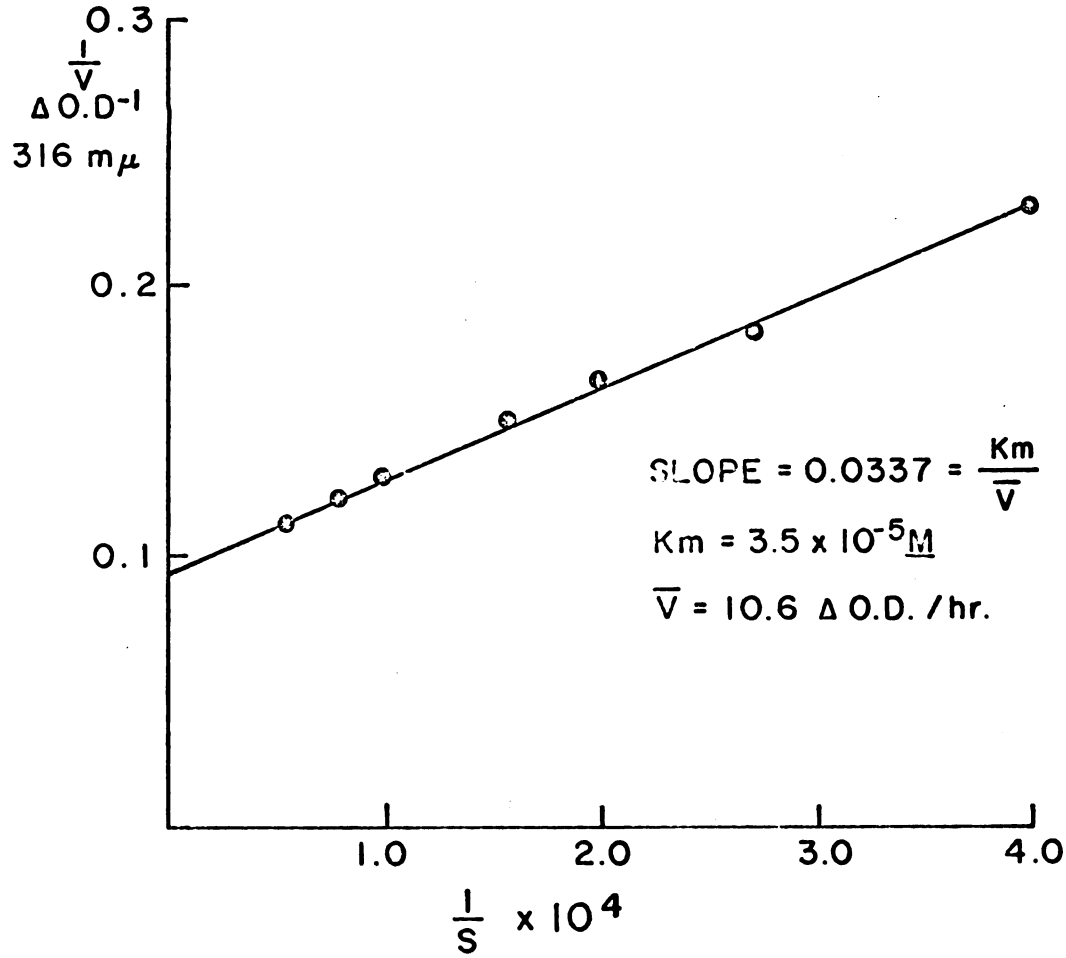
The oxidation of IAAld at an elevated pH was found to be proportional to the concentration of bisulfite ion added. The values plotted for increase in the reaction velocity caused by the addition of SO_3^{-2} ion against the concentration of Na_2SO_3, formed a rectangular hyperbolic curve (Figure 13). Accordingly, a constant similar to the K_m value was calculated for SO_3^{-2} ion by a method described by Lineweaver and Burk (61) and found to be 3.5×10^{-5} M in the presence of 1×10^{-4} M concentration of the IAAld at pH 7.8. The stoichiometric relation between the rate of oxidation and concentration of bisulfite ions suggests that the salt acts as a catalyst in the enzymatic system.

Effect of Enzyme, Substrate, Hydrogen Peroxide, Bisulfite Ion and Sulfite Ion Concentrations on the Formation of a Compound with a Spectral Absorption of 316 and 330 mμ

In the presence of bisulfite ion, the changing of optical density at 244 mμ due to the oxidative breakdown of IAA and IAAld no longer represents the overall reaction. Hence, the rate of the reaction was measured at 316 mμ to determine whether the formation of the new product having

Figure 13. Effect of Na_2SO_3 concentration on the oxidation of IAAld. Reaction mixture contained 1 x 10^{-4} M of IAAld, pH 7.8; concentrations of Na_2SO_3 varied from 0.25 x 10^{-4} M to 2.0 x 10^{-4} M. Reaction rate was followed at 316 mμ.


10
9
8
7
6
5
4
3
2
1
Δ O. D.
at 316 mμ
0.25
0.5
0.75
1.0
1.25
1.5
1.75
2.0
x 10 -4 M Na2 SO3


0.3
1/V
Δ O.D-1
316 mμ
0.2
0.1
SLOPE = 0.0337 = Km/V
Km = 3.5 x 10-5 M
V = 10.6 Δ O.D. / hr.
1.0
2.0
3.0
4.0
1/S x 10 4

the typical double peak, which appeared at 316 and 330 mμ regions, involved any enzymatic reaction.

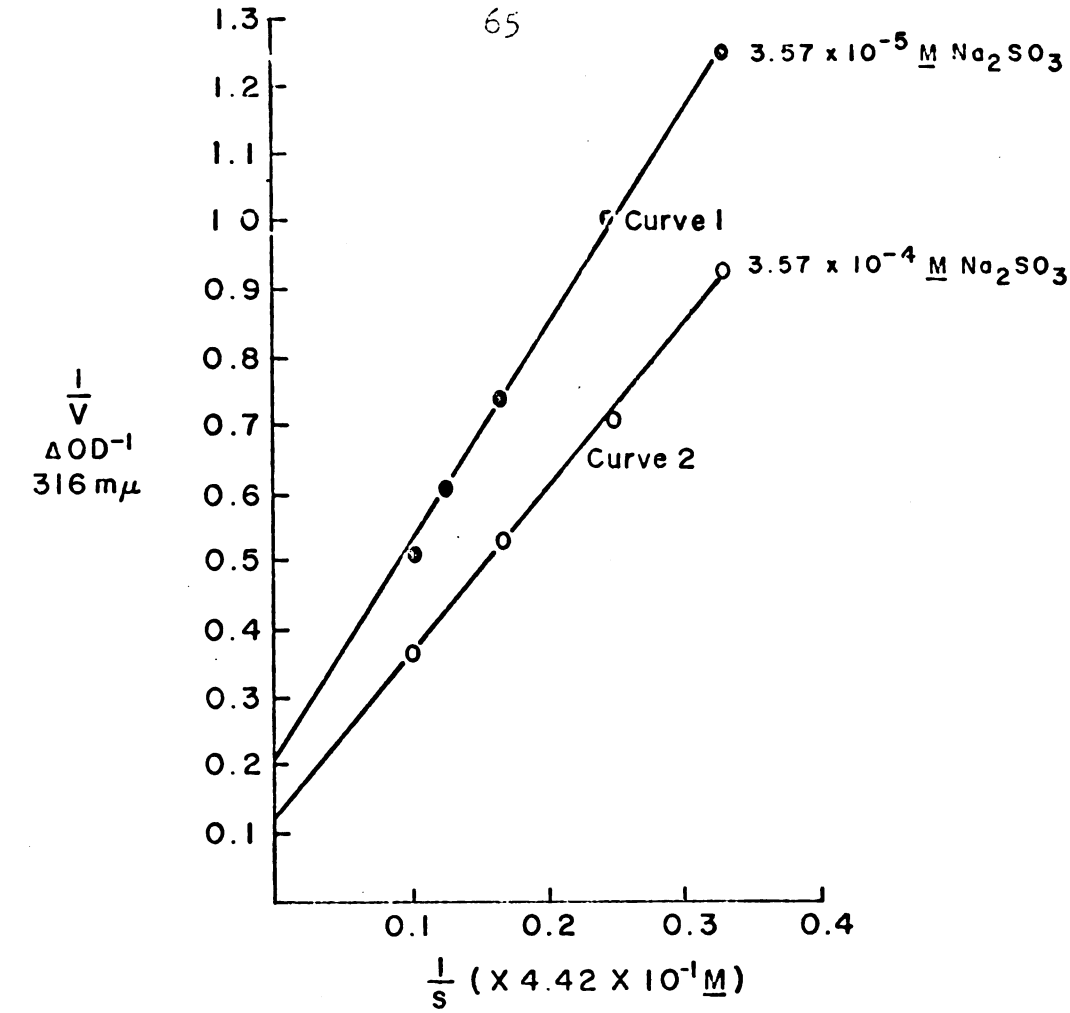
The effect of IAAld concentration on the rate of formation of the compound bearing an absorption at 316 and 330 mμ is shown in Figure 14. The experiments were made in the presence of SO_3^{-2} ion at pH of 7.8. Curve 1 in Figure 14 was obtained by the addition of 3.57×10^{-5} M solution of sodium bisulfite; curve 2 was obtained at a 3.57×10^{-4} M solution of sodium bisulfite. The K_m values for IAAld determined by the reciprocal plots (61) were found to be 1.02×10^{-4} M and 0.048×10^{-4} M, respectively, in the presence of the two different concentrations of sodium sulfite.

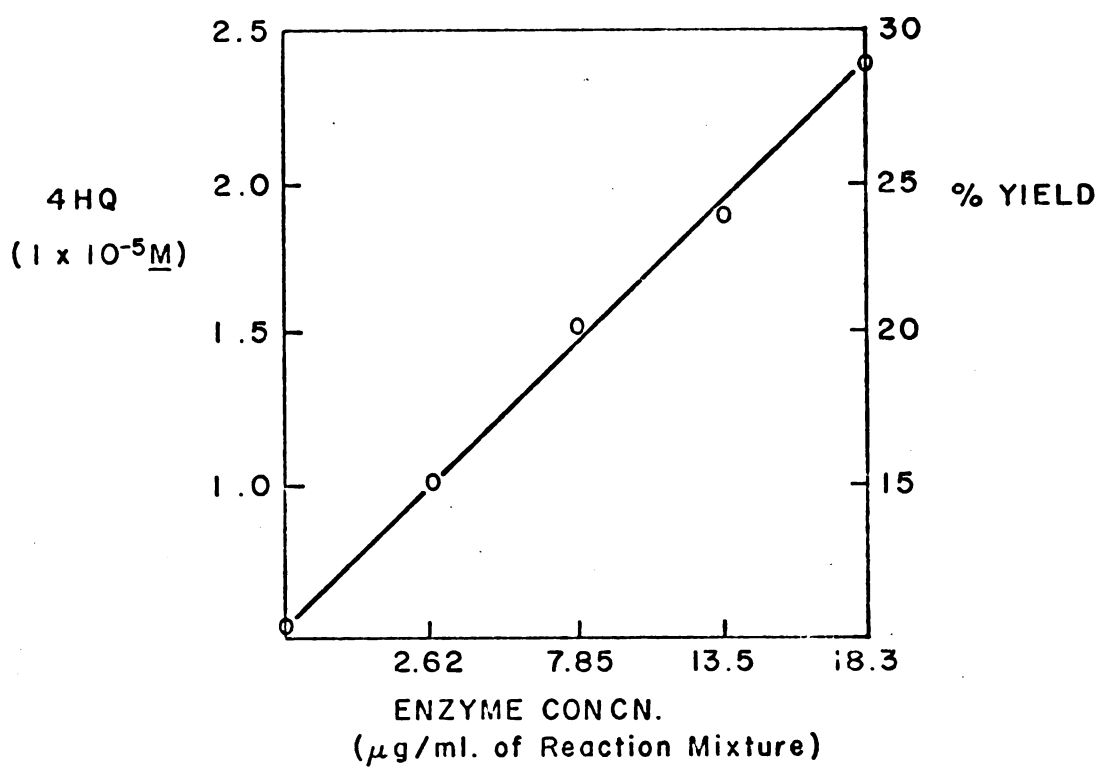
Obviously an increase in the concentration of sodium sulfite in the reaction mixutre had a profound effect on both the V_{max} and the K_m of the reaction system. The tenfold increase in the amount of SO_3^{-2} ions doubled the maximum velocity of the reaction as determined from the spectral changes at 316 mμ. In contrast, a tenfold increase in SO_3^{-2} ions concentration produced a twentyfold decrease in K_m for IAAld. It is clear that the presence of sodium bisulfite had significantly enhanced the affinity of the enzyme system toward the substrate.

The effect of enzyme concentration on the formation of a compound with an absorption spectra of 316 and 330 mμ from the oxidation of IAAld-$NaHSO_3$ is shown in Figure 15. The pH of the reaction mixture was 7.8. The formation of the double peak at 316 and 330 mμ regions was found to

Figure 14. Effect of IAAld concentration on the formation of a compound with spectral absorption at 316 and 330 mμ. Reaction mixture contained 15.7 μg enzyme (RZ = 2.9) per ml, 7×10^{-4} M H_2O_2, 0.05 M phosphate buffer solution, pH 7.8, substrate and bisulfite. Substrate concentrations were varied from 0.438×10^{-4} to 2.17×10^{-4} M. Curve 1 was obtained in the presence of 3.57×10^{-5} M Na_2SO_3; curve 2 in the presence of 3.57×10^{-4} M Na_2SO_3. Initial velocity (Δ O.D./min.) was followed at 316 mμ in a DU-Beckman spectrophotometer.

