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Kinetics of Oxidation of Phenols by Compound II of Horseradish Peroxidase and the Iodination of Tyrosine

by

ADEJARE JOB ADENIRAN

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfilment of the requirements for the degree of Doctor of Philosophy.

Department of Chemistry

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H.B. Dunford, Supervisor

External Examiner

Date Dec. 17, 1987

TO MY PARENTS

ABSTRACT

The rates of reduction of horseradish peroxidase compound II by p-methoxyphenol (4-hydroxyanisole) have been studied from pH 6.0 to 10.5. The kinetics are influenced by an acid group of pK_a 8.7 on compound II probably HIS42. The acidic form of compound II is reactive; the basic form is not. Only the electrically neutral, unionized form of p-methoxyphenol is reactive. Fourteen different phenols were reacted with compound II at either pH 7.6 or pH 7.0 (three of them at both pH's). Rate constants varied from zero for p-nitrophenol to 3.2×10^7 M^{-1} s^{-1} for p-aminophenol. The reactive p- and m-substituted phenols yield a ρ value of -4.6 ± 0.5 when plotted according to the Hammett relation. This compares to the ρ value of -6.9 for similar reactions of horseradish peroxidase compound I (1976, D. Job and H.B. Dunford, Eur. J. Biochem. 66, 607). The difference in sensitivity of compounds I and II to substituent effects on phenols is explained in terms of relative simplicity of the reactions involved as determined by the structures at the active sites.

The rates of non-enzymatic iodination of tyrosine have been investigated at 25.0 ± 0.2°C in the presence, and in the absence of initially added potassium iodide, using the Union Giken stopped-flow spectrophotometer. The apparent second order rate constant for the reaction of molecular iodine with tyrosine varies from 2.1 ± 0.3 M^{-1} s^{-1} at pH 4.44, in the presence of 2.0 mM KI, to $(1.0 \pm 0.2) \times 10^3$ M^{-1} s^{-1} at pH 10.79

in the presence of 20 mM KI. In the absence of initially added iodide ions, the rate constant varies from (8.5±0.5) M^{-1} s^{-1} at pH 3.55 to (1.9±0.3)x10^3 M^{-1} s^{-1} at pH 7.77. The variation of the rate constant with buffer concentration is indicative of general acid catalysis in the low pH range, and general base catalysis in the higher pH. The mechanism of reaction that most fits the results in the presence of added potassium iodide is one in which the unionized phenol is attacked by hypoiodous acid at the iodination step; the deprotonation of the iodinated quinoid intermediate formed is then the rate-determining step.

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CHAPTER ONE

INTRODUCTION

Peroxidases

Peroxidases are enzymes which catalyze oxidation of a variety of substrates, both organic and inorganic, by hydrogen peroxide or by peroxides or peroxyacids of the type ROOH (1). They are widely distributed among higher plants, the richest sources being the sap of fig trees and the root of horseradish (2). In humans peroxidases are found in saliva, the thyroid gland, adrenal medulla, leukocytes and tears (2,3). Some of the better known peroxidases include lactoperoxidase, LPO, from milk, saliva and tears; myeloperoxidase, MPO, from white blood cells; thyroid peroxidase, TPO, from the thyroid gland; chloroperoxidase, CPO, from the fungus Caldariomyces fumago; cytochrome c peroxidase, CcP, from yeast; and horseradish peroxidase, HRP, from the root of horseradish (2,4-6). An up-to-date review of the methods of purification of the common peroxidases has been published (3).

The generally accepted scheme for the sequence of reactions in the catalytic cycle of peroxidases is:

$$P + ROOH \rightarrow \text{Compound I} + ROH$$

$$\text{Compound I} + AH_2 \rightarrow \text{Compound II} + {}^{\bullet}AH$$

[I.1]

$$\text{Compound II} + AH_2 \rightarrow P + {}^{\bullet}AH$$

$$2\,{}^{\bullet}AH \rightarrow A_2H_2 \text{ (or } A_2 + A_2H_4)$$

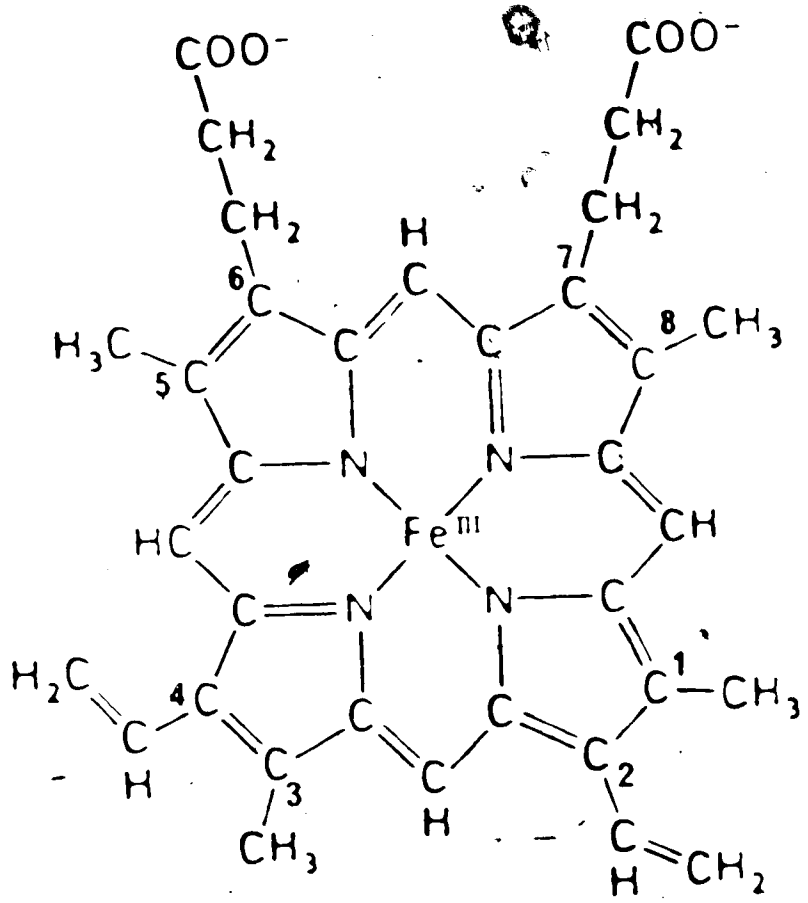
where P represents the native enzyme, compound I and compound II represent the primary and secondary intermediate compounds of the enzyme respectively, and AH_2 represents the reducing substrate, commonly called the hydrogen donor. The overall reaction is:

[I.2] $$ROOH + 2AH_2 \rightarrow A_2H_2 + ROH + H_2O$$

and is known to be a modified form of ping pong kinetics in which one molecule of oxidizing substrate and two molecules of reducing substrate participate (1).

The stability of the intermediates, sometimes with half-life of hours for compound II and minutes for compound I, and the intense environmentally sensitive Soret absorbance at about 400 nm have facilitated the large number of studies that have been carried out on peroxidases (1-3,7,8).

Most peroxidases contain ferriprotoporphyrin IX as prosthetic group (Fig. I.1). The Soret absorbance of peroxidases is due to the conjugated tetrapyrole of this ferric heme. Horseradish peroxidase (EC.1.11.1.7, donor H_2O_2, oxidoreductase) is the most widely studied peroxidase because of its ready availability in pure form. Its primary structure is now known (9,10). Five oxidation states of HRP are known. The three of these which are involved in the



Heme
(Fe-protoporphyrin IX)

Figure I.1 Structure of the heme: the prosthetic group of most peroxidases. The intense environmentally sensitive Soret absorbance at about 400 nm is due to the conjugated tetrapyrole.

normal catalytic cycle are the resting state of the enzyme in which the heme iron is Fe(III); compound I which is formed by the reaction of HRP in the resting state with a hydroperoxide, and which has been shown to have a ferryl type structure, $(R^{\bullet+})Fe(IV)=O$, with a porphyrin π-cation radical and compound II which has a ferryl type structure, $(R)Fe(IV)=O$ (11-13). Thus, compound I contains both oxidizing equivalents of the peroxide. Compound II is formed by a one-electron reduction of compound I as shown in Scheme [I.1], and so it contains only one oxidizing equivalent above the native enzyme.

Reactions of Peroxidases

A comprehensive review of the in vitro reactions of peroxidases has been published (3). Some of the relevant reactions to be considered here include phenol oxidation, and the two-electron reduction of compound I (14). Halide ions and bisulphite ions are the only two types of substrates which are known to reduce compound I of HRP directly to the native enzyme. The behavior of bisulphite ion in the presence of HRP-I has been explained in terms of two possible schemes as follows (15,16):

[I.3] $\underset{\text{Compound I}}{Fe(IV)=O} + HOSO_2^- \longrightarrow \underset{\text{Native enzyme}}{Fe(III)} + SO_4^= + H^+$

or

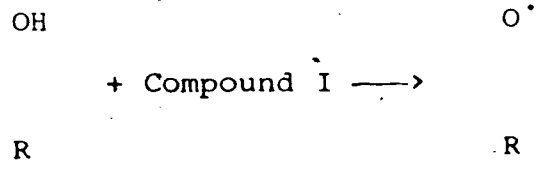
$$\underset{\text{Compound I}}{Fe(IV)=O} + HOSO_2^- + H^+ \longrightarrow \underset{\text{Native enzyme}}{Fe(III)} + SO_3 + H_2O \qquad [I.4]$$

$$SO_3 + H_2O \longrightarrow SO_4^{=} + 2H^+$$

For this reaction, $SO_3^{\cdot -}$ radical has been detected at pH 8.6 where both compound I and compound II have low reactivities towards HSO_3^- (17).

In the absence of a substrate for halogenation, and if compound I of an enzyme has sufficient oxidation potential, halide ions, X^-, react to form hypohalous acid, with a direct conversion of compound I to native enzyme. However, it has been shown that in the presence of a substrate for halogenation, for chloroperoxidase, an enzyme-associated hypohalite, Fe(III)-O-Cl, is the chlorinating agent (18-20). With other peroxidases, the halogenating intermediates are still subjects of intensive investigations.

The reaction of a phenol with either compound I or compound II involves a one-electron transfer and a proton transfer during each elementary step (21);



[I.5]

and similarly for compound II reduction to native enzyme. p-Cresol (21,22) and gnaiacol (23) have been shown to deviate from the general mechanism which depicts the normal

enzymatic cycle (equation [1]) by reducing compound I to the native enzyme, through an intermediate formation of compound II, using only one molar equivalent of phenol.

The observation for a 1:1 HRP and p-cresol reaction mixture at pH 3 to 10.5 has been explained in terms of the mechanism (22):

$$\text{HRP-I} + \text{PC} \rightarrow \text{HRP-II} + \text{PC}^{\bullet}$$

[I.6] $$\text{PC}^{\bullet} \rightarrow \tfrac{1}{2}(\text{PC})_2$$

$$\text{HRP-II} + \tfrac{1}{2}(\text{PC})_2 \rightarrow \text{HRP} + \text{Products}$$

where PC represents p-cresol and PC$^{\bullet}$ represents p-methylphenoxy free radical. The products were identified to be 90% Pummerer's ketone. Thus, the overall reaction is:

[I.7] $$\text{HRP-I} + \text{PC} \rightarrow \text{HRP} + \text{Products}.$$

However, at pH 10.5 where HRP-II is known to be converted into a nonreactive alkaline form, and in presence of 2:1 HRP to p-cresol, an overall 2:1 stoichiometry is observed (22):

[I.8] $$2\text{HRP-I} + \text{PC} \rightarrow 2\text{HRP-II} + \text{Products}.$$

This was explained in terms of the scheme:

$$HRP\text{-}I + PC \rightarrow HRP\text{-}II + PC^{\bullet}$$

[1.9] $$PC^{\bullet} + \tfrac{1}{2}(PC)_2$$

$$HRP\text{-}I + \tfrac{1}{2}(PC)_2 \rightarrow HRP\text{-}II + Products.$$

Active Site of Peroxidase

Most peroxidases consist of heme and glycoprotein, and the structure and intimate interaction between the two components give each peroxidase its unique set of catalytic properties. The heme prosthetic group at the active site has been shown to have a dual role in the catalytic function of peroxidases. The iron, Fe(III), activates the heterolytic cleavage of the peroxide and stores one oxidizing equivalent, while the porphyrin regulates the oxidation-reduction potential and also usually stores one oxidizing equivalent. (In yeast CcP an oxidizing equivalent is stored on the protein, not the heme.) However, in terms of more detailed structure at the active site of peroxidase, much remains to be resolved (3). For example, the type of ligand at the sixth coordination position, and the nature of the halogenating intermediate, are still topics of controversy.