Figure 15. Effect of enzyme concentration on the formation of a compound with spectral absorption at 316 and 330 mμ.


1.3
1.2
1.1
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
3.57 x 10⁻⁵ M Na₂SO₃
Curve 1
3.57 x 10⁻⁴ M Na₂SO₃
Curve 2
1/V
ΔOD⁻¹
316 mμ
0.1
0.2
0.3
0.4
1/S (X 4.42 X 10⁻¹ M)


2.5
2.0
1.5
1.0
30
25
20
15
4HQ
(1 x 10⁻⁵ M)
% YIELD
2.62
7.85
13.5
18.3
ENZYME CONCN.
(μg/ml. of Reaction Mixture)

increase as the concentration of enzyme increased. Extrapolating the enzyme concentration-velocity curve to zero-enzyme concentration it is clearly shown that only a negligible amount of auto-oxidation of the substrate was observed from the intercepting point.

The presence of hydrogen peroxide in the reaction system is required. At the elevated pH values in the presence of bisulfite ions IAAld was oxidized rapidly when a stoichiometric amount of hydrogen peroxide was present. An amount less than the stoichiometric quantity caused incomplete oxidation of the substrates. These results are shown in Figure 16.

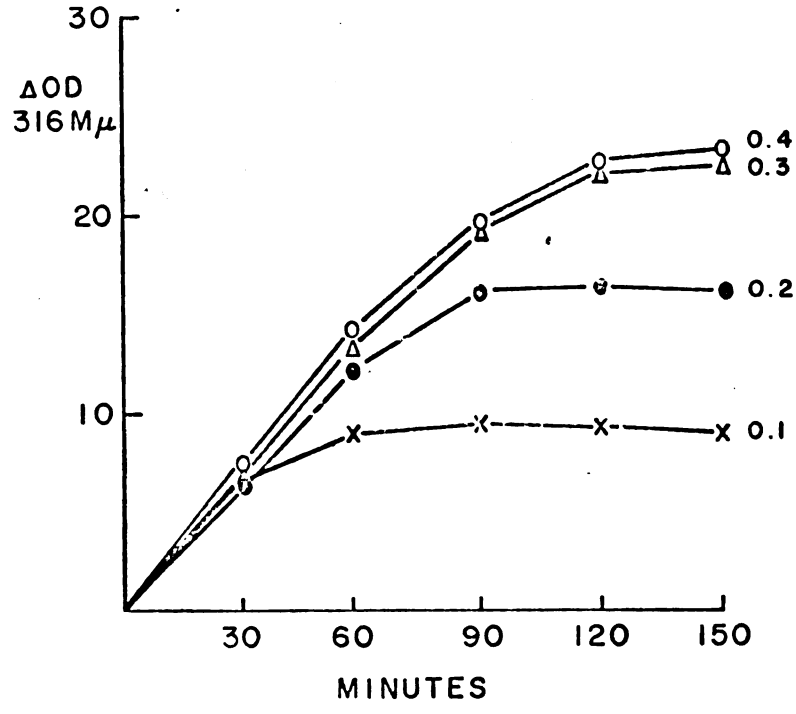
At pH of 7.8, little formation of the compound with an absorption spectra of 316 and 330 mμ was observed in the absence of either one of the components: enzyme, hydrogen peroxide, or bisulfite ion. Heat-treated or trichloroacetic acid-denaturated enzyme had no activity toward the formation of this compound. These results are given in Figure 17.

Effect of 2,4-Dichlorophenol and Manganese Ions on the Oxidation of IAA and IAAld

The effect of 2,4-dichlorophenol and manganese on the oxidation of IAA and IAAld is shown in Figure 18. The experiments were made in the absence of hydrogen peroxide at pH 4.0. At a pH greater than 6 the presence of 2,4-dichlorophenol and manganese had little effect on the peroxidase-catalyzed oxidation of IAA and IAAld, even when hydrogen peroxide was present

Figure 16. Effect of hydrogen peroxide concentration on the oxidation of IAAld-$NaHSO_3$. Reaction mixture contained 0.6 μ mole of IAAld, 0.33 μ mole of Na_2SO_3, 0.05 M phosphate buffer solution, pH 7.8. Curve -x-x- contained 0.1 μ mole of H_2O_2, -●-●- contained 0.2 μ mole of H_2O_2, -△-△- contained 0.3 μ mole of H_2O_2 and -o-o- contained 0.4 μ mole of H_2O_2. Total volume 3.2 ml.

Figure 17. Effect of horseradish peroxidase on the oxidation of IAAld at pH 7.8. Complete system contained 8.3 μg enzyme (RZ = 2.9) per cuvette of 3.2 ml, 1×10^{-4} M IAAld, 3×10^{-4} M H_2O_2 and 1×10^{-4} M Na_2SO_3.


30
ΔOD
316 Mμ
20
10
0.4
0.3
0.2
0.1
30
60
90
120
150
MINUTES

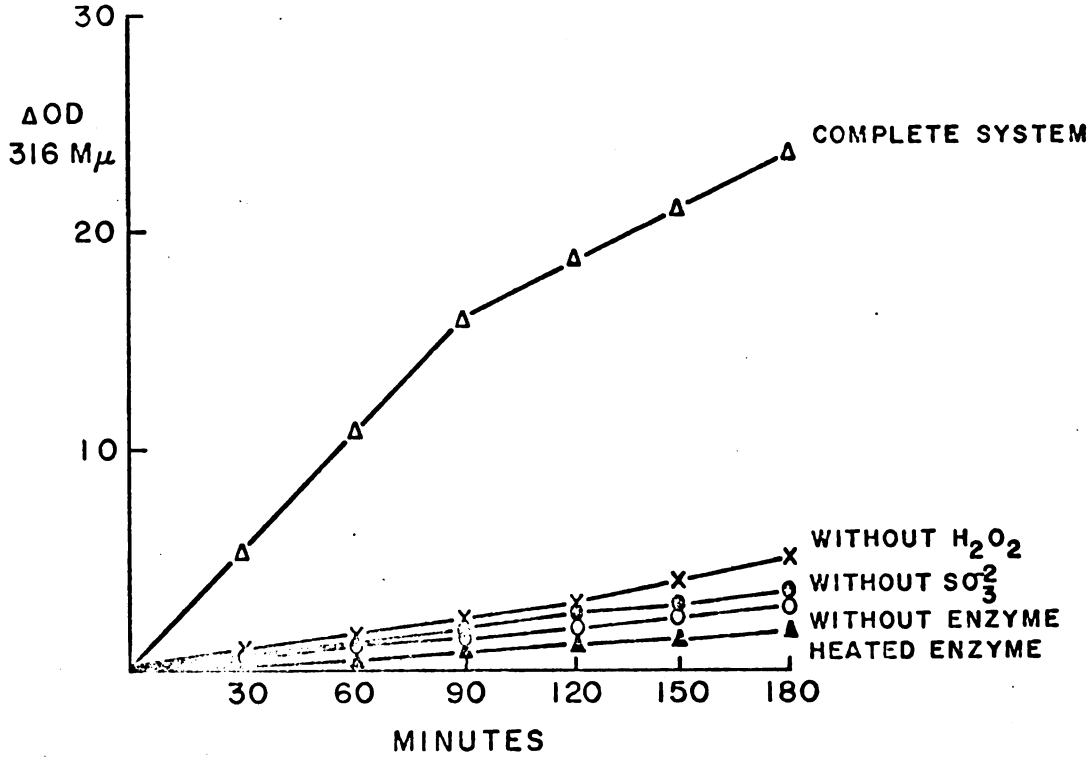
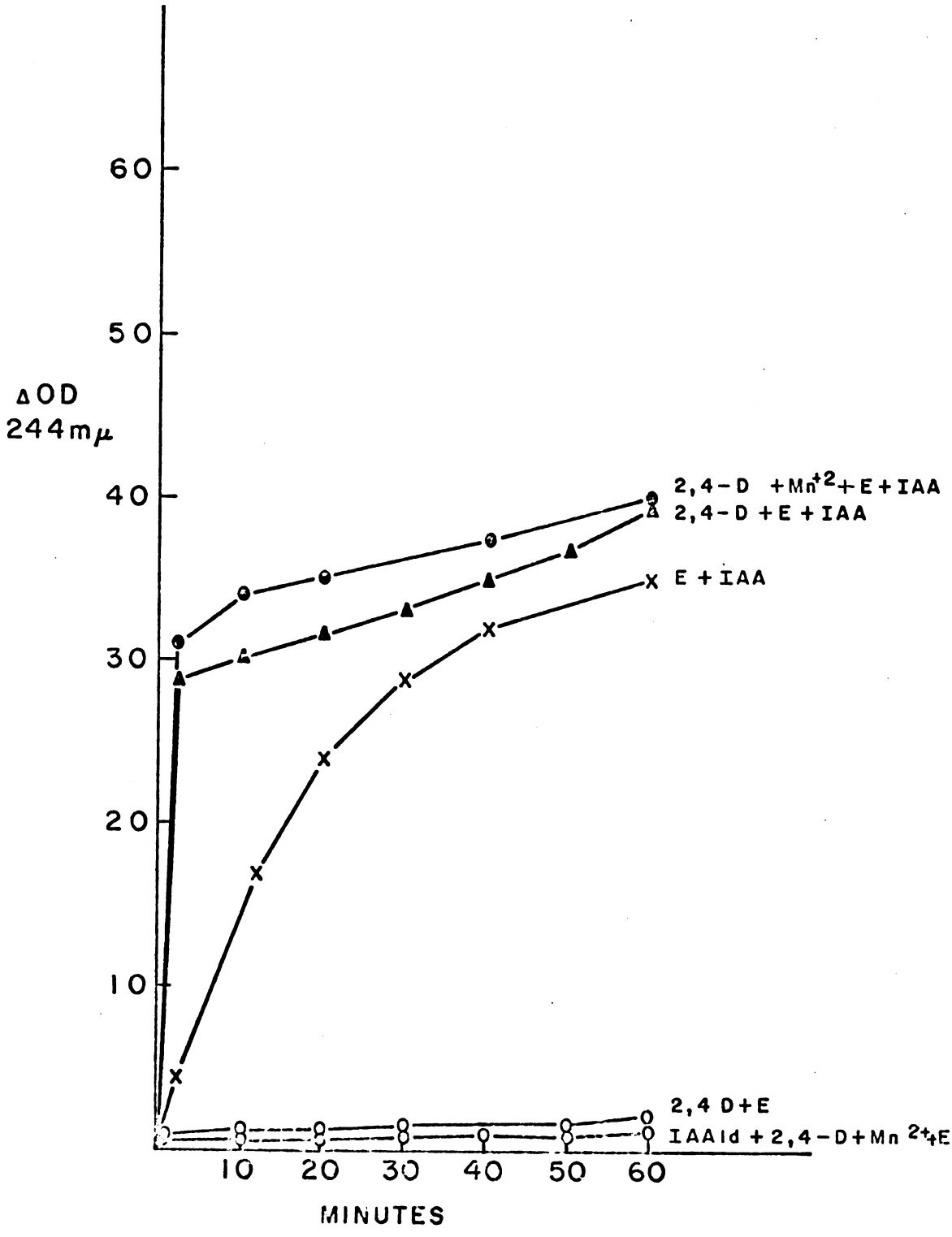

30
ΔOD
316 Mμ
20
10
COMPLETE SYSTEM
WITHOUT H_2O_2
WITHOUT SO_3^2
WITHOUT ENZYME
HEATED ENZYME
30
60
90
120
150
180
MINUTES

Figure 18. Effect of 2,4-dichlorophenol and manganese on the oxidation of IAA and IAAld. Reaction mixture contained 1 x 10^{-4} M substrate (either IAA or IAAld), 1 x 10^{-4} M $MnCl_2$, 1 x 10^{-4} M 2,4-dichlorophenol and 0.2 ml of enzyme (8.3 µg per cuvette, RZ = 2.9). Total volume of the reaction mixture was 3.2 ml per cuvette. Blank contained all reaction components except without addition of enzyme. Reaction mixtures were made in 0.05 M acetate buffer solution, pH 4.0.


60
50
ΔOD
244mμ
40
30
20
10
2,4-D +Mn+2+E+IAA
2,4-D +E +IAA
E +IAA
2,4 D+E
IAAId + 2,4-D+Mn 2++E
10
20
30
40
50
60
MINUTES

in the reaction mixture. In these studies the reaction was followed spectrophotometrically at the 244 mμ region. It was observed that at pH 4.0 the oxidation of IAA was remarkably stimulated in the presence of 2,4-dichlorophenol and manganese, and also in the absence of these cofactors IAA appeared to be an excellent substrate for peroxidase activity.

In contrast to what was observed for IAA, the oxidation of IAAld by the same enzyme system was not influenced, to any extent, by the presence of these two cofactors. Under these conditions IAAld was not oxidized. These observations are contradictory to what is expected. One should recall the observation previously described that IAAld was an excellent substrate for peroxidase activity in the presence of hydrogen peroxide at pH 4.0. Apparently, the diverted behavior of the enzyme toward IAA and IAAld was a result of the slight difference in the chemical structure of these two auxin derivatives. The lack of reactivity of IAAld by the system consisting of 2,4-dichlorophenol, manganese ions, and peroxidase was not simply due to the deficiency of the hydrogen peroxide, because the function of manganese and 2,4-dichlorophenol are closely related to hydrogen peroxide formation required for the peroxidase catalyzed reaction (45).

Identification of the Oxidation Products

Some Preliminary Studies with Thin-Layer Chromatography.--Preliminary studies with thin-layer chromatography indicate

that as a result of the oxidation of IAA, IAAld and IAAld-$NaHSO_3$ several spots could be detected under ultraviolet light. Among them two or three spots could be regarded as common products. One of these common products was called compound X_1, having R_f values of 0.91 (spot A_1) and 0.92 (spot A_2) which were obtained from the oxidative breakdown of IAA and IAAld, respectively. Spot A_1 and spot A_2 derived from compound X_1 resembled each other not only in the R_f values, but also in the absorption spectrum under ultraviolet light (Figure 19). These spots had absorption maxima at 240, 260, and 290 mμ regions. Originally, it was suspected that the compound was a derivative of an oxindole, since oxindole was known to be an established end product resulting from the action of peroxidase on IAA. However, analysis indicated that it was not oxindole.

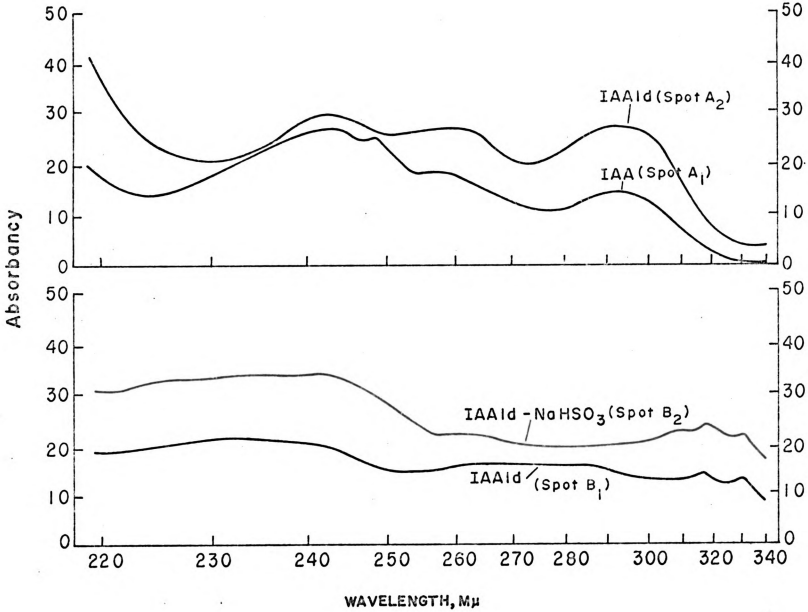
Another common product which was isolated from the peroxidase oxidation of either IAAld or IAAld-$NaHSO_3$ had R_f values of 0.73 (spot B_1 from IAAld) to 0.77 (spot B_2 from IAAld-$NaHSO_3$). The two spots, B_1 and B_2, obtained from IAAld and IAAld-$NaHSO_3$, respectively, gave similar spectra with a characteristic double peak at 316 and 330 mμ regions (Figure 20).

In the preliminary experiments the pH of the reaction mixture was made at 6.1 since the reaction rate of the oxidation of the IAAld and IAA was the lowest at this pH, consequently, additional products may be produced in this reaction.

A significant conclusion which may be drawn from these

Figure 19. Ultraviolet absorption spectra of compound X_1 eluted from spot A_1 and spot A_2. These spots were obtained from the oxidative breakdown of IAA and IAAld, respectively. pH of the reaction mixture was 6.1. Thin-layer: Silica Gel G; Solvent system: Ethyl acetate-2-Propanol-Ammonium hydroxide (45:35:20).

Figure 20. Ultraviolet absorption spectra of compound X_1 eluted from spot B_1 and spot B_2. These spots were oxidation products obtained from IAAld (spot B_1) and IAAld-$NaHSO_3$ (spot B_2), respectively. The pH of the reaction mixture was 6.1. Thin-layer: Silica Gel G; Solvent system: Ethyl acetate-2-Propanol-Ammonium hydroxide (45:35:20).


Absorbancy
50
40
30
20
10
0
IAAld (Spot A2)
IAA (Spot A1)
IAAld - NaHSO3 (Spot B2)
IAAld (Spot B1)
220
230
240
250
260
270
280
300
320
340
WAVELENGTH, Mμ

preliminary studies, is that the presence of sodium bisulfite does not appear to be absolutely necessary for producing compound X_2 from the IAAld, since the peroxidase-catalyzed oxidation of IAAld in the absence of bisulfite ion also gave rise to the same product with the typical double peak at 316 and 330 mμ. The presence of bisulfite ion only stimulated the yield of the X_2. Whether the same is true in case of IAA is unknown, although it was noted above that the presence of bisulfite ion stimulated the formation of X_2 from IAA. The X_1 and X_2 were later identified as IAld and 4-hydroxyquinoline, respectively. Furthermore, for each substrate (IAA, or IAAld, or IAAld-$NaHSO_3$) oxidized at least 5 spots could be detected. In the present work only two of them were identified.

Isolation and Purification of the Compound With a Spectral Absorption of 316 and 330 mμ by Column Chromatography.--For the isolation of the new oxidation product having a typical double peak at 316 and 330 mμ regions, the biosynthetic product was placed on top of the adsorbent in a silicic acid column packed according to the method previously described. The column packing was first developed with a solvent system consisting of 250 ml of hexane and ethyl acetate mixture (50:100); and second, with isopropanol. The samples were collected in two ml portions by an automatic fractionation collector. Portions having similar absorption patterns were combined. The compound having the typical double peak at

316 and 330 mμ remained at the top of the adsorbent during first development and was isolated in a fairly purified form from the second development with isopropanol. It was crystallized in an acetone-ethyl acetate mixture and finally recrystallized several times in pure acetone until a constant melting point was determined.

Identification of Compound X_2 as 4-Hydroxyquinoline.-- The ultraviolet and infrared spectra of compound X_2 are shown in Figure 21 and Figure 22 (A), respectively. The ultraviolet absorption of the compound showed a double peak at 316 and 330 mμ and a broad peak at 234 mμ. Infrared spectrum of the crystalline compound in a KBr pellet showed strong to medium-strong bands at 3250, 3050, 2560, 1650, 1620, 1580, 1500, 1480, 1440, 1360, 1320, 1200, 1140, 1100, 830, 800, 775, 760, 725 and 675 cm^{-1}. The ultraviolet and infrared spectra of the unknown compound were the same as the spectra of an authentic sample of 4-hydroxyquinoline. The infrared spectrum of 4-hydroxyquinoline is shown in Figure 22 (B).

The 4-hydroxyquinoline, which was purchased from Aldrich Chemical Company, was a trihydrate having a melting point of 200-202°. The 4-hydroxyquinoline isolated from the peroxidase reaction mixture was melted at 207-208° (uncorrected). However, after the samples were dried over phosphorous pentoxide, both the authentic and the isolated compound melted at 207-208° (uncorrected) and a mixture of both compounds showed no depression of the melting point. The

Figure 21. Ultraviolet absorption spectrum of compound X_2.
The spectrum was obtained in water solution.
Concentration was 0.2×10^{-4} M.

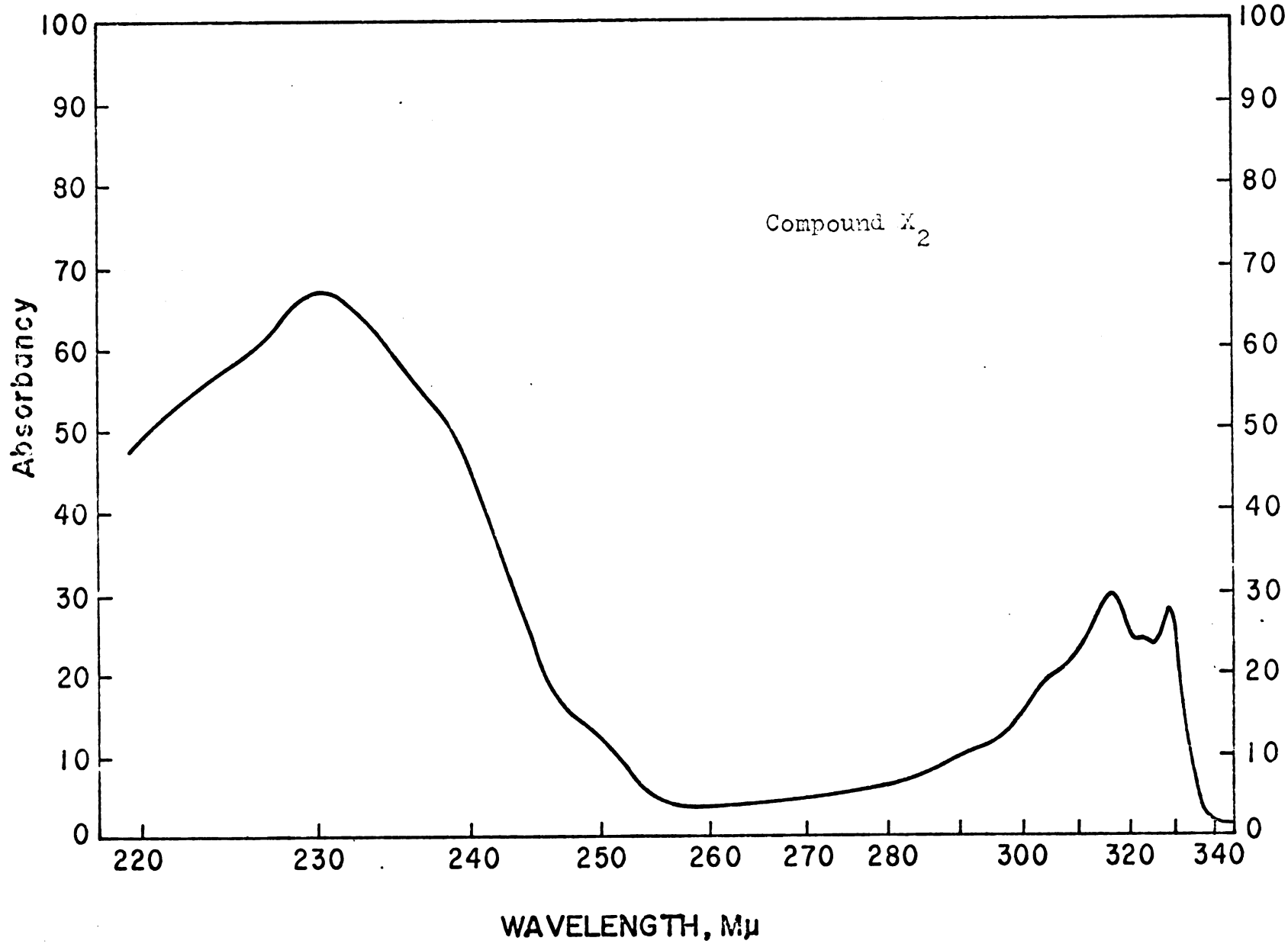

Compound X2
Absorbancy
100
90
80
70
60
50
40
30
20
10
0
220
230
240
250
260
270
280
300
320
340
WAVELENGTH, Mμ

Figure 22. Infrared absorption spectra of compound X_2 (spectrum A) and 4-hydroxyquinoline (spectrum B). Samples were made into potassium bromide pellets.

A
B
WAVENUMBER CM
WAVELENGTH IN MICRONS

molecular weight of the oxidation product determined by mass spectrophotometric method or by a freezing-point depression in acetone was found to be 145.15 and is the same as the calculated value. The compound gave the following percentage on elemental analysis: C 74.47, H 4.90, N 9.68, O 10.95. All available evidence indicates that the oxidation product with the typical double peak in the 316 and 330 mμ regions was 4-hydroxyquinoline which has the following percentage composition: Calcd. C 74.47, H 4.82, N 9.65, O 11.06. The yield of 4-hydroxyquinoline under the experimental conditions used was 20% of the substrate (IAAld) oxidized.

Thin-Layer Chromatographical Identification of Compound X_1 as IAld.--The results of thin-layer chromatography experiments with four different systems are shown in Figure 23. The enzymic oxidation of IAAld was made at pH 4.4. It is evident that the R_f values of the reaction product (compound X_1) on the chromatograms in the different solvent systems correspond to the IAld (Figure 23 and Table 1). The compound X_1 was further identified by ultraviolet spectra which also corresponded to the IAld. The absorption spectrum of the aldehyde isolated from thin-layer chromatography and that of IAld are given in Figure 24.

The isolated IAld gave positive tests with 2,4-dinitrophenylhydrazine and xanthydrol, but responded poorly to Schiff's and Ehrlich's test. These tests correspond with those observed for IAld.