Studies of pH dependence, and the role of protons in the different reactions of horseradish peroxidase have been embarked upon as a means of shedding light on the nature of the active site of the enzyme (1,7,22,23). As pointed out

earlier, the reduction of compound I to compound II, and of compound II to native enzyme can be represented as:

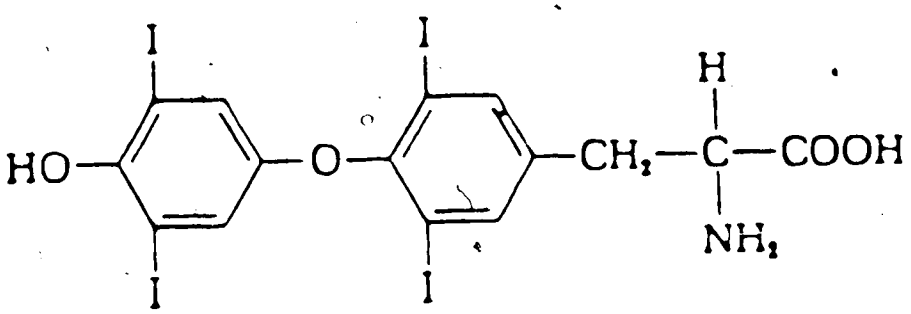
[I.10] Compound I + H^+ + e → Compound II

[I.11] Compound II + H^+ + e → HRP

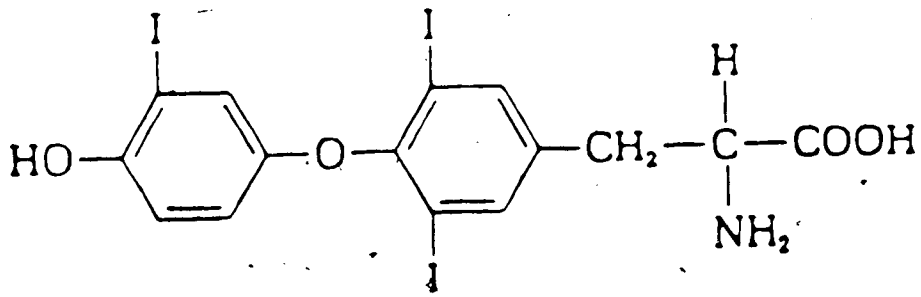
It has been shown from NMR experiments that the proton does not add to the ferryl oxygen, but rather to the apoenzyme (24); it has also been established that an acid group with a pK_a of 8.6 is essential for most reactions of HRP-II. This was first established from kinetic studies on a wide variety of substrates (7) and subsequently confirmed by results from spectrophotometric and proton balance studies (25), and two separate studies on Fe(IV)=O resonance Raman stretching vibrations (26,27). An acid group of pK_a 5.1 to 5.4 has been shown to be important in most one-electron reduction of compound I of HRP (7,8); and the distal His42 has been implicated.

Thyroid hormones.

The hormones of the thyroid gland, thyroxine and L-3,5,3'-triiodothyronine (Fig. I.2) are known to be involved in mammalian body temperature regulation. The mechanism of this process is not fully understood. However, it is known that when these hormones are released, they



Thyroxine (L-3,5,3',5'-tetraiodothyronine)



Triiodothyronine (L-3,5,3'-triiodothyronine)

Figure I.2 The thyroid hormones.

cause an increased basal metabolic rate. This in turn results in an increased heat production, and thus raises the body temperature. The hormones are synthesized from two successive oxidative reactions, both of which are known to be catalyzed by thyroid peroxidase, TPO (28). In the first reaction, ~30 out of ~130 tyrosine residues present in the polypeptide backbone of thyroglobuline are either monoiodinated or diiodinated in a reaction involving I^- and H_2O_2. In a second reaction, ~8 of the iodinated tyrosine are coupled to form thyroxine, (L-3,5,3',5'-tetraiodothyronine), or L-3,5,3'-triiodothyronine. The mechanisms of these reactions have been the subject of many investigations (28-32).

Enzymatic iodination

Two possible mechanisms have been proposed for enzymatic iodination of tyrosine. These are an electrophilic substituion, and a free radical mechanism (33,34). In the electrophilic substitution mechanism, compound I of TPO, formed by the reaction of the native enzyme with H_2O_2, reacts with I^- in a two-electron oxidation to form the iodinium ion, I^+, which then substitutes for hydrogen in position 3 of the phenolic group in a tyrosyl residue. In the free radical mechanism TPO-I is considered to remove an electron from I^- to form an iodine radical, I^o; and one electron from a tyrosyl residue to form a tyrosyl radical. Monoiodotyrosine is then formed by radical addition between I^o and tyrosyl radical (see Fig. I.3).

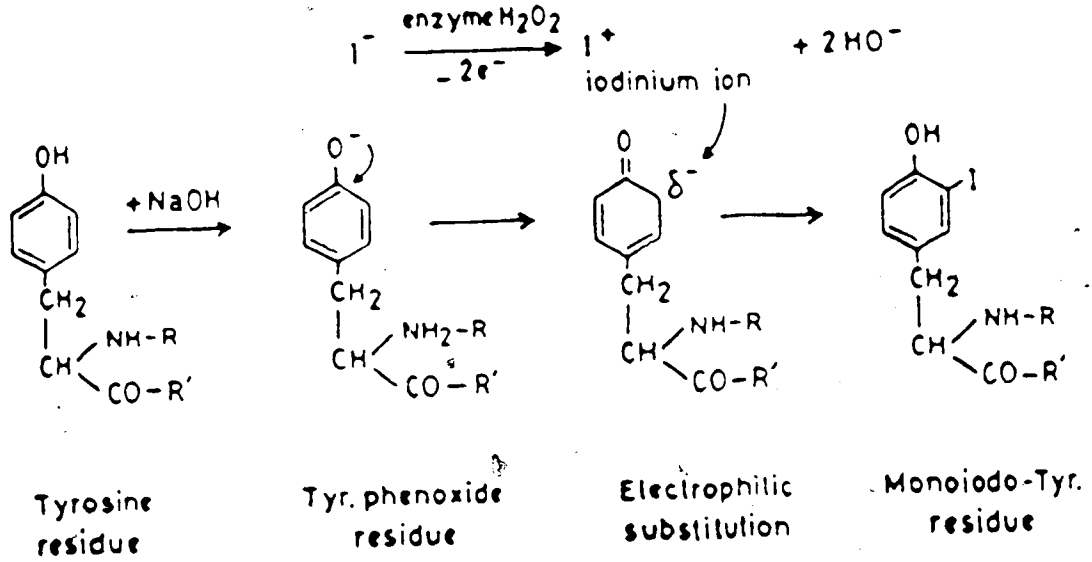


Figure I.3a Proposed mechanism for electrophilic substitution.

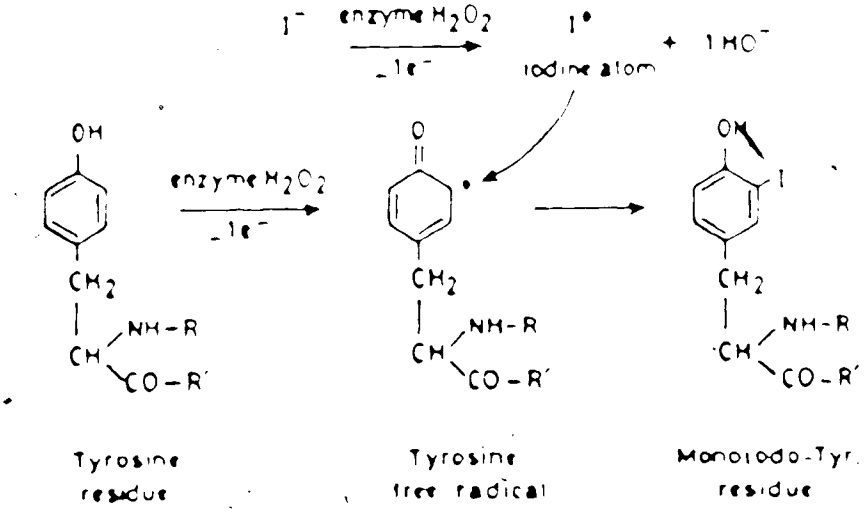


Figure I.3b. Proposed mechanism for free radical addition.

All investigators generally agree, however, that iodide, which is concentrated by thyroid tissue, must be oxidized to a higher oxidation state before it is able to displace hydrogen from tyrosyl residues (7). Only the nature of this oxidized state of iodine has been a subject of controversy. Some workers contend that TPO catalyzes peroxidation of iodide and also participates catalytically in the iodination of tyrosyl residues of thyroglobulin (35-37). Others suggest that after the peroxidation of iodide, the oxidized iodine species may iodinate tyrosyl residues in a nonenzymatic process (38).

As a result of the report that halogenation catalyzed by chloroperoxidase goes via an enzyme-bound halogenium cation intermediate (39), a similar mechanism involving enzyme-associated iodinium cation as intermediate was proposed for iodination reactions of thyroid peroxidase, lactoperoxidase and horseradish peroxidase (35,36,40-43). This hypothesis was given support by the observation that HRP-I is reduced directly to native enzyme by iodide ions in a 2-electron process, without an observable intermediate formation of compound II (44,45); and the observation that gluthathione, GSH, inhibited the iodination reaction in the synthesis of thyroxine (36,46-49). The inhibition was attributed to the reaction of GSH with the enzyme-iodinium cation.

It has been suggested that HRP and lactoperoxidase, LPO, have two binding sites in their catalysis of iodination of tyrosine: I^- binds to one, and tyrosine to the other (37,50-52). Iodination is then considered to occur on the enzyme surface either by the oxidation of I^- to I^+, which then reacts with the bound tyrosine by electrophilic substitution; or by the oxidation of both tyrosine and I^- to free radicals which then couple. It was observed that D-tyrosine is iodinated at a rate which is 1.5 times faster than L-tyrosine, suggesting that iodination involves a complex of the enzyme with the phenolic compound and that the complex formation is stereospecific (49). A site for an iodide acceptor substrate on TPO, and on HRP was demonstrated (37,53,54). Peroxidase-catalyzed iodination of proteins has been shown to have iodide (35,37,54) and pH (44,49,52) requirements different from those for non-enzymatic iodination. Thus, enzymatic iodination is known to be inhibited at those iodide concentrations necessary for enzymatic I_2 formation, and proceeds at those pH's where chemical iodination by I_2 in the presence of I^- does not occur. In one study, the pH dependences of three different reactions of iodine were compared: viz., chemical iodination of Glu-Tyr-Glu by I_2 in the presence of I^-, enzymatic iodination of Glu-Tyr-Glu catalyzed by TPO, and enzymatic oxidation of I^- to I_2 catalyzed by TPO (36). The

pH dependences were found to differ in all three cases, thus, favoring the conclusion that free iodine is not the primary iodinating species in enzyme catalyzed iodination reactions. In another study solutions of I_2 in I^- were added to reaction mixtures containing tyrosine and the LPO in the absence of peroxide at pH 5.0 and 7.4. No iodination was observed (52). In the same study it was also found that at low pH values where I_2 is stable, and where the spontaneous reaction of I_2 with phenols is known to be very slow, LPO catalyzes iodination in the presence of peroxide (52). The rate of iodination in this low pH region was found to be nearly identical whether I_2 or I^- is employed as substrate. This was interpreted to reject the suggestion that I_2 is an obligatory intermediate in enzymatic iodination reaction (38).

However, in a kinetic study of the oxidation of iodide ions by HRP-I and by HRP-II, a 1:1 ratio was observed between enzyme reacted and iodine formed (44). This was interpreted to indicate that oxidized iodine species does not form a complex or compound with the enzyme. A TPO-associated iodinating intermediate was later generated by incubating equimolar amounts of the enzyme, $^{125}I^-$, and H_2O_2 at pH 5.6 (36). The intermediate was isolated by column chromatography and shown to be capable of iodinating the tripeptide Glu-Tyr-Glu without additional H_2O_2 and $^{125}I^-$.

Thus, evidence was provided for the existence of an enzyme-associated iodinating intermediate. It then appears that this observation (36) and the earlier kinetic results on the oxidation of iodide ions by HRP-I and HRP-II (44) could only be reconciled by assuming that the step at which I_2 is produced from the enzyme-associated intermediate is much faster than the step at which the intermediate is formed. Thus, the fraction of the oxidized form of iodine which remains bound would be very small. This conclusion would be supported by the results of a series of steady state, stopped-flow and rapid scan studies on the chlorination of 2-chlorodimedone by peracetic acid, catalyzed by chloroperoxidase (19,20). From detailed kinetic and spectral analyses it was concluded that the most probable mechanism for chlorination catalyzed by CPO is one in which the substrate is chlorinated by enzyme-bound hypochlorite, Fe(III)-OCl (20). However, in a kinetic study of three different reactions of iodine catalyzed by TPO, and by LPO, viz., oxidation of I^- to I_2, iodide dependent catalatic degradation of H_2O_2, and iodination of tyrosine, the results were interpreted to indicate that LPO behaves differently from TPO (55). It was suggested that while an enzyme-bound hypoiodite intermediate is the iodinating agent with thyroid peroxidase, it is hypoiodous acid generated in the case of lactoperoxidase (55).

Biological defense mechanism.

Besides their roles in the synthesis of the hormones, thyroxine and triiodothyronine, peroxidase-catalyzed iodination reactions play important roles in biological defense mechanisms. Antimicrobial activity of peroxidases is due to their ability to catalyze H_2O_2-dependent oxidation of halide ions or SCN^- to produce powerful oxidizing agents (56). The oxidizing agent produced makes an electrophilic attack on microbial components, resulting in chemical modification of essential enzymes, transport systems and other functional components. Most aspects of antimicrobial actions can be correlated with chemical modification of such nucleophilic components as sulfhydryl groups and aromatic amino acid residues (56). Even though H_2O_2 is a powerful oxidizing agent, it reacts slowly with biological materials; and most cells have enzymes that could decompose H_2O_2 rapidly. Thus peroxidase-catalyzed oxidation of halides and thiocyanate conserves the oxidizing power of H_2O_2 in forms that react more rapidly, and for which the target cell may have no defence (3).