Figure 23. Thin-layer chromatogram of compound X_1 obtained by oxidation of indole-3-acetaldehyde. Plates: Eastman Chromatogram Sheet (Type K 301, R2 silica gel without fluorescent indicator). Solvent systems were (1) n-propanol-hexane (1:4); (2) chloroform-acetic acid (95:5); (3) benzene-acetic acid-water (4:1:1); and (4) n-butanol-hexane (18% n-butanol). Authentic spots were marked as "A" and the spots from oxidation of indole-3-acetaldehyde were marked as "S". Spraying reagent: 2,4-dinitrophenylhydrazine in ethanol.

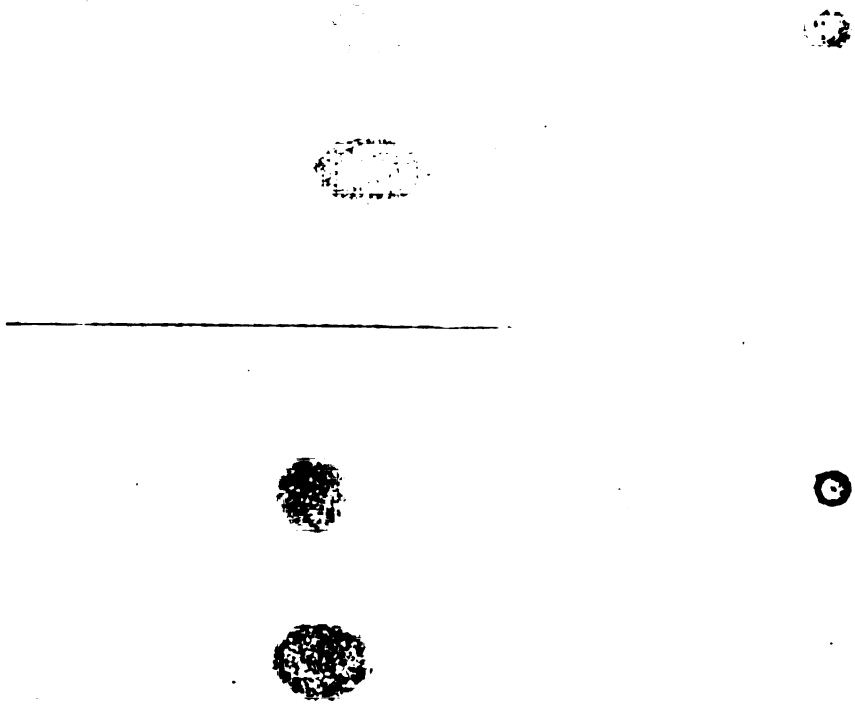
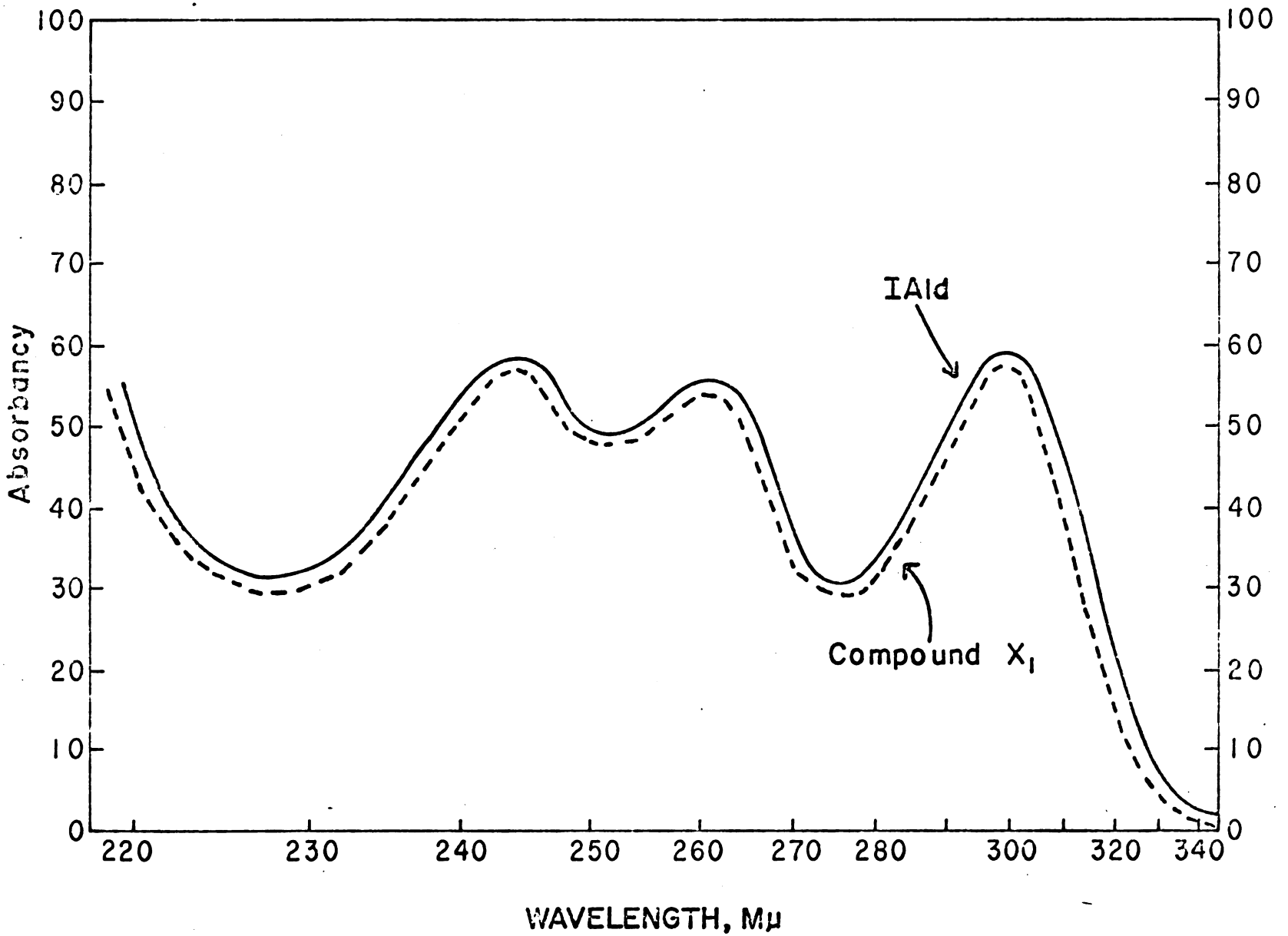


Table 1. R_f Values of IAld and Isolated IAld

Thin-Layer Plates	Solvent System	IAld	Isolated IAld	IAAld
Eastman Chromatogram Sheet	n-Propanol-Hexane (1:4)	0.76	0.79	---
"	Chloroform-Acetic Acid (95:5)	0.80	0.82	---
"	Benzene-Acetic Acid-Water (4:1:1)	0.69	0.70	---
"	n-Butanol-Hexane (18% n-Butanol)	0.62	0.62	---
Silica Gel F_{254}	n-Butanol-Hexane (18% n-Butanol)	0.50	---	0.76

Figure 24. Ultraviolet absorption spectrum of the compound X_1 and IAld. The absorption spectrum was determined in water solution. Concentration was 0.5×10^{-4} M.


100
90
80
70
60
50
40
30
20
10
0
Absorbancy
IAld
Compound X1
220
230
240
250
260
270
280
300
320
340
WAVELENGTH, Mμ

The yield of IAld obtained by the peroxidation of IAAld was approximately 50%. About 0.5×10^{-4} M of IAld was formed by the oxidation of 1×10^{-4} M of IAAld. The yield was calculated from the spectral changes at 289 mμ when the reaction reached the final steady state.

Oxidation of 1-Methyl-IAAld by Horseradish Peroxidase

The spectrophotometric changes of 1-methylindole-3-acetaldehyde during the course of oxidation was more complicated than that observed for IAA and IAAld. Both the rate of the reaction and the pattern of oxidation product were greatly influenced by the pH of the reaction mixture. The optimal pH for the oxidation of 1-methyl-IAAld was in the range of about 4.0, and the reaction rate decreased as the pH was increased above 6.5. A similar stimulatory effect of bisulfite ions on the oxidation of 1-methylindole-3-acetaldehyde at high pH values was observed.

One of the major oxidation products was found to be 1-methyl-IAld. Its formation from the peroxidase oxidation of 1-methyl-IAAld was identified by thin-layer chromatography in different solvent systems. However, the formation of 1-methyl-IAld from 1-methyl-IAAld could not be shown spectrophotometrically under ultraviolet light, since 1-methyl-IAAld and 1-methyl-IAld have very similar ultraviolet absorption spectra. 1-Methyl-IAld has a single absorption maximum at 288 mμ while 1-methyl-IAAld has λ_{max} at 292 mμ. The

distinguishable difference between these two absorption spectra is the absorption minimum. 1-Methyl-IAAld shows a trough at 247 mμ and 1-methyl-IAld has a trough at 261 mμ. During the course of peroxidase oxidation of 1-methyl-IAAld, some new peaks were found to appear at 249, 260 and 330 mμ regions which neither represent the formation of IAld nor that of 1-methyl-4-hydroxyquinoline.

Apparently, other than 1-methyl-IAld and 1-methyl-4-hydroxyquinoline, other oxidation product or products were formed as a result of the oxidative degradation of 1-methyl-IAAld catalyzed by the peroxidase system. The spectroscopic changes of 1-methyl-IAAld during the course of the oxidation is shown in Figure 25. Because the characteristic absorption peak appeared in the region 240 to 250 mμ, the formation of a derivative of oxindole is not improbable. However, additional work will be required to identify the structural characteristics of this unknown substance.

The enzymatic oxidation of 1-methyl-IAAld was made at pH 4.4. The results from the thin-layer studies are summarized in Figure 26 and Table 2. It is clear that the R_f values of the reaction product correspond quite well with 1-methyl-IAld. The spots were identified by spraying the thin-layer plates with 2,4-dinitrophenylhydrazine reagent. Furthermore, if the reaction was made at higher pH values in the presence of bisulfite ion, the formation of 1-methyl-4-hydroxyquinoline was not shown by thin-layer chromatography on a plate having a fluorescence background. The possibility

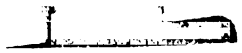


Figure 25. Changes in ultraviolet absorption spectrum during enzymatic oxidation of 1-methylindole-3-acetaldehyde (1-methyl-IAAld). 0.1 ml enzyme (17 μg per cuvette of 3.2 ml), 3 ml of 1.07×10^{-4} M 1-methyl-IAAld in 0.05 M acetate buffer solution, pH 4.85, and 0.1 ml of 1×10^{-2} M H_2O_2 solution. Total volume of the reaction mixture was 3.2 ml per cuvette. Records were begun at 340 mμ. Curves were taken at 0, 5, 10, 15, 20, 30, 45, 60, 75, 90, 105 and 120 minutes, each record was completed in 1 minute. Final concentration of 1-methyl-IAAld was 1×10^{-4} M and that of H_2O_2 was 3×10^{-4} M.

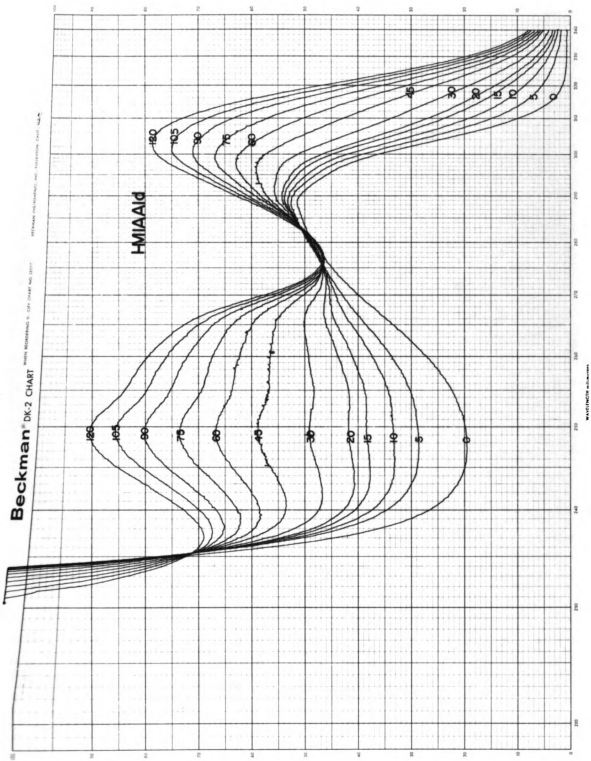

Beckman DK-2 CHART
HMIAAld
180
105
90
75
60
45
30
20
15
10
5
0

Figure 26. Thin-layer chromatogram of 1-methylindole-3-carboxaldehyde obtained by oxidation of 1-methylindole-3-acetaldehyde. Plates: Eastman Chromatogram Sheet (Type K301, R2 silica gel without fluorescent indicator). Solvent system were (1) n-propanol-hexane (1:4); (2) chloroform-acetic acid (95:5); (3) benzene-acetic acid-water (4:1:1); and (4) n-butanol-hexane (18% n-butanol). Authentic spots of 1-methylindole-3-carboxaldehyde were marked as "A" and the spots from oxidation of 1-methylindole-3-acetaldehyde were marked as "S". Spraying reagent: 2,4-dinitrophenylhydrazine in ethanol.