The most studied enzymes in connection with the body's defense against invading micro-organisms, parasites and tumor cells are myeloperoxidase and lactoperoxidase (3,57). MPO is located in the polymorphonuclear leukocyte, and LPO is detected in human saliva, milk and tears (57,58-60). MPO

utilizes chloride ions and, to a lesser degree, iodide; LPO utilizes thiocyanate primarily even though it can also catalyze the oxidation of iodide ions. MPO can utilize SCN^- when present along with LPO in the oral cavity (3). It has been suggested that MPO produces a variety of reactive substances including halogens, hypohalous acids and chloroamines (59,61-64) while both LPO and MPO produce hypothiocyanous acid, HOSCN (58,65) and that these agents are responsible for the toxic effects on target cells and molecules. However, in addition to halide-dependent antimicrobial actions, MPO has been associated with O_2-dependent toxicity, mediated through superoxide anion, hydroxyl radical, or singlet oxygen (59,66).

Nonenzymatic iodination reactions.

A great number of studies have been carried out on nonenzymatic halogenation reactions in an effort to help to understand peroxidase-catalyzed halogenation reactions. After the nitronium ion, NO_2^+, was isolated and shown to be the nitrating agent in aromatic substitution reactions (67) the search for an analogous positive halogen species, as active halogenating agent, was intensified (68). For uncatalyzed halogenation by molecular chlorine or bromine, it was claimed that the active halogenating species was Cl_2 or Br_2 (69). However, in a study of the bromination of sodium m-anisole sulphonate with hypobromous acid (70), in

the presence of mineral acid, the rate law was found to be of the form

[I.12] $V = k(ArH)(HOBr)(H^+)$

where ArH is the aromatic compound. This was interpreted to be a result of the formation of a positive bromine species by the reaction

[I.13] $HOBr + H^+ \rightleftharpoons Br^+ + H_2O$

The existence of a positive iodinium ion was demonstrated in the study of iodine solution in concentrated H_2SO_4 and oleum (71); it was accounted for by the equation:

[I.14] $I_2 + H_2S_2O_7 + 3SO_3 \rightleftharpoons 2I^+ + 2HS_2O_7^- + SO_2$

No evidence was found for the presence of Br^+ or Cl^+ in oleum solutions. However, in several separate studies it was demonstrated that the species, H_2OBr^+, formed by the protonation of hypobromous acid in acidic solution, is a more powerful electrophile than Br_2 (72-76).

Until very recently no work had been done with HOI as a reagent for iodination because it is very unstable compared to the other hypohalous acids, HOBr and HOCl. However, from

one of the early studies on iodination of phenols using molecular iodine in phosphate, and acetate buffers, it was concluded that the active iodinating agent was either HOI, attacking unionized phenol, or I^+, attacking the phenolate ion (77). A general acid catalysis was observed and interpreted in terms of CH_3COOI or $HIPO_4^-$, formed between the buffer acid and iodine, taking part as an iodinating agent (77). Evidence was shown by other workers that acetyl hypoiodite and acetylhypobromite are the active halogenating agents in some aromatic ring halogenations (78). General acid-base catalysis was studied between pH 5 to 7 for the iodination of phenol, and the results were interpreted to indicate general base catalysis (79). Mechanisms of iodination proposed in subsequent studies on iodination reactions of phenols and related compounds were based on the assumption that the reaction is general base catalyzed (80-85).

Hydrolysis of iodine in aqueous solution.

The system of iodine in aqueous solution has been shown to be complex (86); this is a result of the number of oxidation states available to iodine. Many studies have been carried out to determine the most important iodine species present in aqueous solution under different conditions. An increased interest in kinetic and equilibrium studies of I_2 hydrolysis has now been brought

about by the need for designing effective measures to prevent the release of radioactive iodine species into the environment following a nuclear reactor accident (87). As a result, equilibrium constants have now been determined for most of the known equilibria in aqueous iodine solution, and kinetic parameters have been determined for many of them (86-89).

From temperature-jump relaxation studies on the hydrolysis of iodine (86,88,89) a mechanism has been proposed in which the formation of HOI is controlled by the disproportionation of I_2OH^- (89). The proposed mechanism is shown in scheme [I.15]:

$$I_2 + H_2O \rightleftharpoons I_2OH^- + H^+$$

[I.15] $$I_2 + OH^- \rightleftharpoons I_2OH^-$$

$$I_2OH^- \rightleftharpoons HOI + I^-$$

The existence of I_2OH^- as an intermediate was confirmed by the results of a Raman spectroscopic study of the disproportionation of hypoiodite in basic solutions (87). In the latter study the mechanism proposed for the disproportionation of elemental iodine, I_2, to iodate and iodide in basic aqueous media is shown in scheme [I.16] (87):

$$I_2OH^- \underset{\text{Rapid}}{\rightleftharpoons} IO^- + I^- + H^+$$

$$IO^- + I^- + H_2O \underset{\text{Rapid}}{\rightleftharpoons} I_2OH^- + OH^-$$

$$IO^- + IO^- \xrightarrow{\text{slow}} IO_2^- + I^-$$

[I.16]

$$IO^- + I_2OH^- \xrightarrow{\text{slow}} IO_2^- + 2I^- + H^+$$

$$IO^- + IO_2^- \xrightarrow{\text{fast}} IO_3^- + I^-$$

$$IO_2^- + I_2OH^- \xrightarrow{\text{fast}} IO_3^- + 2I^- + H^+$$

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CHAPTER TWO

HAMMETT $\rho\sigma$ CORRELATION FOR REACTIONS OF HORSERADISH PEROXIDASE COMPOUND II WITH PHENOLS[1]

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[1]A version of this chapter has been published. Dunford, H.B. and Adeniran, A.J. (1986) Arch. Biochem. Biophys. 251, 536-542.

Introduction

Since the period following the discovery of the oxidized intermediates of horseradish peroxidase (EC 1.11.1.7, donor-H_2O_2 oxidoreductase) used in its catalytic action (1,2) much work has been done in an effort to establish details of the mechanism of its peroxidatic action. A scheme for horseradish peroxidase catalysis of the oxidation of a variety of substrates by H_2O_2 or by organic peroxides of the type ROOH which is generally accepted is as follows (3,4):

[II.1] $HRP + H_2O_2 \rightarrow HRP\text{-}I + H_2O$

[II.2] $HRP\text{-}I + AH_2 \rightarrow HRP\text{-}II + \cdot AH$

[II.3] $HRP\text{-}II + AH_2 \rightarrow HRP + \cdot AH + H_2O$

[II.4] $2 \cdot AH \rightarrow A_2H_2$ (or $A_2 + A_2H_4$)

Horseradish peroxidase, compound I and compound II are represented by HRP, HRP-I and HRP-II respectively, while the oxidizable substrate (organic or inorganic) has been represented by AH_2. Subsequent studies have focused upon establishing a molecular description of events occurring at the heme active site of the enzyme during its catalytic action (see for example (5,6)).

The complete amino acid sequence of horseradish peroxidase is now known (7) and the pK_a values obtained from

kinetic studies (6) and from spectrophotometric titration studies (8) for compound I and compound II have been explained in terms of the ionization of heme-linked groups.

Phenols are widely used as antioxidants either because of their ability to scavenge free radicals or in some cases to react directly with the oxidant (9). The prime example of physiological significance is the fat-soluble α-tocopherol (Vitamin E) (10). p-Methoxyphenol, also known as 4-hydroxyanisole, exhibits action against malignant melanomas (11). Phenols are highly reactive with peroxidases. Thus they are excellent substrates for mechanistic studies (12,13). Lignin formation is catalyzed by peroxidase from phenolic monomers (14-16). It has been suggested that peroxidases could be used commercially to remove phenol contaminants from water supplies (17-19). The phenolic side chains of tyrosine residues can be coupled in a peroxidase-catalyzed reaction, providing cross-links in a variety of biopolymers (20,21).

In this study fourteen water-soluble phenols are reacted with compound II of horseradish peroxidase. In one case the pH-rate profile is obtained. The results are correlated, where possible, using the Hammett $\rho\sigma$ relation (22).

Experimental

Materials

Horseradish peroxidase, Grade I, obtained from Boehringer Mannheim Canada, as an ammonium sulfate suspension, was dialysed extensively against water purified in a Milli-Q water purification system. The enzyme concentration was determined spectrophotometrically at 403 nm using a molar absorptivity of 1.02×10^5 M^{-1} cm^{-1} (23). A fresh stock solution of compound II of the enzyme was prepared each day by addition of one equivalent of $K_4Fe(CN)_6$ and one equivalent of H_2O_2 to the native enzyme solution. The concentration of the resultant solution of compound II was between 1 and 3 μM. The concentration of $K_4Fe(CN)_6$ solution was determined by weight while that of H_2O_2 was determined by a spectrophotometric method involving the conversion of I^- to I_3^-, using HRP as catalyst (24).

Solutions of most substrates were prepared by dissolving a weighed amount of the compound in an appropriate volume of purified water each day to make fresh stock solutions of ~10 mM concentration. The solutions were stored in brown bottles to prevent photochemical reactions. p-Aminophenol, however, was first dissolved in a small amount of 1 M HNO_3 before being diluted with purified water. In a similar way, p-hydroxybenzoic acid was

dissolved in a small amount of 1 M NaOH. Acid or base was added to increase the solubility of each of the substrates. The substrate solution for each run was then made by diluting portions of the 10 mM stock solution to give concentrations of 10 μM to 70 μM, using a buffer of pH 7.6. In the case of p-methoxyphenol buffers of pH 6.0 to pH 11.0 were used in the determination of the pH-rate profile. At least ten-fold excess of substrate over the enzyme was always used so that the reaction occurred by a pseudo-first order process.

Buffers, Ionic Strength Control.

Stock buffer solutions used for both enzyme and substrate were made up to ionic strength, μ = 0.11 M by adding 0.1 M KNO_3 to a buffer which was 0.01 M (final concentration). For solutions pH 6 to pH 8, phosphate buffers were used. Tris buffers were used between pH 8 and pH 9, while carbonate buffers were used above pH 9.

Kinetic Experiments.

The kinetic measurements, with the exception of the experiments with m-hydroxybenzaldehyde as substrate, were all made on a Union Giken Stopped-Flow Spectrophotometer Model RA-601. The 1 cm cell was thermostated at 25.0°C for all the measurements. For m-hydroxybenzaldehyde, the kinetic measurements were made on a Cary 219

spectrophotometer for which the 1 cm cell was also thermostated at 25.0°C.

One of the reservoirs of the stopped-flow spectrophotometer was filled with the compound II solution, freshly prepared prior to the experiment, and the other reservoir was filled with the substrate. The reaction was then followed by monitoring the disappearance of compound II at 420 nm, the wavelength of maximum difference between the spectra of compound II and native enzyme (see Fig, II.1). Between [redacted]o nine experiments were made for each substrate concentration and the data stored directly in an on-line computer memory.

For experiments using the Cary spectrophotometer, 3 mL of freshly prepared solution of compound II in buffer was measured into a cuvette and placed in the observation chamber. The reference cuvette was filled with distilled water. A calculated amount of the stock solution of the substrate was measured onto the tip of a Teflon micro-spatula. This was mixed rapidly with the enzyme and the instrument was started as soon as possible. The relative absorbance at regular time intervals was then measured manually from the trace obtained from the spectrophotometer. The observed pseudo-first order rate constant, k_{obs}, for each experiment, either on the stopped-flow apparatus or the Cary spectrophotometer was calculated through the use of

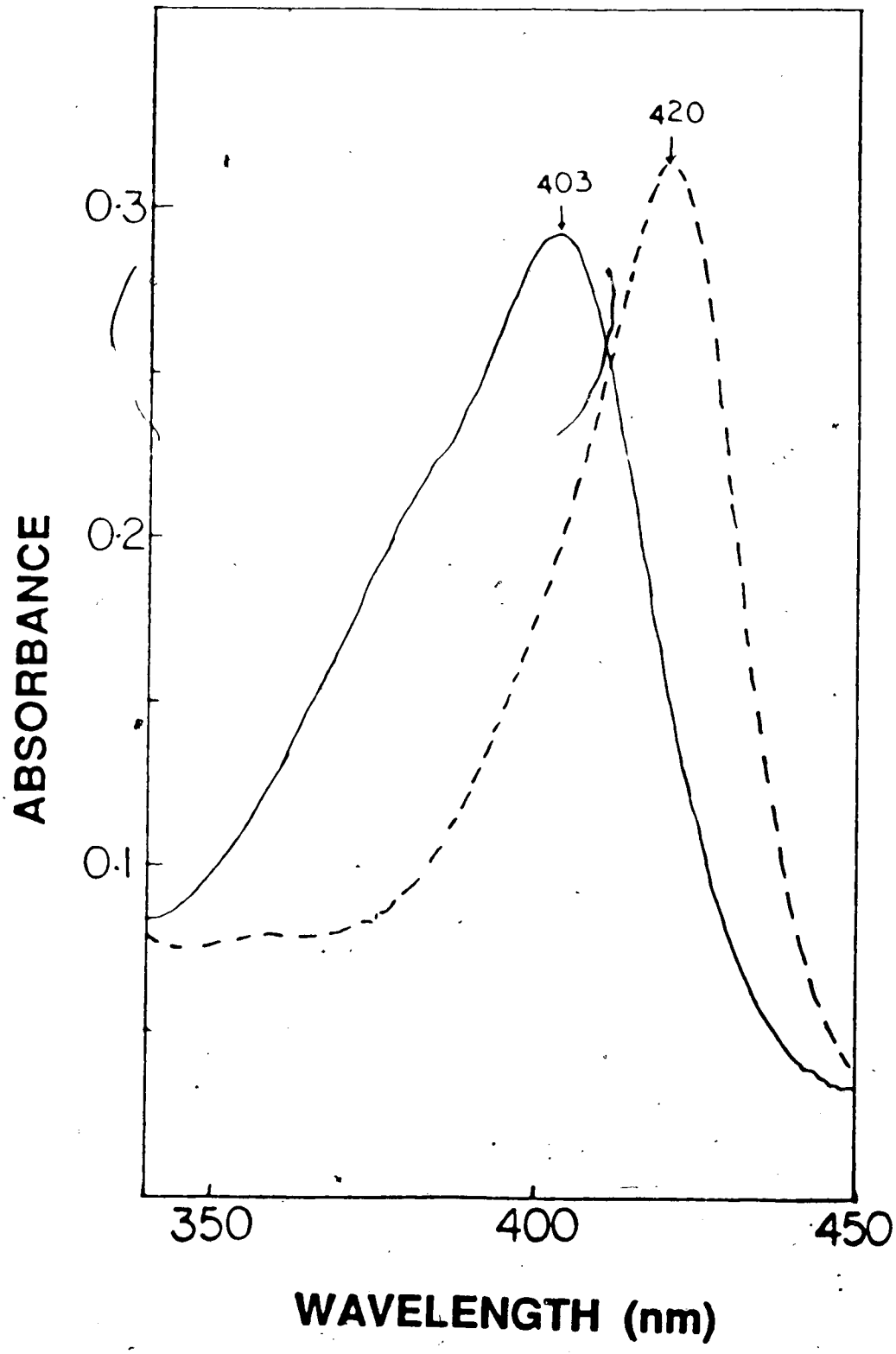


Figure II.1 Spectra of HRP and HRP-II in the Soret region; pH 9.75, 2.9 μm HRP ; ——— HRP; ---- HRP-II

a non-linear least squares computer program based on Gauss' method.

Results

Pseudo-first order traces were observed when substrate concentration was in excess over that of compound II. Examples are shown in Fig. II.2. The apparent second order rate constant, k_{app}, was found to be related to the observed pseudo-first-order rate constant, k_{obs}, by the expression:

[II.5] $$k_{obs} = k_{app}[S]$$

where [S] is the substrate concentration. Thus k_{app} was determined from the slopes of plots of k_{obs} against substrate concentration (Fig. II.3). The slopes were obtained using the method of linear least squares regression. In a typical result obtained with p-methoxyphenol (4-hydroxyanisole) the slope of a plot according to equation (II.5) yields a k_{app} value of $(9.2\pm0.2)\times10^5$ M^{-1} s^{-1}. The intercept was zero. Data were obtained at pH 7.0, 25.0°C and ionic strength 0.11 M.

pH Dependence

A log k_{app} vs. pH plot is shown in Fig. II.4 for the reaction of p-methoxyphenol with compound II. The simplest mechanism which could be found to fit the experimental data is

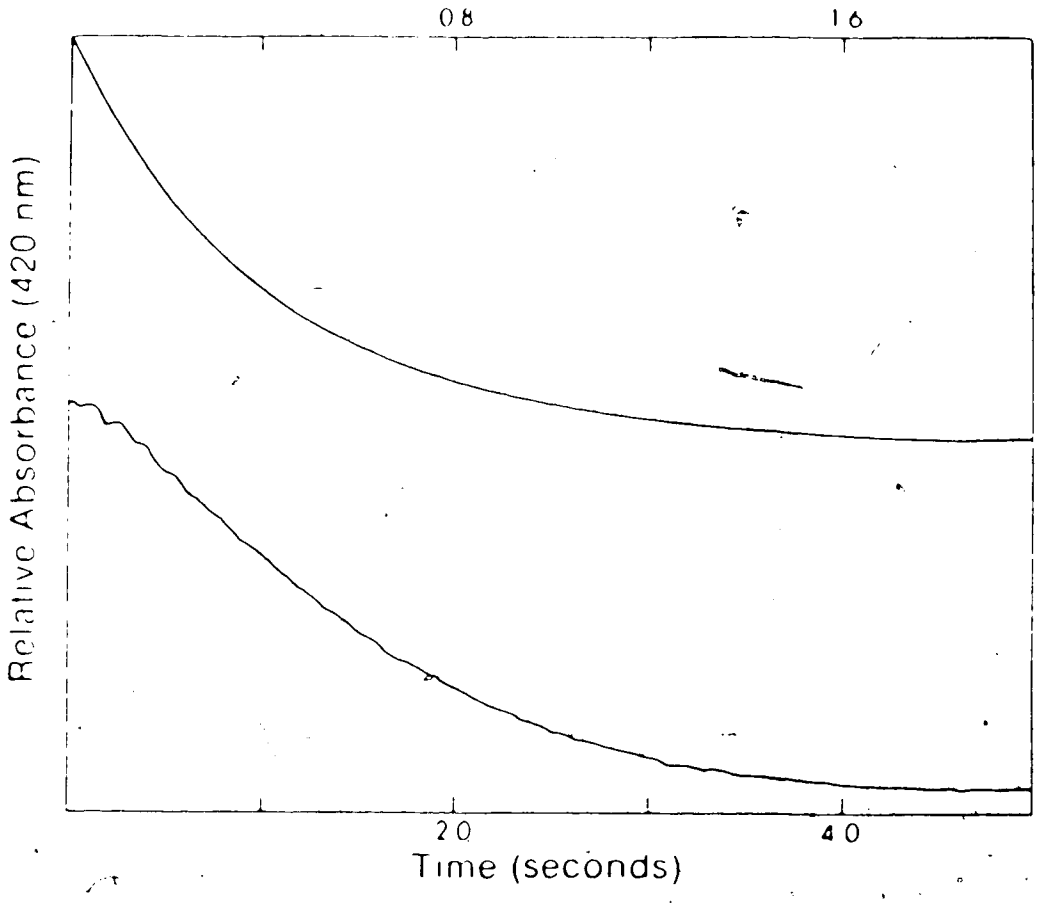
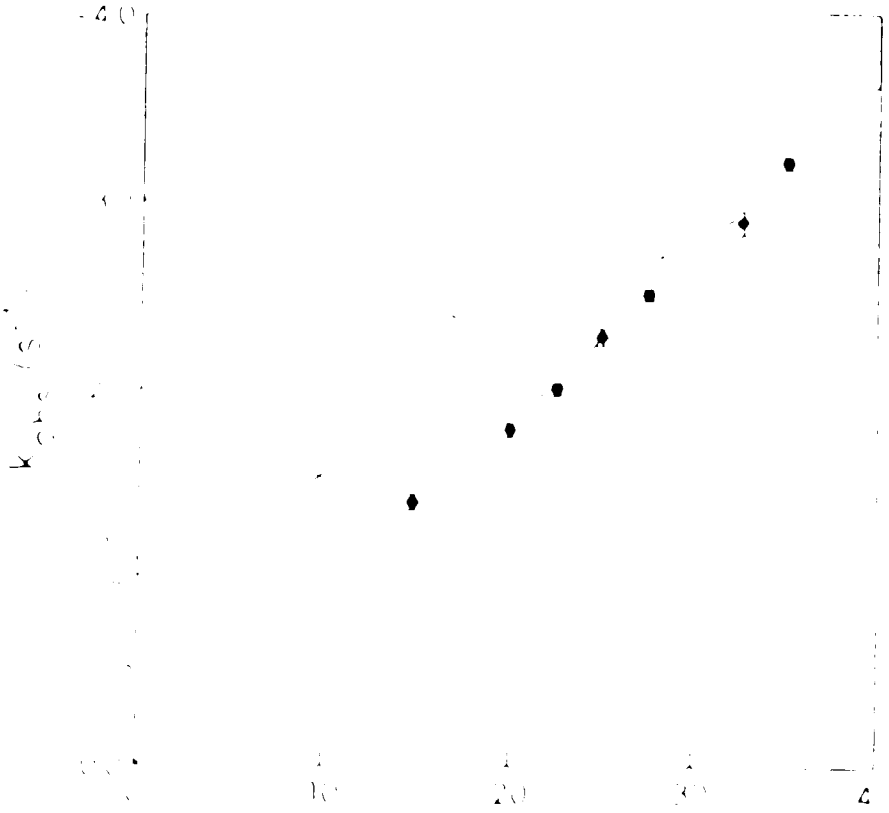


Figure II.2. Reaction traces showing the pseudo-first-order disappearance of compound II. Because of first-order conditions absolute absorbances are not required to obtain accurate k_{obs} values. Top curve and abscissa: [phenol] 10.0 μM, [compound II] 1.22 μM. Bottom curve and abscissa: [m-CHO phenol] 10.0 μM, [compound II] 1.41 μM. pH 7.0, ionic strength 0.11, 25.0°C.



[S] μM

Figure II.2. Observed first order rate constant versus substrate concentration for the reaction of horseradish peroxidase compound II with p-methoxyphenol (4-hydroxyanisole). The slope yields k_{app}, the apparent second order rate constant, $(9.2 \pm 0.2) \times 10^5$ M^{-1} s^{-1}. Data obtained at pH 7.0, 25.0°C, ionic strength 0.11 M.

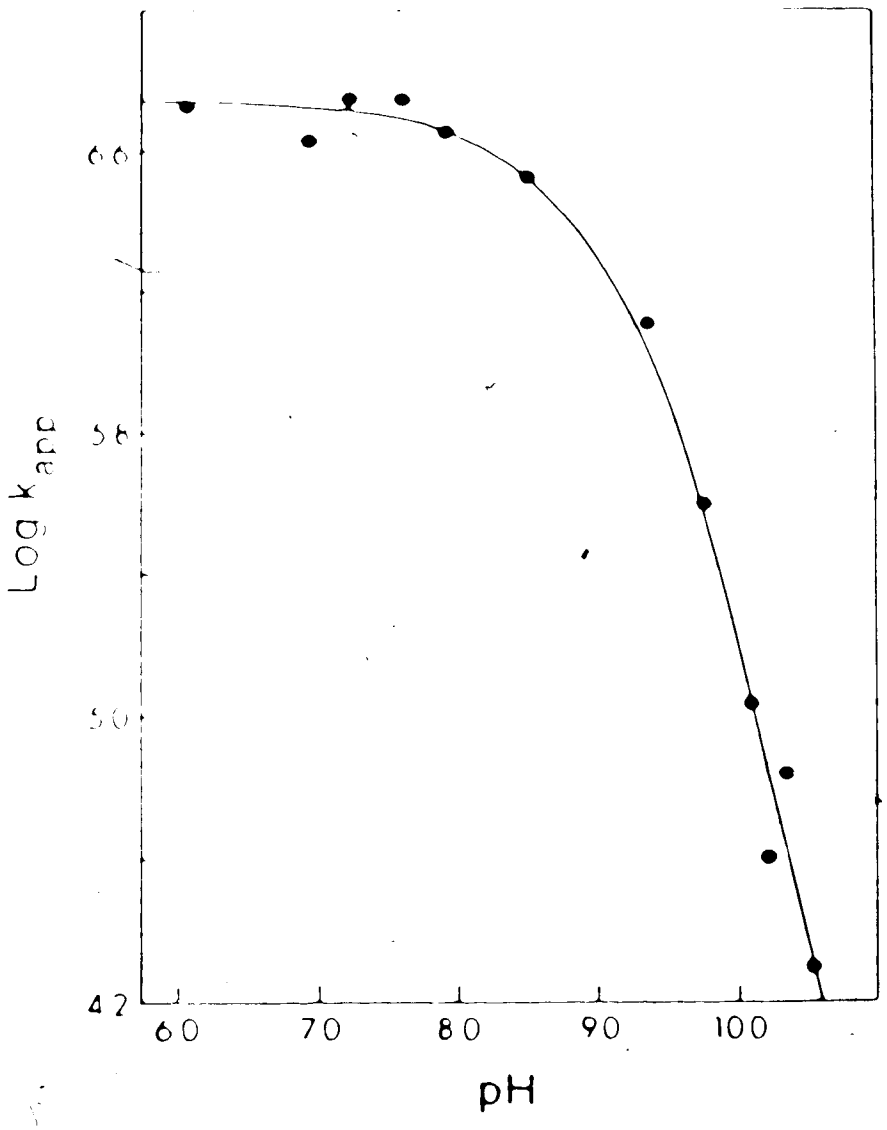
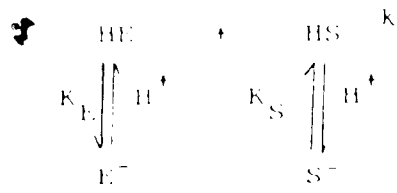


Figure II.4. pH-rate profile for the reaction of horseradish peroxidase with p-methoxyphenol (4-hydroxy-anisole) at 25.0°C and ionic strength 0.11 M.

[II.6]



where HE is a protonated form of compound II and HS is unionized p-methoxyphenol. Equation (6) leads to

[II.7]

$$k_{app} = k\left[1 + \frac{K_E}{[H^+]}\right]\left[1 + \frac{K_S}{[H^+]}\right]$$

where k_{app} is the experimentally determined pH-dependent second order rate constant and k is defined in equation [II.6]. K_E and K_S are acid dissociation constants of compound II and substrate respectively. A best-fit to the experimental data was obtained using a non-linear least squares program in which k and K_E were adjustable parameters with an independently determined value of K_S introduced as a constant. From the fit, using $K_S = 6.31 \times 10^{-11}$ (25), $k = (5.5 \pm 0.6) \times 10^6$ M^{-1} s^{-1} and $K_E = (1.9 \pm 0.3) \times 10^{-9}$ or $pK_E = 8.72$. The best fit line computed from these constants is shown in Fig. II.4.