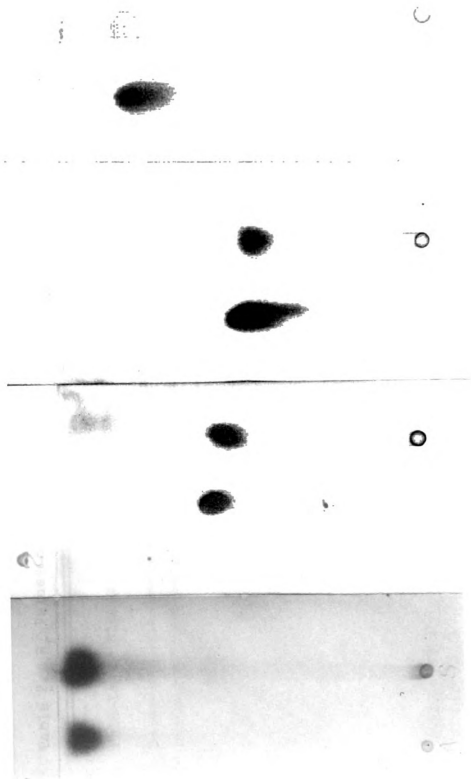


Table 2. R_f Values of 1-Methyl-IAld and Isolated 1-Methyl-IAld

Thin-Layer Plates	Solvent System	1-Methyl-IAld	Isolated 1-Methyl-IAld	1-Methyl-IAAld
Eastman Chromatogram Sheet	n-Propanol-Hexane (1:4)	0.94	0.94	---
"	Chloroform-Hexane (95:5)	0.56	0.54	---
"	Benzene-Acetic Acid-Water (4:1:1)	0.46	0.45	---
"	n-Butanol-Hexane (18% n-Butanol)	0.78	0.80	---
Silica Gel F_{254}	n-Butanol-Hexane (18% n-Butanol)	0.30	---	0.83

of the formation of 1-methyl-4-hydroxyquinoline cannot be ruled out if appropriate reaction conditions and better chromatographical technique can be developed. At this point, it should be mentioned that 1-methyl-IAAld was not as stable as IAAld during chromatographical analysis since it undergoes spontaneous decomposition to form 1-methyl-IAld, and two other unidentified compounds. Therefore, it may be possible that because of the structural characteristics of 1-methyl-IAAld, the formation of 1-methyl-IAld and the other unidentified compounds are energetically more favorable than is 1-methyl-4-hydroxyquinoline.

Possible Mechanism of Peroxidase Oxidation of IAA and IAAld

The effect of bisulfite ion on the formation of peroxide-peroxidase complexes is shown in Figures 27 and 28, respectively. The experiments presented in Figures 27 and 28 were made aerobically at pH 7.68 in 0.15 M phosphate buffer solution. Curve A, Figure 27, represents the optical absorption of the normal peroxidase solution which had a maximum peak at 402 mμ. Curve B is the absorption curve of complex II which had a maximum absorption at 418 mμ and was formed when a small amount of hydrogen peroxide was added to the free enzyme solution (14, 15). Curve C was obtained when a small amount of IAA was added to the solution containing the peroxidase and hydrogen peroxide. The transformation of the hydrogen peroxide-peroxidase complex, curve B, to free

Figure 27. Effect of hydrogen peroxide, indole-3-acetic acid, and sodium bisulfite on the absorption spectra of horseradish peroxidase. 0.15 M phosphate buffer solution, pH 7.68. A. Free enzyme. B. A plus 0.05 ml of 0.3% H_2O_2. C. B plus 0.05 ml of IAA (2 mg). D. C plus 0.03 ml of 1 x 10^{-3} M $NaHSO_3$. Enzyme: 0.85 mg per cuvette (3 ml).

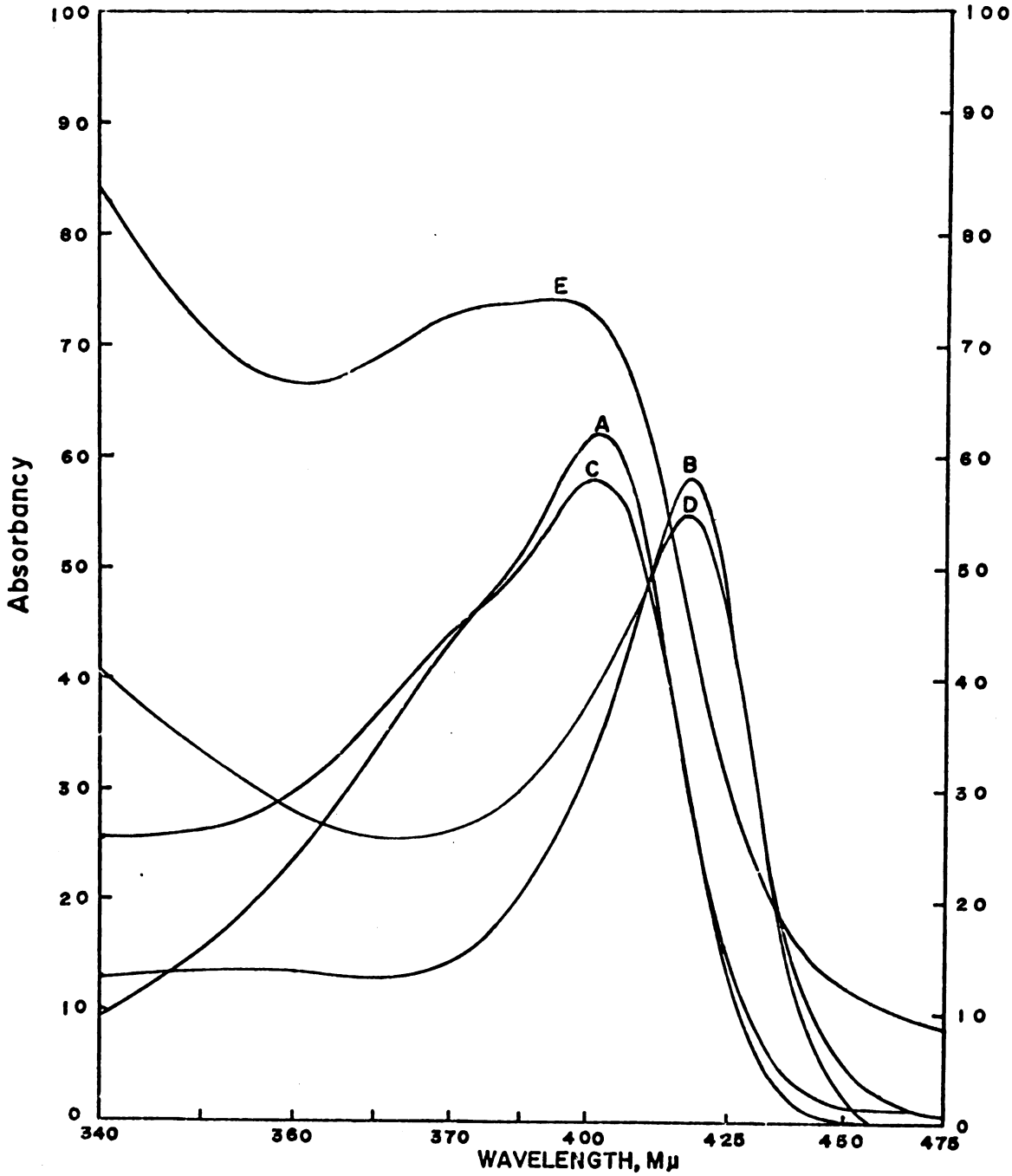
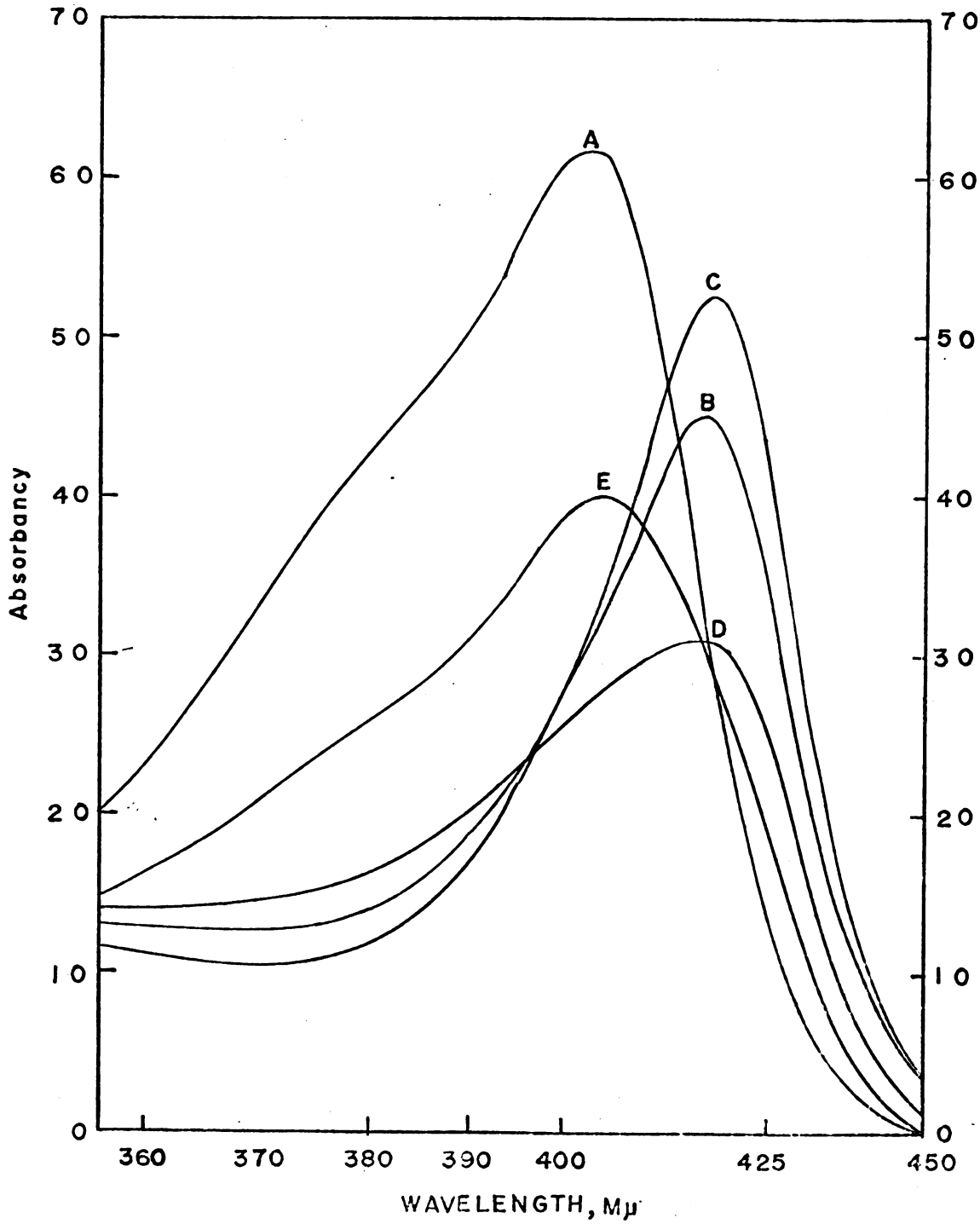

Absorbancy
100
90
80
70
60
50
40
30
20
10
0
340
360
370
400
425
450
475
WAVELENGTH, Mμ
A
B
C
D
E

Figure 28. Effect of hydrogen peroxide and sodium bisulfite on the absorption spectra of horseradish peroxidase. 0.15 M phosphate buffer solution, pH 7.68. A. Free enzyme. B. A plus 0.05 ml of 0.3% H_2O_2. C. B plus 0.03 ml of 1 x 10^{-3} M $NaHSO_3$. D. 30 min. after the addition of $NaHSO_3$. E. Two hours later after the addition of $NaHSO_3$. Enzyme: 0.85 mg per cuvette (3 ml).


70
60
50
40
30
20
10
0
Absorbancy
A
B
C
D
E
360
370
380
390
400
425
450
WAVELENGTH, Mμ

enzyme, curve C, occurred instantaneously. Apparently the addition of IAA had reduced the complex II back to free enzyme and prevented its further formation.

Furthermore, it is also interesting to note that further addition of a small amount of sodium bisulfite to the solution represented by curve C would convert the free enzyme (curve C) to complex II (curve D) again. At this point the enzyme complex mixture did not remain in a steady manner, but the solution underwent further transformation as represented by curve E in a consecutive manner which was different from the instantaneous change of B to C. It is possible that the transition of B to C is due to the inhibitory effect of IAA toward the formation of complex II, whereas the transformation of D to E is an oxidation and reduction process. Because bisulfite ion is also the substrate of the enzyme system (18) it reversed the inhibitory effect of IAA under the alkaline pH condition and induced the formation of the complex II which is essential for the further oxidation of IAA. The observation that complex II is essential for the peroxidase-catalyzed reactions is in agreement with that reported previously by Chance (14, 15).