Hammett Plot

Second order rate constants determined for a variety of meta- and/or para-substituted phenols reacting with horseradish peroxidase compound II at pH's 7.6 and 7.0 are listed in Table II.1. Also listed are Hammett σ values. The rate constants measured at two pH values are identical

TABLE II.1 Rate Constants for the Reactions of Horseradish Peroxidase Compound II with m- and p-Substituted Phenols at 25.0°C and Ionic Strength 0.11 M. Hammett σ Values.[a]

Substituents	$k_{app}(M^{-1} s^{-1})$		
	pH 7.6	pH 7.0	σ
1. m-CHO	$(3.2\pm0.4)\times10^{3}$	-	0.38
2. p-SO_3^-	-	$(1.4\pm0.1)\times10^{4}$	0.38
3. p-COO^-	$(1.9\pm0.7)\times10^{4}$	-	0.35
4. p-Cl	$(2.4\pm0.2)\times10^{4}$	-	0.23
5. m-C_2H_5O	$(5.0\pm0.3)\times10^{4}$	$(5.3\pm0.3)\times10^{4}$	0.15
6. H	$(3.0\pm0.2)\times10^{5}$	$(2.86\pm0.04)\times10^{5}$	0
7. 3,4-$(CH_3)_2$	$(4.9\pm0.2)\times10^{5}$	-	0.083
8. p-CH_3[b]	$(1.01\pm0.04)\times10^{6}$	$(1.00\pm0.04)\times10^{6}$	-0.076
9. p-CH_3O	$(5.6\pm0.3)\times10^{6}$	$(4.3\pm0.9)\times10^{6}$	-0.13
10. p-OH	$(1.25\pm0.06)\times10^{7}$	-	-0.36
11. p-NH_2	$(3.20\pm0.04)\times10^{7}$	-	-0.66

[a] For refs see (12).

[b] σ-Values taken from ref (26).

within experimental error, so that all measurements have been made in a pH-independent region, as exemplified in detail for p-methoxyphenol (Fig. II.4) and in an earlier study on p-cresol (26). A mean rate constant, k_H, of 2.93×10^5 M^{-1} s^{-1} for unsubstituted phenol reacting with compound II (Table II.1) is used as the reference for the Hammett plot of $\log(k_X/k_H)$ vs. σ shown in Fig. II.5 (k_X is the rate constant for the substituted phenol). From the slope of the plot a ρ value of -4.6 ± 0.5 is obtained, compared to the value of -6.9 measured for compound I reactions with phenols (12). Since p-nitrophenol is unreactive it is not listed in Table II.1. The value for p-aminophenol is not included in Fig. II.5 for reasons discussed below.

Discussion

pH Dependence

The pH-rate profile for the reaction of compound II of horseradish peroxidase with p-methoxyphenol is similar to that observed earlier in a kinetic study of the reaction of compound II with p-cresol (26). The value of 8.7 for the pK_a of compound II obtained from the p-methoxyphenol reaction is close to the value of 8.6 obtained from kinetic studies on a wide variety of substrates (6) and of 8.5 obtained from spectrophotometric and proton balance studies

(8).

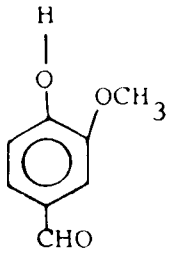
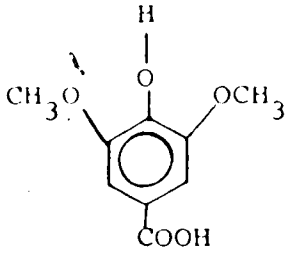
With increasing pH the plot of the log k_{app} vs pH has a slope which changes from zero to -1 to -2, as compound II and substrate each lose a proton (Fig. II.4). The data are entirely consistent with a protonated form of compound II reacting with the unionized p-methoxyphenol. The phenoxide ion appears to be unreactive.

Substituent Effects

(a) Steric Factors

A comparison of rates for the reactions of compound II with the following phenols:

[II.8]

	4-hydroxyanisole	vanillin	syringic acid
k_{app} ($M^{-1}s^{-1}$)	5×10^6	1.3×10^6 (27)	6.8×10^2 (27)
			

indicates little if any steric factor for a single o-substituent. However the steric factor is appreciable for syringic acid (where the comparison should be to p-hydroxybenzoic acid).

(b) Electronic Factors, the Hammett Correlation

The Hammett plot in Fig. II.5 shows a reasonable correlation of rate with σ values. Thus the electron-donating or electron-withdrawing effect of m- and p-substituents is dominant in determining the reactivity of the compounds listed in Table 1. This would appear to rule out any possible effect on K_M values. In any case these could not be determined because no saturation effects were observed (equation II.5). Electron-donating groups (negative σ) enhance the rate and the converse is also true. As in the case of phenols reacting with compound I, a consistent picture emerges of the unionized phenol donating an electron to the oxidized enzyme compound, accompanied by simultaneous loss of a proton. Evidence for the latter is obtained by lack of correlation with the Brown-Okamoto equation (plot not shown) for the compound II results (28); also by use of the diffusion-controlled limit for rapidly reacting phenols (29). Hydrogen bonding of the phenol to a basic group of the enzyme may facilitate the reaction (29); otherwise one would expect the phenolate anion to be more reactive.

The rate constant for p-aminophenol does not fit the plot shown in Fig. II.5. It deviates to the left of the plot as occurred with its reaction rate with compound I. In the latter case the possibility of partial diffusion control was discussed (12). However the reaction with compound II

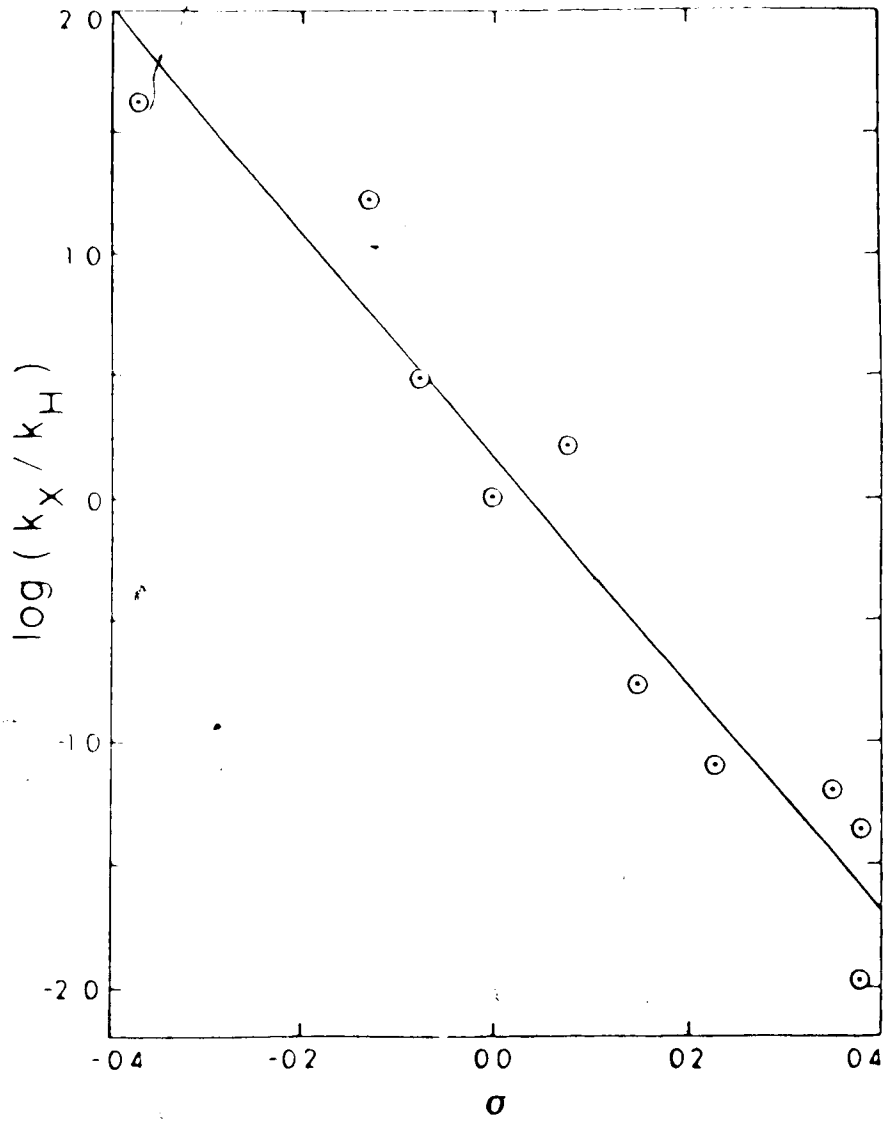


Figure II.5. Hammett plot for reactions of horseradish peroxidase compound II with m- and p-substituted phenols. k_X is rate constant for the substituted phenol, k_H for phenol.

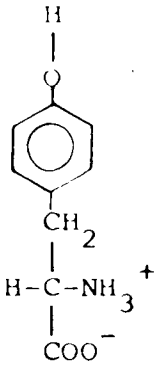
is considerably slower. The simplest explanation appears to be that p-aminophenol reacts as a substituted aniline rather than a substituted phenol, despite the lower reactivity of anilines compared to phenols (12). Perhaps the substrate has a preferred binding orientation which enhances the probability of electron donation from the $-NH_2$ substituent.

The ρ value of -4.6 obtained from the reactions of phenols with compound II indicates the strong enhancement of rate by electron-donating substituents on the substrate. However it is two-thirds of the value of -6.9 obtained from compound I reactions with phenols (12). The difference in reactivity of compounds I and II cannot be accounted for by redox potentials. These are very similar for the two compounds in the region of pH 7 (30). However an explanation might be found in knowledge of the structures of compounds I and II. Compound I is a porphyrin π-cation radical (31,32). Thus it is a highly aromatic electron-deficient species which provides an attractive target for the electron-donating phenol. The one-electron reduction of compound I to compound II also involves proton addition to the protein moiety (8,33). Whether this proton could be derived directly from a phenol in a simple Brønsted acid-base reaction is an attractive bit of speculation. When compound II is reduced to the native enzyme, the ferryl oxygen atom is converted to water which is lost from the

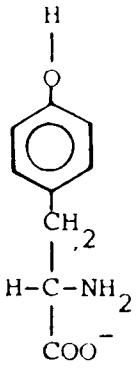
inner coordination sphere of the iron (34). This process involves bond-breaking and bond-forming reactions, so that factors other than the electron-donating ability of the substrate are also important. Thus compound I reduction is a simpler chemical process than compound II reduction. This makes compound I more sensitive to substrate reactivity, hence a higher negative ρ value for compound I substrates.

Earlier results obtained on tyrosine oxidation by compounds I and II are more difficult to compare because of the more complicated nature of the reactions (35,36). There are two reactive substrate species:

[II.9]



A



B

with species B being more reactive as expected, since an electron-withdrawing proton has been lost. Pertinent rate constants for discussion of a $\rho\sigma$ correlation are listed in Table II.2. The difference in log k between species A and B is -0.92 for compound I and -0.60 for compound II. This

TABLE II.2 Rate Constants (M^{-1} s^{-1}) for Tyrosine Species[a] (A and B, defined in Equation [9]) with Compounds I and II.

	Compound I	Compound II
A	4.8×10^4	5×10^2
B	4×10^5	2×10^3

[a]Data obtained from refs. (36,37).

correlates so closely to the corresponding ρ values of -6.9 for compound I and -4.6 for compound II that it would appear to be a coincidence. See Appendix. A much more severe test is to use the rate data to interpolate from Fig. 3 of ref. (12) and to extrapolate from Fig. II.5 of the present paper to obtain absolute σ values. The average values obtained for σ are (+0.5±0.2) and (+0.3±0.2) for the p-substituents of species A and B defined in equation II.9.

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CHAPTER THREE

KINETIC STUDY OF THE IODINATION OF TYROSINE BY MOLECULAR IODINE

Introduction

As steps toward an understanding of the in vivo iodination of tyrosine within the thyroglobulin molecule, and the enzyme-catalyzed antimicrobial iodination process, many studies have been carried out on non-enzymatic iodination of phenols. The attention has been focussed particularly on identifying the active iodinating agent from all possible species present in an aqueous solution of iodine. From a study of the iodination of phenol in acetate and phosphate buffers it was concluded that the iodination step was either the attack of unionized phenol by hypoiodous acid, or the attack of ionized phenol by a positive iodine species, I^+, (1). It was suggested that acetyl hypoiodite is the iodinating agent in a study of the reaction of pentamethylbenzene in an acetic acid solution containing iodine and mercuric acetate (2). Some other workers have also suggested that the complex formed by combination of the buffer base with iodine could act as an active iodinating agent (1,3,4,5).

From an isotope effect study on the iodination of phenol it was observed that the rate of iodination of 2,4,6-trideuterophenol was about four times slower than the iodination of phenol which was not isotopically substituted (6). A mechanism was therefore proposed involving a rapid and reversible attack of molecular iodine on ionized phenol

to give an iodinated quinoid intermediate, iodocyclohexadienone. The observed isotope effect was then considered to have arisen from the deprotonation of the intermediate as the rate-determining step. A study of the iodination of a series of substituted tyrosine was interpreted to support the iodination step of the above mechanism: attack of ionized tyrosine by molecular iodine (7). In the same study it was observed that O-methyl-L-tyrosine and aqueous iodine do not undergo reaction. This was interpreted to support the hypothesis that the OH group of substituted tyrosine was ionized: i.e., it was the anion that was reactive with I_2 (7).