Figure 28 shows the direct effect of bisulfite ion on the formation of complex II in the absence of IAA. Curve A of the Figure represents the optical absorption of the free enzyme and curve B is that of complex II. In the absence of IAA the addition of sodium bisulfite, at first, enhanced complex II formation and then reduced complex II through a

redox process back to the original free enzyme (Curve E). In Figure 28, Curve C was made immediately after the addition of bisulfite, Curve D was taken 30 minutes after the addition of bisulfite, and Curve E was obtained 2 hours later.

These data indicate that during the course of bisulfite oxidation, complex II was reduced in a consecutive manner. Consequently, the free enzyme was released. This contrasts with the case when IAA was oxidized under the same condition. When IAA was oxidized at pH 7.68 in the presence of bisulfite ion, the final absorption of enzyme was also changed, possibly indicating the change of the enzyme conformation due to the interaction of the enzyme molecule with IAA.

Yamazaki and Souzu (114) reported that IAA can inhibit the oxidation of guaiacol by peroxidation. As reported (114), the inhibition of oxidation of guaiacol by IAA could only be observed under anaerobic conditions. However, the inhibitory effect of IAA toward the formation of complex II was found under aerobic conditions at alkaline pH values. It was assumed by the same authors that the inhibitory effect of IAA toward the oxidation of guaiacol possibly was due to the inactivation of the enzyme by interaction of the enzyme molecule with the free radicals generated from IAA. If the same explanation can be applied in the case of IAA oxidation, it is possible that the inactivity of peroxidase toward IAA at pH above neutrality is due to the inhibitory effect of IAA toward the formation of complex II as was observed in Figure

27. This seems to constitute a typical example of substrate inhibition of enzyme action. It is interesting to note that at the physiologically important pH range the oxidation of IAA by "auxin oxidase" is controlled by auxin itself.

DISCUSSION

DISCUSSION

The possibility that 4-hydroxyquinoline is produced as an oxidation product of IAA has been discussed by Manning and Galston (64). They believed that 4-hydroxyquinoline was not formed as an oxidative product in the "auxin oxidase" catalyzed oxidation of IAA. However, our work definitely shows that 4-hydroxyquinoline, which has typical absorption at 316 and 330 mμ, can be formed from peroxidase-catalyzed oxidation of either IAA or IAAld. However, in order to detect its formation, specific reaction conditions must be employed. Apparently there exist, simultaneously, at least two pathways for the oxidative breakdown of IAA or IAAld catalyzed by the peroxidase system. These pathways compete with each other and the inhibition of the one may result in the stimulation of the other. Although 4-hydroxyquinoline is not formed as a major product in peroxidase oxidation of IAA, the probability of such a transformation seems to be always present. Once favorable conditions are established, its formation can be detected easily.

Our work partially supports Kenten's viewpoint (44), i.e., that the oxidation of IAAld catalyzed by peroxidase may result in the formation of IAld as one of the major products. However, the results indicate that the peroxidase-catalyzed reactions depended mainly on the reaction conditions.

Varying the pH and the presence or absence of certain cofactors may change the pathways. These observations that the change of pH will result in the change of the reaction pathways have not been reported previously for any other enzyme.

The effect of the bisulfite ion on the enzymatic system might be accomplished in several different ways. First, like hydrogen peroxide, it might participate directly in the reaction as one of the enzyme substrates; second, it might change the structural character of the side chain of the indole compounds either of the substrate, IAAld or the product, IAld; and finally, the bisulfite ion might have a profound effect on the structural characteristics of the enzyme. Because of the large differences between the pH-activity curves in the presence and absence of the bisulfite ion, it seems that the effect of the ions might have a direct influence on the catalytic behavior of the enzyme. This was found to be the case. In this thesis, direct evidence has been obtained to illustrate how the peroxidase molecule is involved in the bisulfite-stimulated oxidation of IAA (see Figures 25 and 26).

As far as the pH effect is concerned, Maclachlan and Waygood (62) has proposed a free radical mechanism to explain the inactivity of the peroxidase system above neutrality. The proposed reaction mechanism is shown by:

$$ICH_2COO^- + Mn^{3+} \longrightarrow ICH_2COO^- + Mn^{2+} \quad (1)$$

$$ICH_2COO^{\bullet} \longrightarrow ICH_2^{\bullet} + CO_2 \quad (2)$$

$$ICH_2^{\bullet} + O_2 \longrightarrow ICH_2OO^{\bullet} \quad (3)$$

$$ICH_2OO^{\bullet} + ROH \xrightarrow{\text{Peroxidase}} ICH_2OOH + RO \quad (4)$$

$$RO^{\bullet} + Mn^{2+} + H^+ \longrightarrow ROH + Mn^{3+} \quad (5)$$

From the suggested mechanism it is evident that Waygood attributed the lack of reaction above neutrality to the instability of the free radical intermediates, since, according to the mechanism, the generation of the free radicals ($RO^{\bullet}$) involves the utilization of hydrogen ions. In our reaction system no manganese was added; thus Waygood's explanation is unlikely. Furthermore, there is some degree of uncertainty involved in the proposed mechanism: first, Waygood's mechanism does not involve hydrogen peroxide (see equation 3). Second, Waygood's mechanism is based almost entirely on the initial rate of nonenzymatic reactions (equations 1, 2, 3) and consequently neglects the importance of the involvement of the enzymatic process. The general mechanism of peroxidase-catalyzed reactions has been studied extensively by Chance (14, 15). He has shown that the peroxidase-catalyzed reactions depend on the formation of chemical complexes between peroxidase and H_2O_2. In the present work we were also able to show how the peroxide-peroxidase complexes are involved in the enzyme-catalyzed reaction. It is apparent that the inhibitory effect of IAA toward the formation of compound II may account for, at least partially, the instability of the

enzymatic system at pH values above neutrality. Since Waygood's mechanism was also criticized by Ray (76), there is no need to repeat in detail all of his statements here.

Recently, Fridovich (17) reported that certain nitrogenous compounds such as ammonia, pyridine, imidazole, and hydrazine can stimulate the peroxidase-catalyzed oxidation of anisidine. In contrast to stimulators of the monophenol type, activation of the enzyme system by these ligands resulted in changing the pK_a of the enzyme protein from 6.5 to 9.5. Similar results were observed in the present investigation. It is known that the optimal pH for the Mn^{3+}-monophenol system is in the range of 3.5 to 4.5. However, the optimal pH for the thyroxine activated oxidation of epinephrine and norepinephrine is between 7 and 8 (50), and thyroxine at pH 5.6 was inhibitory to the oxidation of iodide catalyzed by the same peroxidase system. Another interesting observation reported by Kirdani and Layne (47) was that the oxidation of norethynodrel by horseradish peroxidase was increased as the pH was increased from 6.0 to 8.5.

The conclusion drawn from these observations is that the optimal pH for peroxidase-catalyzed reactions depends on both the chemical properties of the substrates and those of the stimulators. Yamazaki and Souzu (114) has classified peroxidase substrates into two groups: redogenic substrates and oxidogenic substrates. The redogenic substrates are characterized by their capacity to reduce ferricytochrome c in the presence of hydrogen peroxide and peroxidase, a

property not possessed by the oxidogenic group.

By analogy, it seems that peroxidase substrates and its stimulators as well can be divided into two groups according to the optimal pH at which they react. One group reacts with an alkaline enzyme system, whereas the other reacts with an acidic one. The results are possibly the consequence of the amphoteric character of the enzyme molecules. Thus, we may say that peroxidase may assume two conformational forms: Form I is predominant in acidic medium and Form II in alkaline medium. Compounds like thyroxine, bisulfite, and nitrogenous ligands are a group which can activate the enzyme system with alkaline Form II, whereas the Mn^{3+}-phenol group is active toward the acidic Form I.

From this assumption, it is not unreasonable to predict that a compound which stimulates enzyme Form I might inhibit enzyme Form II. The phenomena was apparently observed in the present experiments. Therefore, it appears that at the physiologically important pH range (near neutral), the action of the peroxidase system requires the presence of an appropriate cofactor such as bisulfite ion, ammonia, thyroxine, etc. The presence of the one of such cofactors may induce the enzyme protein to assume a proper conformational form essential for the enzyme activity toward the specific substrate.

However, in our work it has been shown that peroxidase activity directly involves the heme group of the enzyme

molecule. The inhibitory effect of IAA toward the formation of compound II at alkaline pH under aerobic condition may involve an electron transfer process in which the hematin-peroxide complex is an essential intermediate.

Recently Yamazaki et al. (113) established that free radicals participate in certain peroxidase-catalyzed reactions. In fact, by using the technique of paramagnetic resonance, they were able to detect the formation of free radicals of dihydroxyfumarate, ascorbate, hydroquinone, and IAA from peroxidase-catalyzed reactions. The proposed free radical mechanism of Yamazaki et al. (113) is as follows:

$$\text{Peroxidase} + H_2O_2 \xrightarrow{k_1} \text{Compound I} \qquad (1)$$

$$\text{Compound I} + AH_2 \xrightarrow{k_2} \text{Compound II} + AH^{\bullet} \qquad (2)$$

$$\text{Compound II} + AH_2 \xrightarrow{k_3} \text{Peroxidase} + AH^{\bullet} \qquad (3)$$

$$2AH^{\bullet} \xrightarrow{k_d} A + AH_2 \text{ (or HA-AH)} \qquad (4)$$

In the above mechanism AH_2 is the substrate electron donor, $AH^{\bullet}$ the free radical intermediate, and A is the oxidized product.

From the above formulation, the free radical concentration at steady state should be

$$[AH^{\bullet}]_S = \left[\frac{k_3 [E] [AH_2]}{k_d}\right]^{1/2}$$

The fundamental concept of this formulation is based on two propositions: that the free radicals are formed from substrates, and that the formation of free radicals depends on an enzymatic process. The results obtained by Yamazaki from electron paramagnetic resonance spectra indicate that the concentration of free radical formation is proportional to $[E]^{1/2}$ in accordance with the mechanism indicated above. The same mechanism was also suggested by George (24). The results obtained by Yamazaki are completely inconsistent with Waygood's formulation which depends basically on the auto-oxidation process. It is evident that auto-oxidation processes are not necessarily related to the enzyme-catalyzed reactions.

Aerobic oxidation of sulfite ions has been used by Alyea and Backstrom (2) and also by Fridovich and Handler (18) as a sensitive detector for the occurrence of free radicals. In the presence of their respective substrates, the enzymes, xanthine oxidase, aldehyde oxidase, and horseradish peroxidase, were found capable of initiating the oxidation of bisulfite ion. The mechanism involved in bisulfite oxidation was nearly identical to that found in bioluminescence (101) caused by xanthine oxidase acting on xanthine in the presence of luminol or lucigenin, in that both of these two processes depend on the generation of free radicals. As reported (18), the oxidation of the bisulfite ion initiated by horseradish peroxidase required the presence of both hydrogen peroxide and a peroxidizable

substrate. Either substrate by itself caused no oxidation of the sulfite. Accordingly, since no free radical can be generated in the transfer of an electron pair, they suggested that the free radical was not generated from the interaction of hydrogen peroxide with peroxidase, but from the oxidation of a peroxidizable substrate through a successive univalent electron transfer.

In view of the important observations reported by Alyea and Backstrom (2), Fridovich and Handler (17, 18), George (24, 25), and Yamazaki et al. (113, 114), it is clear that the stimulatory effect of the bisulfite ion toward the oxidation of IAA and IAAld by the peroxide-peroxidase system occurs because the bisulfite anions are capable of being oxidized through a chain reaction which is initiated by the enzyme system. This type of reaction is essential for the production, maintenance, and propagation of the free radicals required for the catabolism of the substrates at the elevated pH values. Furthermore, during such free radical transfer processes the formation of peroxide-peroxidase intermediate is essential for the overall reaction sequence. In other words, the overall process of the free radical chain is under the strict control of the enzymatic system. This is reflected in the changes of V_{max} and K_m of the enzymatic system in the presence of bisulfite anions. In addition, the fact that the presence of bisulfite ion can change the oxidative products may also attribute to the free radical mechanism, since the change of the environmental factors such as pH or enzyme

conformation may have changed the distribution of the single electron about the substrate molecules.

Such environmental changes may lead to the increased formation of a product which originally was only produced in a small quantity. The observation by Stutz (90) that cytochrome c and a cytochrome oxidase containing homogenate of rat liver can stimulate the formation of IAld from IAA is another example to support such a hypothesis. At this point, it should be recalled that Yamazaki and Souzu (114) have divided the peroxidase substrates into two groups based on their capacity to reduce ferricytochrome c in the presence of hydrogen peroxide and peroxidase; and that Fridovich and Handler (18) noted that in the presence of ferrocytochrome c cytochrome oxidase can initiate the oxidation of sulfite ion vigorously. Hence the presence of the cytochrome c and cytochrome oxidase may have changed the electron wave function of the IAA free radical, thereby facilitating the elimination of the carboxyl carbon and stimulating the yield of IAld. A similar explanation may be applied to 4-hydroxyquinoline since its formation is greatly stimulated by the existence of a strong free radical generating system, i.e., a bisulfite-peroxide-peroxidase system.

If Yamazaki *et al.* (113) mechanism is correct, in case of IAA oxidation, the following reactions may be suggested:

$$\text{HRPO} + H_2O_2 \xrightarrow{k_1} \text{Complex I} \quad (1)$$

$$\text{Complex I} + \text{IAA} \xrightarrow{k_2} \text{Complex II} + \text{IAA}' \quad (2)$$

$$\text{Complex II} + \text{IAA} \xrightarrow{k_3} \text{HRPO} + \text{IAA}' \quad (3)$$

$$\text{IAA}' + Fe^{3+} \xrightarrow{k_d} \text{IAA}'' + Fe^{2+} \quad (4)$$

In the above mechanism IAA' represents free radicals of IAA and IAA" represents IAA oxidation products. Apparently, this mechanism is only applicable in the acidic conditions, at pH values above neutrality the oxidation of IAA is inhibited because of the inhibitory effect of the IAA toward the formation of the compound II. In other words, at alkaline pH values IAA itself inhibits reactions (1) and (2) but not reactions (3) and (4). However, when bisulfite anions are also present, these ions are also the substrate of the peroxide-peroxidase system, so they can act as a bridge to induce the reproduction of the compound II, thereby activating the overall system. Such an idea is depicted as follows:

$$\text{HRPO} + H_2O_2 \xrightarrow{k_1} \text{Complex I} \quad (1)$$

$$\text{Complex I} + HSO_3^- \xrightarrow{k_2'} \text{Complex II} + (HSO_3^-) \quad (2)$$

$$\text{Complex II} + \text{IAA} \xrightarrow{k_3} \text{HRPO} + \text{IAA}' \quad (3)$$

$$\text{IAA}' + Fe^{3+} \xrightarrow{k_d} \text{IAA}'' + Fe^{2+} \quad (4)$$

In the above formulation, (HSO_3^-) stands for bisulfite free radicals. Since bisulfite itself can also be oxidized

through the same mechanism, the oxidation of bisulfite by the peroxidase system may follow the scheme:

$$\text{HRPO} + H_2O_2 \xrightarrow{k_1'} \text{Complex I} \qquad (1)$$

$$\text{Complex I} + HSO_3^- \xrightarrow{k_2'} \text{Complex II} + (HSO_3^-)\cdot \qquad (2)$$

$$\text{Complex II} + HSO_3^- \xrightarrow{k_3'} \text{HRPO} + (HSO_3^-)\cdot \qquad (3)$$

$$(HSO_3^-)\cdot + Fe^{3+} \xrightarrow{k_d'} HSO_4^- + Fe^{2+} \qquad (4)$$

Since at this alkaline pH k_3' is much smaller than k_3 (the transition of D to E in Figure 25 took only 30 minutes, but it took more than 3 hours for the transition of B to E in Figure 26), the presence of bisulfite results in the stimulation of the oxidation of IAA.

Although the formation of a small amount of IAld has been reported from the oxidation of IAA by peroxidase from various plant sources, the major oxidation product was not IAld but 3-methylene oxindole. In our work 3-methylene oxindole rather than IAld was formed as one of the major products in the peroxidase-catalyzed oxidation of IAA. However, in contrast to the observation noted for IAA, the oxidation of IAAld by the same system yielded as high as 50% of IAld in the reaction mixture. These results suggest that IAA and IAAld were degraded by the same peroxidase system in different ways and that IAA was not an intermediate product in the course of peroxidase-catalyzed oxidation of IAAld. Apparently there is a common factor in the peroxidase-

catalyzed oxidation of either IAA or IAAld. As a result of oxidative breakdown of IAA and IAAld, the oxidation products in general are one carbon atom less than the original compound, and the missing carbon atom is generally derived from the terminal carbon atom of the side chain. Thus it appears that there is a general principle which is governing the course of the enzymatic reaction. This principle may be closely related to the chemical property of the free radical generated during the course of the catalyzed reaction.

A two-step sequential reaction mechanism (IAA to A to B) has been suggested by Ray (78) to explain some results which he had obtained in a series of spectroscopic studies on the course of peroxidase oxidation of IAA. Furthermore, based on some results obtained in the present work, the author suggests that Ray's intermediate compound A, either generated from IAA or from IAAld may have a structural feature in common.

Recently, Hinman and Lang (36) suggested an "epoxide mechanism" to explain the oxidation of IAA to 3-methylene oxindole or indole-3-carboxaldehyde as catalyzed by highly purified horseradish peroxidase. Reviewing the results obtained in the present work, it is worthwhile to point out the following about the mechanism proposed by Hinman and Lang.

1. They proposed that the aldehydic oxygen in the IAld molecule is derived directly from H_2O_2, whereas the oxygen at 2-position of 3-methylene

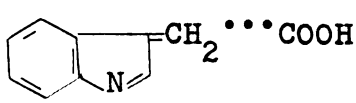
oxindole is derived from the water molecule in the reaction mixture.

2. According to the proposed mechanism of Hinman and Lang, they further proposed that in the formation of 3-methylene oxindole from the hypothetical epoxide precursor, the epoxide oxygen is finally lost to the reaction mixture again. It appears that the major feature of this scheme designed for 3-methylene oxindole is simply a sequence of the two steps of hydration and dehydration of the epoxide intermediate.

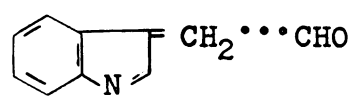
3. Finally, their experiments indicated that the immediate precursor for the formation of 3-methylene oxindole is oxindole-3-carbinol. According to their observations, the formation of oxindole-3-carbinol is an enzyme-catalyzed process, whereas the transformation of oxindole-3-carbinol to 3-methylene oxindole is nonenzymatic. In their experiments it took 76 minutes for the oxindole-3-carbinol absorption peak to appear, but 23 hours for the spontaneous transformation of this oxindole-3-carbinol to the typical absorption of 3-methylene oxindole. Under our experimental conditions the formation of 3-methylene oxindole is not a slow reaction. The typical peak of 3-methylene oxindole can be observed within one or two hours and the

rate of its formation depends on the enzyme concentration. The more enzyme present the faster is the appearance of this peak. The inconsistency between the observation of the writer and the latter investigators may be that the enzyme used by Hinman and Lang had a rather low activity (RZ = 0.64), whereas the RZ of the horseradish peroxidase used in our experiments was between 2.0 and 3.0. Nevertheless, there are at least two paths in the formation of 3-methylene oxindole: one by the spontaneous dehydration of oxindole-3-carbinol (a slow process); and the other by direct addition of one oxygen atom to the free radical intermediate compound A (a fast process).

The chemical nature of Ray's intermediate compound A is unknown. However, our evidence indicates that during the course of enzyme-catalyzed oxidation of either IAA or IAAld there may be a transient existence of a 3-methylene indolenine-like structure as indicated below:



Indole-3-acetic Acid



Indole-3-acetaldehyde

Yamazaki and Sauzu (114) noted that electron density of the IAA free radical generated from the peroxidase-

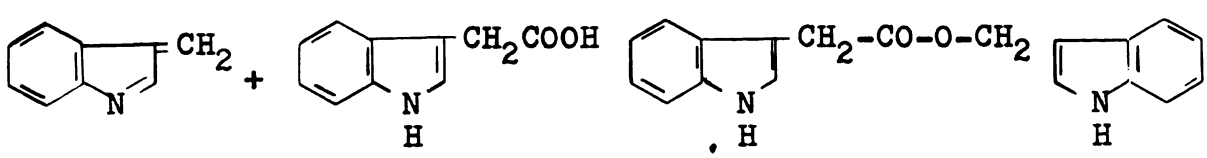
catalyzed oxidation of IAA was highest at the 3-position, therefore the above diagrams of IAA and IAAld free radicals are only hypothetical. However, a single electron in a molecule does not have a localized charge, but has a distributed wave function. A change in the environment may change the distribution of the electron within the molecule. Consequently, a single electron of the IAA or IAAld free radical could be distributed among three possible sites in the molecule. One site is located at the methylene carbon of the side chain, and the other two at the 2- and 3-positions of the indole ring. The course of the reaction is then determined by the following factors:

1. The nature of the carbonyl carbon.--Since the carboxyl group of the acetic acid side chain of IAA is more stable than the aldehydic carbonyl group of IAAld, the carboxyl group stabilizes the methylene hydrogens of the side chain of IAA, whereas the same hydrogens are activated by the aldehydic group in IAAld. Consequently, because of this activation, the methylene group of IAAld becomes more susceptible to attack by the oxidizing agent (in this case peroxide-peroxidase complex) and thereby results in the formation of IAld as the major product.
2. The polarity of the -N=C- group in the indolenine intermediate.--According to Witkop and Patrick (110), the polarity of the -N=C- group of the indolenine ring is determined by the acidity of

the reaction medium. The presence of H^+ will enhance the additive ability of the -N=C- group, whereas the hydroxide anions will reverse this effect. Thus under alkaline conditions the 2-position of the pyrrole rings of either IAA or IAAld becomes less active toward the approaching oxidizing agent (in this case peroxide-peroxidase complex), consequently leaving the other two positions as possible oxidation sites. Indeed, under alkaline conditions as a result of the oxidation of IAAld, the major products were found to be a mixture of 4-hydroxyquinoline and indole-3-carboxaldehyde.

The evidence for the existence of the indolenine-like free radical is as follows:

1. From the peroxidase-catalyzed oxidation of the IAA reaction mixture, Hinman and Lang (36) have isolated an ester which had the following structure and possibly is formed by addition of IAA to 3-methylene-indolenine:



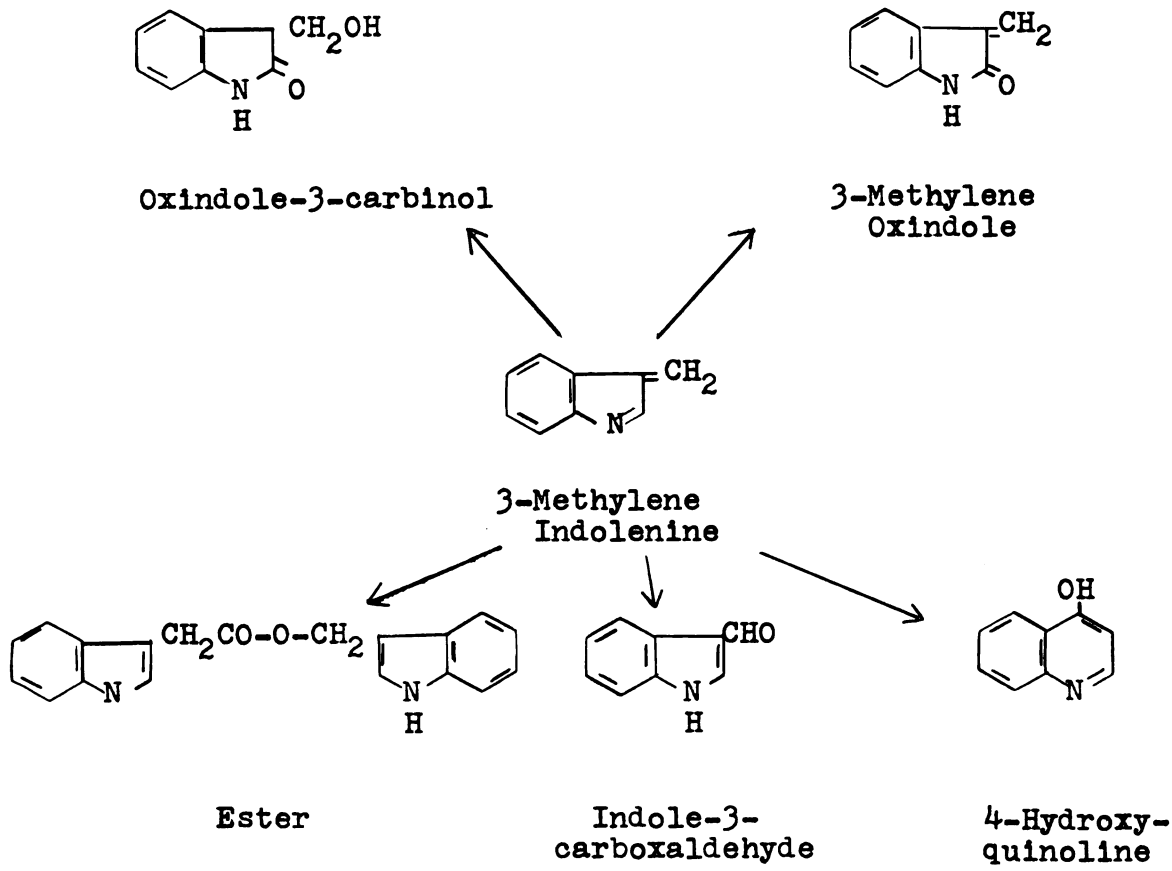
3-Methylene Indolenine | Indole-3-acetic Acid | Ester