Most of these earlier studies were carried out within small pH ranges and none was carried out in the absence of initially added iodide ions. Also, none of the steady state kinetic studies, involving initial rate method, have been done with a rapid reaction technique such as stopped-flow spectrophotometry because the instrument does not give absolute absorbance changes. Most of such earlier steady state, initial rate studies have therefore been carried out at low, and neutral pH's, but not at high pH where reaction could be very fast. This difficulty has been overcome in this study by calibrating the oscilloscope traces obtained from the stopped-flow spectrophotometer for each kinetic run. This method has the advantage, over transient kinetic

techniques, of avoiding complications that might arise from side reactions of intermediates involved. The controversy about the nature of the iodinating species and the phenolic species involved at the iodination step remains essentially unresolved. In the present studies, the rate of iodination of tyrosine has been investigated in the presence of initially added potassium iodide over the pH range 4.5 to 10.8; and in the absence of initially added iodide ions over the pH range 3.5 to 7.8. The nature of acid-base catalysis has also been investigated in acetate buffer over the pH 4.4 to 5.2, and in carbonate buffer over the pH 9.3 to 9.9.

Definition of Terms

HTOH: L-Tyrosine in the unionized form; the first H is in the ortho position; it is replaced by I upon iodination.

HTO^-: Ionized form of L-tyrosine.

Materials and Methods

Crystalline iodine of reagent grade was obtained from Al-Don Chemicals, Inc. L-Tyrosine was obtained from Eastman Kodak Co., and potassium iodide of analytical grade was obtained from BDH Chemicals. All reagents were used without further purification.

Stock solutions of iodine were prepared by stirring a suspension of 0.2 g crystalline iodine in 50 mL of purified

distilled water made to pH 4 by addition of sulphuric acid. The flask was wrapped with aluminum foil to prevent photochemical oxidation, and the suspension was stirred for 10 to 12 hours. The saturated solution of iodine was then filtered through glass wool and stored in a brown bottle. Concentrations of resultant stock solutions, measured at 460 nm where absorptivity of iodine is 7.46×10^2 M^{-1} cm^{-1} (9), were between 1.1 mM to 1.3 mM. The concentration was checked each day and found to be constant to 5% within three days, after which the solution was discarded. Stock solutions of tyrosine, 20 mM, were prepared by dissolving calculated amount of L-tyrosine in 1 mL of 1 M NaOH and then making up to 50 mL with purified distilled water. The solution was then stored in a brown bottle. A fresh stock solution was prepared for kinetic runs at each pH. Solutions of iodine, and tyrosine for each kinetic run were made up in buffers containing potassium iodide. The presence of iodide ions serves to suppress the formation of IO_3^- and it also provides the advantage of monitoring the reaction by the absorbance change of I_3^- at 353 nm. The initial concentrations of iodine and tyrosine were 1.5 to 20 μM, and 10.0 to 800 μM respectively.

All kinetic measurements were carried out with a Union Giken stopped-flow spectrophotometer Model RA601. The reaction of molecular iodine with L-tyrosine in the presence

of initially added iodide ions was followed spectrophotometrically at 353 nm, the wavelength of maximum absorbance of the complex I_3^- formed between iodine and iodide ions. A molar absorptivity of 2.55×10^4 (M^{-1} cm^{-1}) for I_3^- was used (8). For single experiments at each pH value the buffer concentration was kept at 0.010 M to 0.015 M. However, at pH's at which the acid-base catalysis was extensively studied, the buffer concentrations were varied from 0.010 M to 0.100 M. For all reactions in the presence of initially added potassium iodide, the ionic strength was made up to 0.40 M using potassium sulphate in the low pH range, and potassium nitrate at the higher pH.

The relative absorbance changes determined from oscilloscope traces obtained from the stopped-flow experiments were calibrated by using two or more solutions of iodine in potassium iodide for which the absorbances were previously measured on a Cary 219 spectrophotometer.

Absence of initially added iodide ions.

For the study of the reaction of molecular iodine with tyrosine in the absence of initially added iodide ions the consumption of iodine was followed at 460 nm using a molar absorptivity of 7.46×10^2 (M^{-1} cm^{-1}) for molecular iodine (9). The initial concentrations of molecular iodine were 0.20 mM at the low pH's and 0.3 mM at higher pH's, while the

tyrosine concentration was varied from 5.0 μM to 40.0 μM at each pH. The total ionic strength was maintained at 0.10 M again using potassium sulfate or potassium nitrate as described above.

Results

A set of typical oscilloscope traces from which rates of reaction are determined is shown in Fig. III.1. Fig. III.2 shows a typical plot of initial rate of reaction ($M\ s^{-1}$) against initial concentration of tyrosine. A similar plot of rate against initial concentration of molecular iodine is also linear (result not shown). Thus the reaction is overall second order, first order with respect to both tyrosine and molecular iodine.

Presence of potassium iodide.

The apparent second order rate constants ($M^{-1}\ s^{-1}$) at different pH's in the presence of added potassium iodide are shown in Table III.1. The effect of buffer concentration at constant pH, and (acid)/(base) ratio of 1, was studied for five different buffers (Fig. III.3a to Fig. III.3e). For acetate, phosphate and carbonate buffers at pH's 4.73, 6.81 and 9.85 respectively, the plots of k_{app} against buffer concentration are linear with positive slopes and, with the exception of phosphate, they gave finite intercepts; a zero

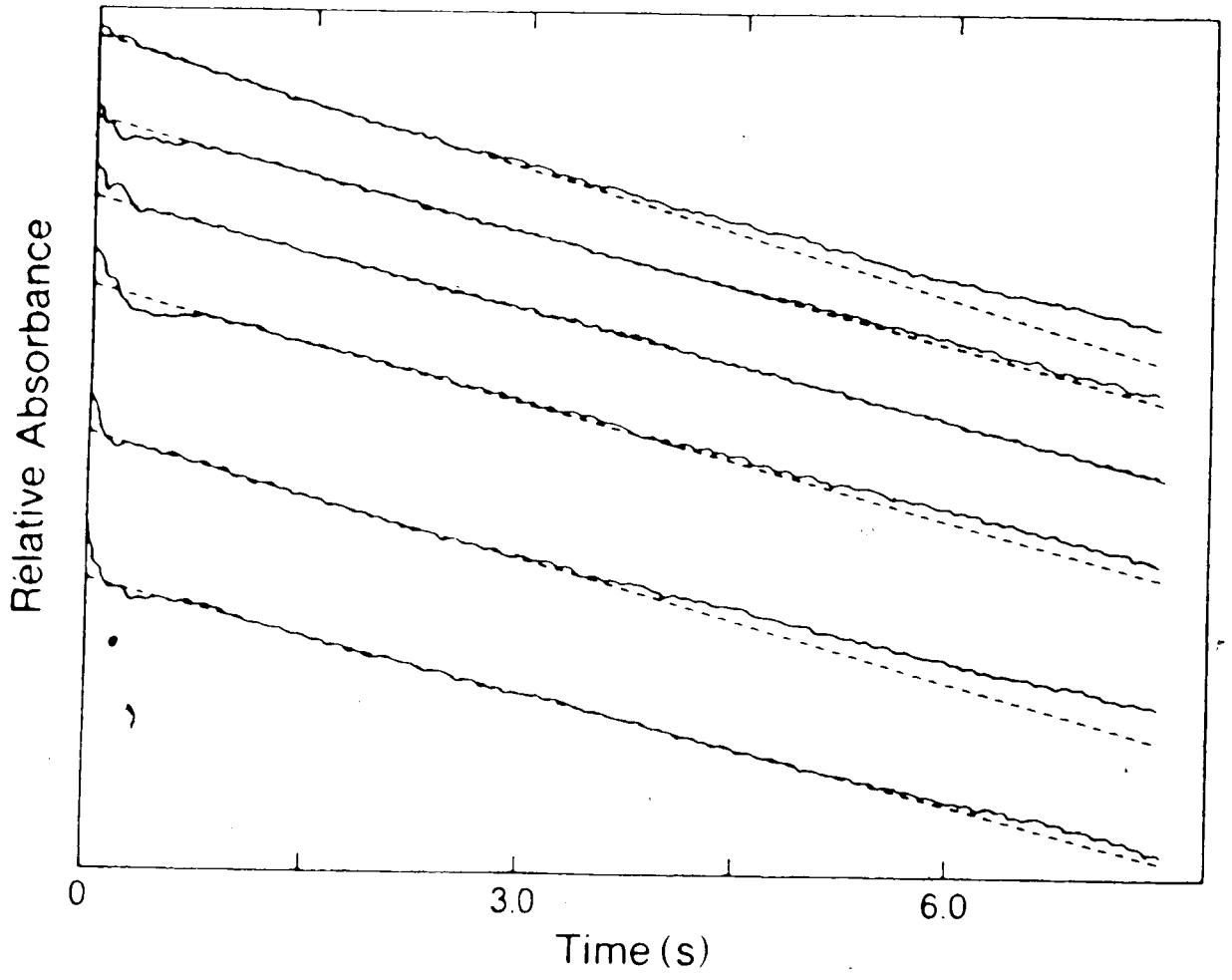


Figure III.1 Typical oscilloscope traces of initial change in absorbance with time at 353 nm; conditions of reaction were: 4.0 mM KI, 55 μM tyrosine, 3.0 μM I_2, pH 8.14, ionic strength 0.40 M, 25.0°C. V = $(6.4 \pm 0.2) \times 10^{-8}$ M s^{-1}.

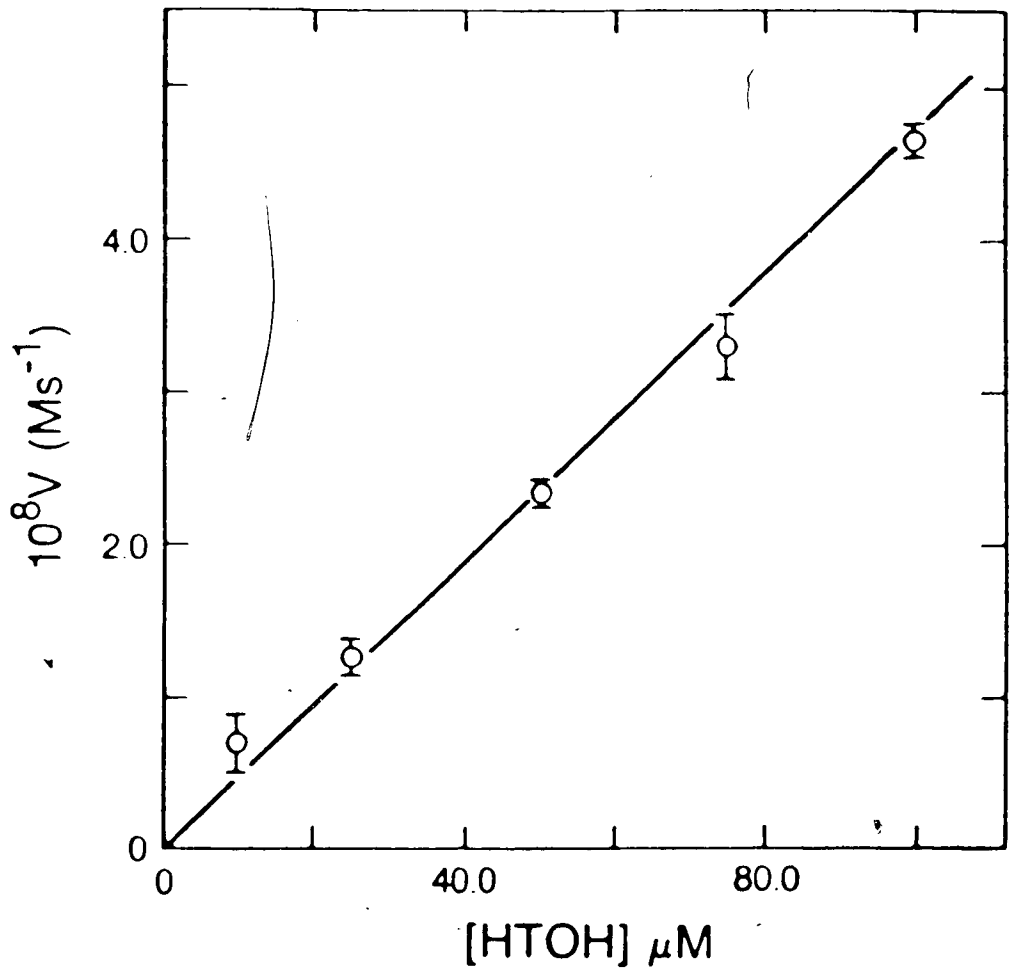


Figure III.2 A typical plot of zero-order rates of reaction against tyrosine concentration. The slope of this plot gives the pseudo-first order rate constant, k_{obs} (s^{-1}) which is related to the apparent second order rate constant by $k_{obs} = k_{app}\,[I_2]_o$, where $[I_2]_o$ is the initial concentration of molecular iodine. Conditions of reaction were: 4.0 mM KI, 3.0 μM I_2, pH 7.47, ionic strength 0.4 M, 25.0°C; $k_{obs} = (4.3 \pm 0.2) \times 10^{-4}\ s^{-1}$.