2. The methylene side chain of the intermediate 3-methylene indolenine is also the side chain of

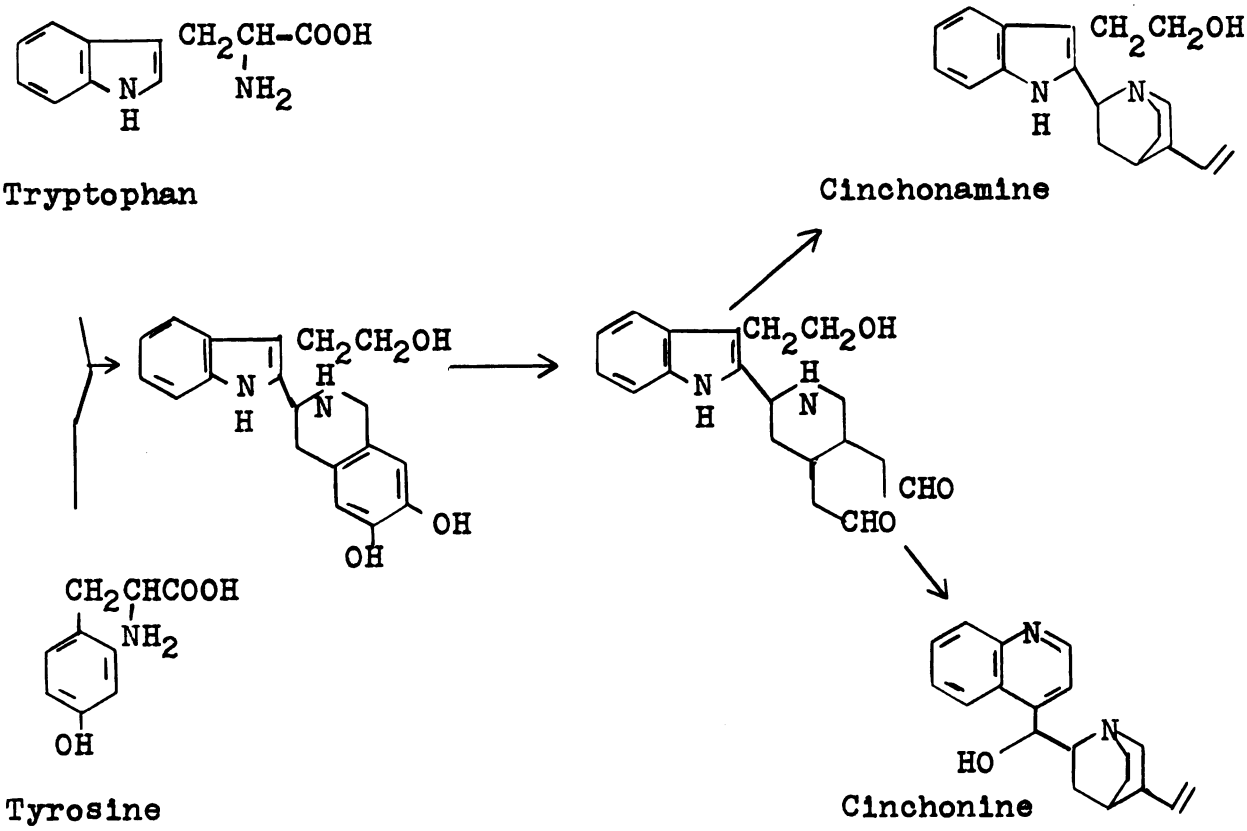
3-methylene oxindole, which is a major oxidation product from IAA.

3. The functional group which can stabilize the indolenine structure greatly stimulates the α-carbon elimination process. For instance, during chromatographical analyses, 1-methyl-IAAld can undergo spontaneous auto-oxidation to give 1-methyl-IAld at room temperature in the presence of atmospheric oxygen.

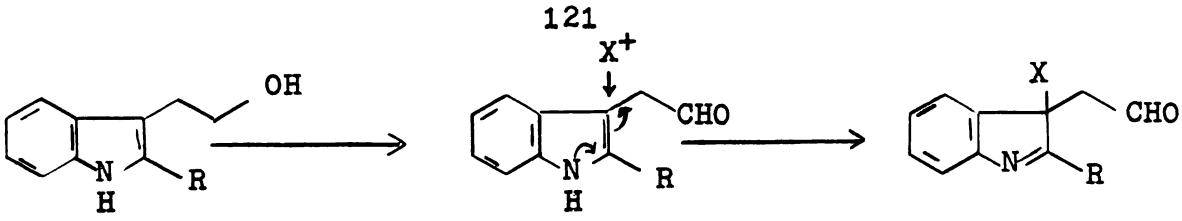
Therefore, the possible routes for the oxidation of IAA or IAAld are the following:



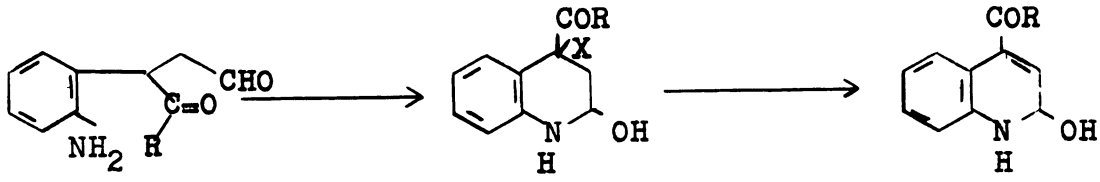
Goutarel *et al.* (31) suggests that two naturally occurring alkaloids cinchonamine and cinchonine are biochemically related. A hypothetical pathway for production of cinchonamine and cinchonine is shown below:



Recently, Van Tamelen and Haarstad (104) suggested that cinchonamine could be the precursor of cinchonine. Using 2-methyl-IAAld as a model intermediate, they suggested a sequence of reactions for the possible manner of transformation of cinchonamine into cinchonine. The suggested reaction scheme follows:

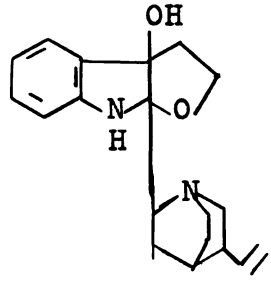


Cinchonamine



Cinchonine

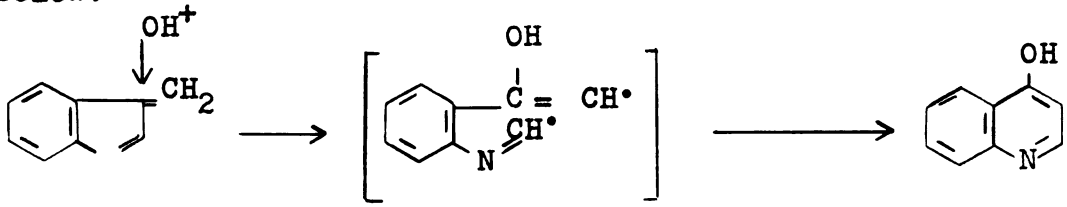
Based on the above working hypothesis, they were able to synthesize 4-acetylquinoline directly from 2-methyl tryptophan by treatment of the tryptophan derivative with two equivalents of sodium hypochlorite. The key hypothetical intermediate of the proposed reaction scheme is a 2-methyl-indole-3-acetaldehyde derivative. The natural occurrence of quinamine constitutes support for the proposed electrophilic attack at the 3-position. In the above scheme, X^+ represents the agent OH^+ or an equivalent.



Quinamine

The sequence of the reactions proposed is very similar to the well-known kynurenine pathway for the tryptophan metabolism except that the cleavage of the indole ring occurs at 1, 2-position and no carbon is lost during the ring expansion process.

In the transformation of IAAld to 4-hydroxyquinoline a similar mechanism, suggested by Van Tamelen and Haarstad (104), may be followed with a net loss of carbon atom at 2-position. In view of the results obtained in the present work, it appears that a common feature of peroxidase-catalyzed oxidation of IAA and IAAld is the elimination of the first carbon of the side chain. If this is also true in the case when 4-hydroxyquinoline is produced, an alternative pathway of transformation of IAAld into 4-hydroxyquinoline is suggested. An alternative mechanism which has an analogy among other organic reactions is the rearrangement shown below:



3-Methylene Indolenine

4-Hydroxy-quinoline

Since no direct evidence has been available to support either mechanism proposed, further studies of this problem are of great interest.

The formation of 1-methyl-4-hydroxyquinoline from

1-methyl-IAAld could not be verified in the present work. However, this naturally occurring alkaloid was first isolated from Echipses by Greshoff (33) in 1900 as the chloride. Consequently it was called echipsin. Possibly, the procursor of echipsin is either IAAld or 1-methyl-IAAld. From results obtained in our work we suggest that echipsin may be directly produced from methylation of 4-hydroxyquinoline rather than from 1-methyl-IAAld.

This discussion shows that it is possible that plant peroxidase may have played an important role in biosynthesis of some physiologically important plant alkaloids as cinchonine, quinine, and echipsin. All these processes may require the participation of certain cofactors such as bisulfite ion, ammonia or other normal metabolites present in plant systems.

Recently it was reported that abnormally large quantities of IAld have been detected in the urine of schizophrenic patients (80). The presence of peroxidase system in nervous systems has been reported (11). It is possible that peroxidase is also involved in these biologically important processes.

The reason why manganese and 2,4-dichlorophenol have no effect on the peroxidase-catalyzed oxidation of IAAld in the acidic pH range is unknown. Further studies of this aspect are required.

Klapper and Hackett (48) demonstrated that commercial horseradish peroxidase contains several isozymes. However, the five purified enzyme components have been shown to have

identical catalytic activities and the amino acid compositions of three of the five purified isozymes are similar. It is unlikely that the formation of 4-hydroxyquinoline is a result of structural differences between these isozyme components.

SUMMARY

SUMMARY

In a series of spectrophotometric studies on the oxidation of indole-3-acetic acid (IAA), indole-3-acetaldehyde (IAAld), and indole-3-acetaldehyde sodium bisulfite addition product (IAAld-$NaHSO_3$) catalyzed by horseradish peroxidase, it was found that these substances each followed a different pattern of oxidative pathway. Either qualitatively or quantitatively the peroxidase-catalyzed oxidation of IAA and its analogs are pH dependent. At a pH range from 3.7 to 4.5, the conversion of IAA to 3-methylene oxindole, as reported previously, was confirmed in the present work; whereas the oxidation of IAAld under the same conditions gave rise to as high as 50% yield of indole-3-carboxaldehyde (IAld). The oxidation of IAAld-$NaHSO_3$ at pH between 3.7 and 4.4 followed the same pattern as that observed for free IAAld. As the pH was increased above 6.2, a differential behavior of the enzymatic system was noted, i.e., the oxidation of IAA and IAAld decreased continually as the pH was increased, whereas the oxidation of IAAld-$NaHSO_3$ increased greatly as the pH increased above 6.2.

One of the interesting observations is that at alkaline pH values as result of oxidative breakdown of IAAld-$NaHSO_3$, a new product having a double peak at 316 and 330 mμ regions was always observed. The presence of bisulfite ion was found

to be stimulatory toward the oxidation of the auxins catalyzed by the horseradish peroxidase. In the presence of 1 x 10^{-4} M of IAAld K_m calculated for bisulfite ion was found to be 3.5 x 10^{-5} M. Increasing bisulfite concentration from 3.57 x 10^{-5} to 3.57 x 10^{-4} M increased the V_{max} of the system two-fold and reduced the K_m of the system twentyfold. Apparently the presence of bisulfite ion greatly enhanced the affinity of horseradish peroxidase toward its substrates at elevated pH values. The present work definitely shows that 4-hydroxyquinoline is the oxidation product in horseradish peroxidase-catalyzed oxidation of IAAld. However, a change of pH and the presence or absence of a cofactor greatly influenced the peroxidase-catalyzed oxidation of IAA and IAAld. In the present work the formation of 4-hydroxyquinoline from peroxidation of IAAld was established. The evidence was obtained by the direct isolation of the oxidation product in crystalline form.

The mechanism of the bisulfite ion stimulated oxidation of IAA and IAAld was also studied. The results indicated that at alkaline pH values the presence of the bisulfite ion stimulated the formation of peroxidase complex II which is essential for peroxidase-catalyzed reactions.

Another interesting observation is that during the course of peroxidase oxidation of IAA the indole ring was altered by the oxidizing agent, whereas when IAAld was oxidized the ring remained intact. This is not true when the bisulfite ion is added to the reaction mixture. At alkaline pH values in the presence of bisulfite ion the oxidation of

IAAld will result in the expansion of a five-membered indole ring to a six-membered quinoline ring. Whether this is true in case of the oxidation of 1-methylindole-3-acetaldehyde (1-methyl-IAAld) is unknown. No definite results have been obtained to indicate that 1-methyl-4-hydroxyquinoline is an oxidation product of 1-methyl-IAAld. Apparently, there is no single rule which governs the peroxidase-catalyzed oxidation of auxins, although a common feature in the peroxidase-catalyzed oxidation of IAA, IAAld, and 1-methyl-IAAld was the elimination of the terminal carbon of the side chain. 1-Methyl-IAld was found to be one of the major products in the peroxidase oxidation of 1-methyl-IAAld.

The reason why the presence of bisulfite can divert the oxidation pathway in the course of peroxidase-catalyzed oxidation of IAA and IAAld is unknown. Some possible mechanisms are discussed.

The inhibitory effect of IAA toward oxidation of guaiacol was recently reported. Our investigations are similar to the previous observation, i.e., IAA is inhibitory to the peroxidase system. Furthermore, our work indicates that the inhibitory effect of IAA toward the enzymatic system is directly related to the formation of peroxide-peroxidase complexes.

Another interesting observation is that the presence of manganese and 2,4-dichlorophenol had no effect on the peroxidase-catalyzed oxidation of IAAld under either acidic or alkaline pH conditions.

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