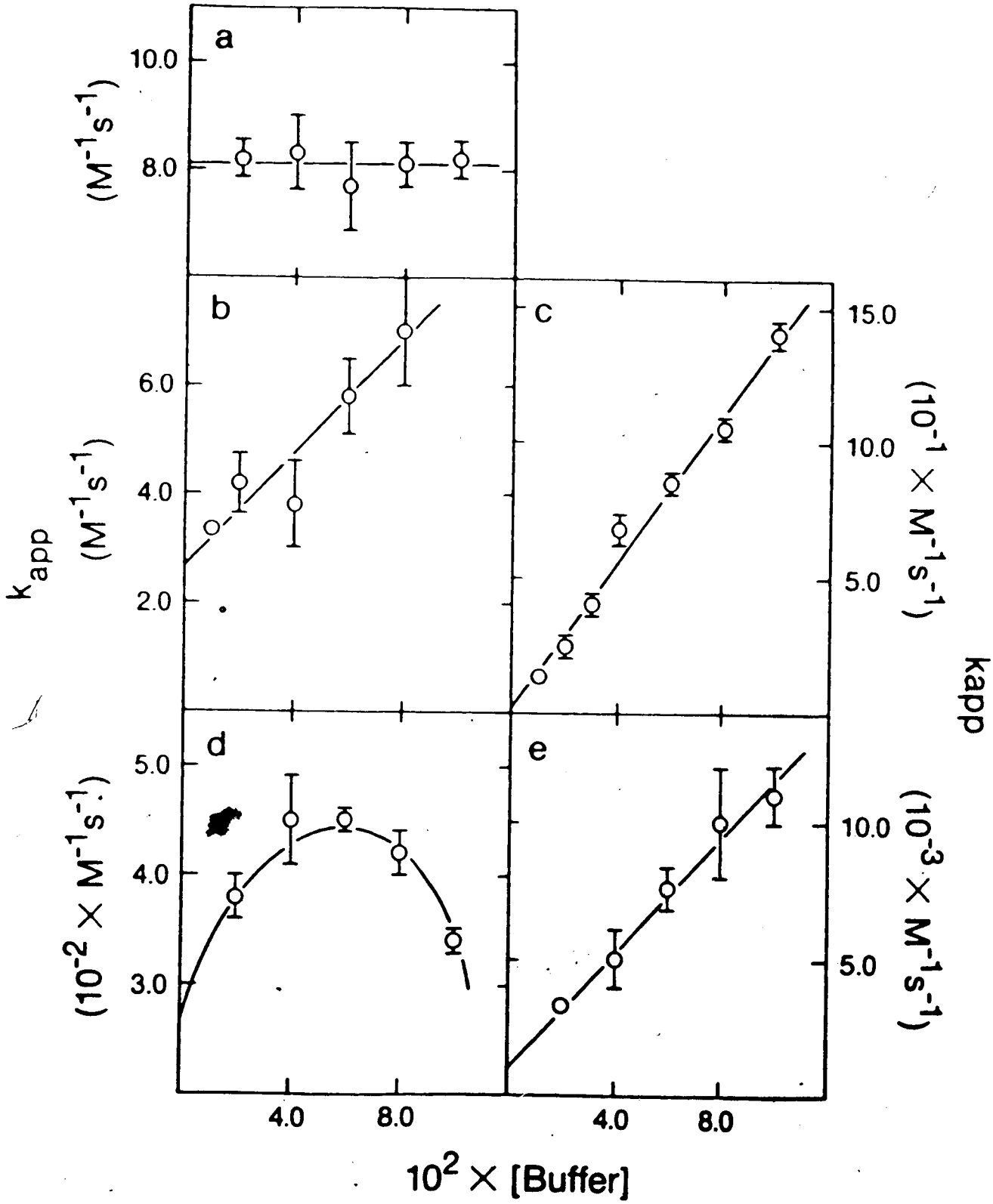

a
b
c
d
e
10.0
8.0
6.0
4.0
2.0
15.0
10.0
5.0
5.0
4.0
3.0
10.0
5.0
4.0
8.0
4.0
8.0
k_{app} $(M^{-1}s^{-1})$
$(10^{-1} \times M^{-1}s^{-1})$
$(10^{-2} \times M^{-1}s^{-1})$
$(10^{-3} \times M^{-1}s^{-1})$
kapp
$10^2 \times$ [Buffer]

Table III.1. Apparent second order rate constants, k_{app}, at different pH and iodide ion concentrations. Ionic strength was kept constant at 0.4 M.

pH	(I^-) mM	k_{app} $M^{-1}\ s^{-1}$	Buffers[a]
4.44[b]	2.00	2.1±0.3	Ac
4.53[b]	2.00	8.1±0.1	Cit
4.63[b]	2.00	2.7±0.9	Ac
4.98[b]	2.00	3.1±0.2	Ac
5.16[b]	2.00	3.6±0.2	Ac
5.38	4.00	9±2	Cit
6.19	4.00	$(2.0\pm0.3)\times10^1$	Cit
6.55	4.00	$(2.9\pm0.3)\times10^1$	Ph
6.81[b]	4.00	2±4	Ph
6.90	2.00	$(8.0\pm0.1)\times10^1$	Ph
7.24	4.00	$(3.7\pm0.6)\times10^1$	Ph
7.47	4.00	$(1.43\pm0.07)\times10^2$	Ph
7.72	4.00	$(2.0\pm0.2)\times10^2$	Ph
8.14	4.00	$(4.7\pm0.3)\times10^2$	Tris
8.36[b]	4.00	2.7×10^2	Tris
9.01	10.0	$(5.7\pm0.2)\times10^2$	Ca
9.26[b]	20.0	$(2\pm1)\times10^2$	Ca
9.58	4.0	$(3.3\pm0.3)\times10^3$	Ca
9.76[b]	20.0	$(5\pm3)\times10^2$	Ca
9.78	50.0	$(8.5\pm0.8)\times10^1$	Ca
9.85[b]	20.0	$(1.1\pm0.3)\times10^3$	Ca
10.06	100.0	$(5.0\pm0.6)\times10^1$	Ca
10.79	20.0	$(1.0\pm0.2)\times10^3$	Ca

(a) Designation of the buffers used are: Ac - Acetate; Ca - Carbonate; Cit - Citrate; Ph - Phosphate; Tris - Tris-(hydroxymethyl)methylamine.

(b) Values of k_{app} at these pH's were obtained by extrapolation to zero buffer concentrations. Generally the initial concentration of molecular iodine ranged from 2.0 μM at low pH to 10 μM at high pH.

intercept (within experimental error) was obtained in phosphate buffer. A similar plot for citrate buffer at pH 4.53 is linear with a zero slope, giving a constant value of $8.1\pm0.1\ M^{-1}\ s^{-1}$ for k_{app}. For tris buffer (tris-(hydroxymethyl)methylamine) at pH 8.36 a maximum is obtained at 0.055 M buffer concentration and extrapolation to zero buffer concentration gives a value of $2.7\times10^{2}\ M^{-1}\ s^{-1}$. The values of k_{app} at pH 4.53, 4.73, 6.81, 8.36 and 9.85 listed in Table III.1 and six others so indicated in the table, were obtained by extrapolation to zero buffer concentration. All other values in the table were determined at 0.01 M or 0.015 M buffer concentration.

Acid-base catalysis was studied more extensively at low pH's in acetate buffer and at high pH's in carbonate buffer. The variations of rate with buffer concentration are shown in Fig. III.4 and Fig. III.6. A plot of slopes at different pH's taken from Fig. III.4 against the $(CH_3COOH)/(CH_3COO^-)$ ratio gives values of $(4.4\pm0.8)\times10^{1}\ M^{-2}\ s^{-1}$ for k_{HB} and $(3\pm4)\ M^{-2}\ s^{-1}$ for k_B (Fig. III.5) (see Appendix III.B for derivation). For lower concentrations of carbonate buffer a similar plot against the $(CO_3^{=})/(HCO_3^{-})$ ratio gives values of $(7.2\pm0.8)\times10^{4}\ M^{-2}\ s^{-1}$ for k_B and $(-5\pm4)\ M^{-2}\ s^{-1}$ for K_{HB} (Fig. III.7). A complication appears for the highest ratio of $(CO_3^{=})/(HCO_3^{-})$ as shown by the curvature in the plot shown in Fig. III.7. We have no

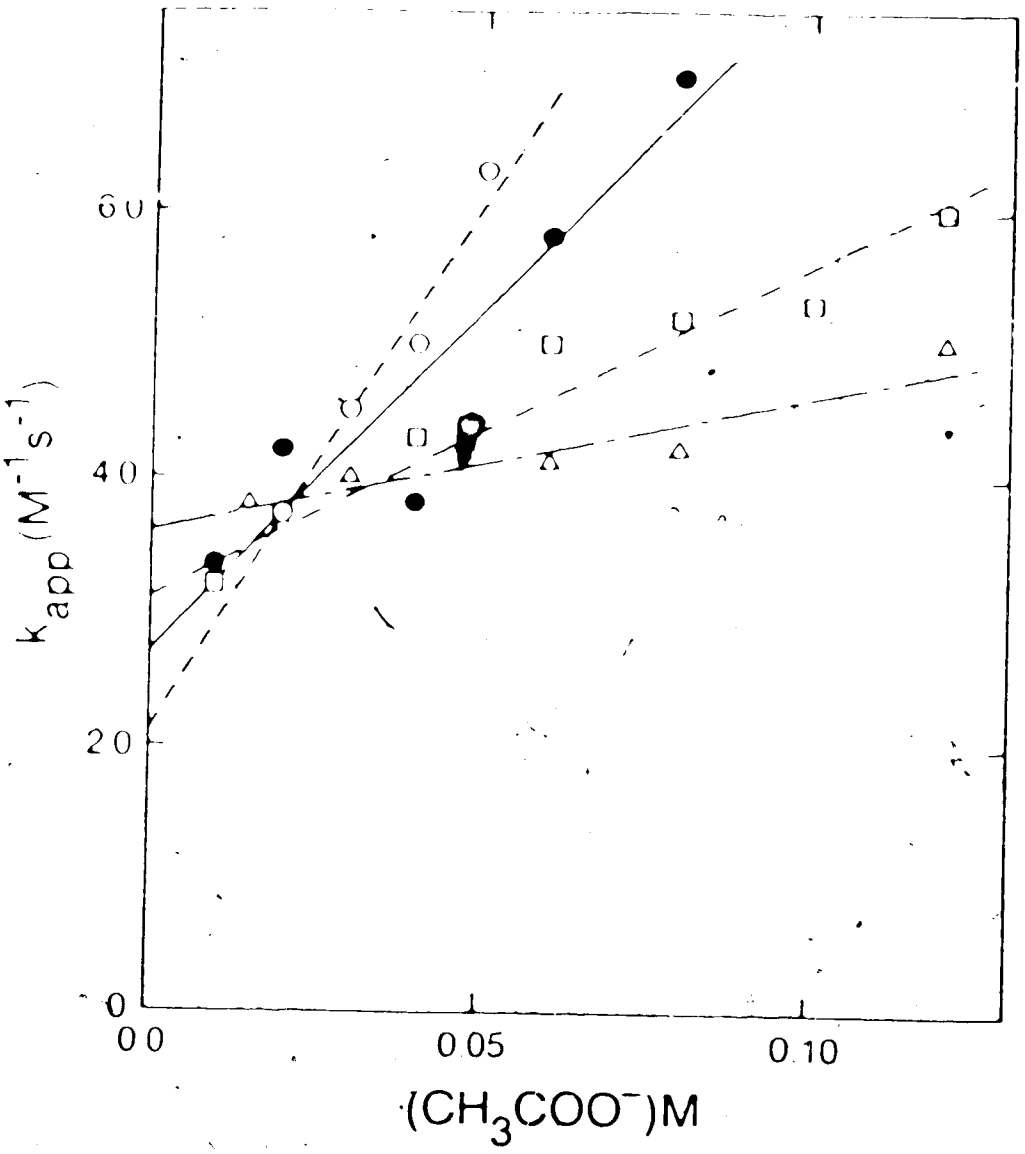


Figure III.4 Variation of rate constant, k_{app}, with buffer base concentration, acetate buffer. -O-O- pH 4.44; -●-● pH 4.63; -□-□- pH 4.98; -△-△- pH 5.16.

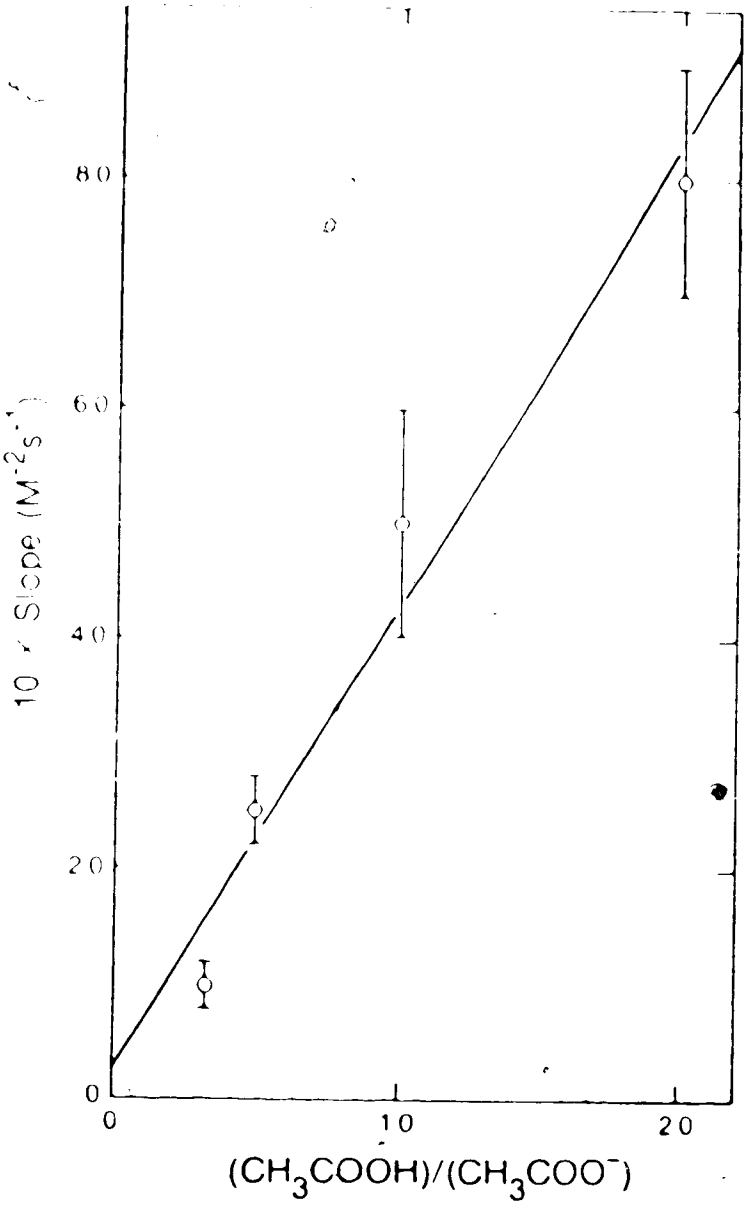


Figure III.5 Plot of slopes from Fig. III.4 against $(CH_3COOH)/(CH_3COO^-)$ ratio. The slope gives $k_{CH_3COOH} = (4.4 \pm 0.8) \times 10^1$ M^{-2} s^{-1} and the intercept gives $k_{CH_3COO^-} = 0$ within experimental error.

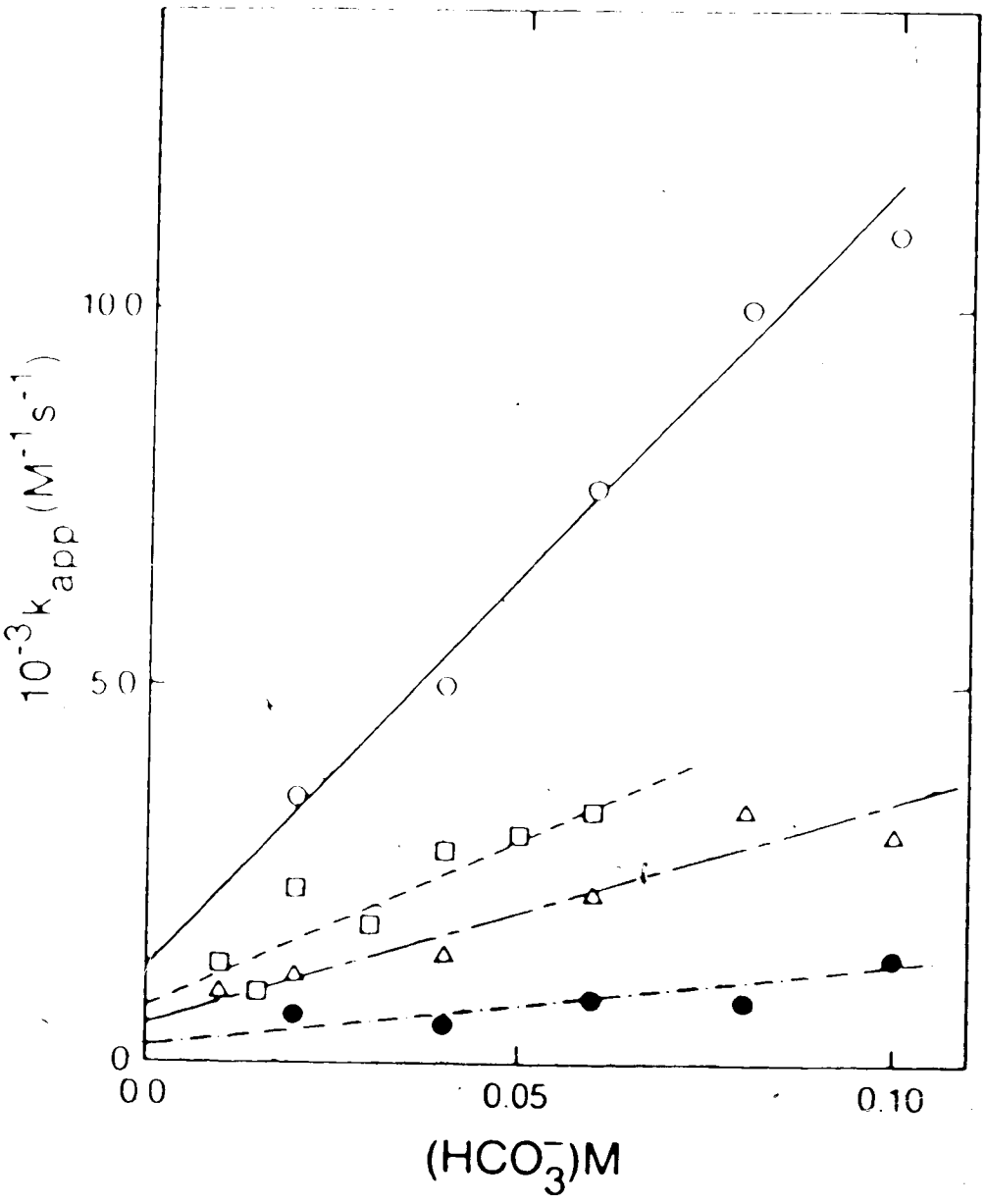


Figure III.6 Variation of rate constant, k_{app}, with buffer acid concentration; carbonate buffer. -O-O- pH 9.86; -□-□- pH 9.82; -△-△- pH 9.76; -●-●- pH 9.26.

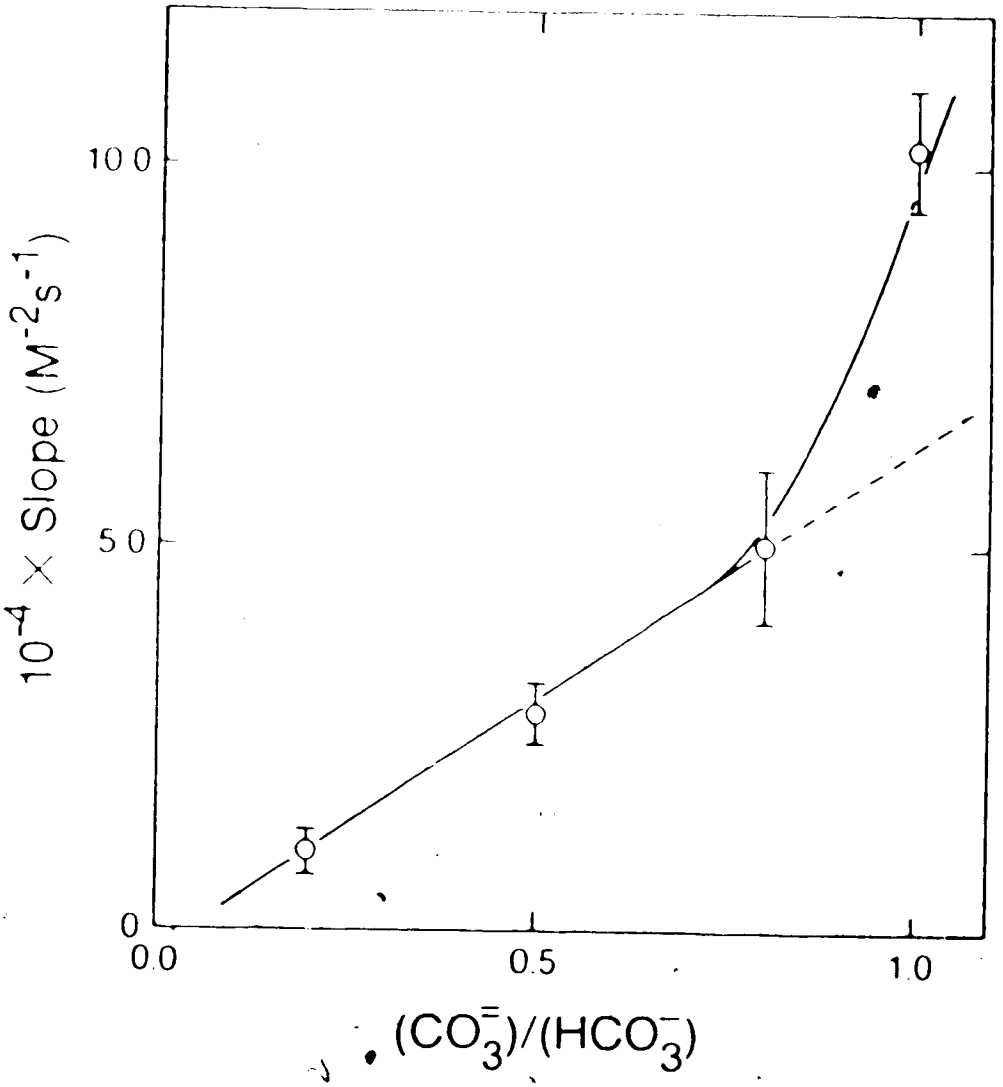


Figure III.7 Plot of slopes from Fig. III.6 against $(CO_3^=)/(HCO_3^-)$ ratio. The slope gives $k_{CO_3^=}$ = $(7.2\pm0.8)\times10^4$ M^{-2} s^{-1} and the intercept gives $(-5\pm4)\times10^3$ M^{-2} s^{-1}, which is essentially zero within experimental error.

explanation for this effect, but we are confident it is real because the results were duplicated. Thus it appears that at low pH's a general acid catalysis is operative while at high pH a general base catalysis operates.

Absence of initially added potassium iodide.

The reaction is also first order with respect to both tyrosine and molecular iodine in the absence of initially added iodide ions. The apparent second order rate constants, k_{app}, in the absence of added potassium iodide at different pH's are shown in Table III.2. The formation of IO_3^- which is known to be inert, reduces the concentration of molecular iodine, and the effect increases with pH. k'_{app} is the value of the rate constant after correction for the conversion of I_2 to IO_3^-. The correction is negligible up to pH 5 but becomes quite large beyond pH 6. Above pH 7.8 the iodine color (from an initial I_2 concentration of 300 μM) was completely bleached in the absence of initially added iodide ions and so no reaction was observable.

Discussion

Acid-Base Catalysis:

Results of the present studies indicate that the iodination of tyrosine by molecular iodine in aqueous solution involves general acid catalysis at low pH, and

Table III.2. Apparent second order rate constants, k_{app}, for the reaction of molecular iodine with tyrosine in the absence of added iodide ions; $(I_2)_o$ = 0.2 mM.

pH	k_{app} $M^{-1}\ s^{-1}$	k'_{app}[b] $M^{-1}\ s^{-1}$	Buffers[c]
3.55	$(8.5\pm0.5)\times10^{1}$	$(8.5\pm0.5)\times10^{1}$	Cit
3.76	$(5.0\pm0.5)\times10^{1}$	$(5.0\pm0.5)\times10^{1}$	Cit
3.85	$(7\pm2)\times10^{1}$	$(7\pm2)\times10^{1}$	Cit
4.59	$(1.3\pm0.1)\times10^{2}$	$(1.3\pm0.1)\times10^{2}$	Ac
4.75	$(4.6\pm0.4)\times10^{2}$	$(4.8\pm0.4)\times10^{2}$	Ac
5.15	$(7\pm2)\times10^{2}$	$(8\pm2)\times10^{2}$	Ac
5.44	$(1.02\pm0.05)\times10^{3}$	$(1.24\pm0.06)\times10^{3}$	Ac
5.61	$(3.1\pm0.4)\times10^{2}$	$(4.1\pm0.5)\times10^{2}$	Ac
5.94	$(1.2\pm0.2)\times10^{3}$	$(2.2\pm0.4)\times10^{3}$	Ph
6.31	$(3.0\pm0.5)\times10^{3}$	$(1.1\pm0.2)\times10^{4}$	Ph
6.56	$(9.22\pm0.03)\times10^{2}$	$(1.21\pm0.04)\times10^{4}$	Ph
6.93[a]	$(1.1\pm0.3)\times10^{3}$	$(2.8\pm0.8)\times10^{4}$	Ph
7.44	$(1.7\pm0.3)\times10^{3}$	$(6\pm1)\times10^{5}$	Ph
7.77[a]	$(1.9\pm0.3)\times10^{3}$	$(2.1\pm0.3)\times10^{6}$	Ph

(a) Initial concentration of molecular iodine was 0.3 mM at these pH's.

(b) k'_{app} are values obtained by correcting k_{app} for I_2 used up in IO_3^- formation.

(c) The notations for the buffers are as indicated in Table III.1.

Table III.3. Values of the catalytic constants[a] in acetate and carbonate buffers.

Buffer[b]	k_{HB} (M^{-2} s^{-1})	K_{B^-} (M^{-2} s^{-1})
Acetate	$(4.4 \pm 0.8) \times 10^{1}$	$(0.3 \pm 0.4) \times 10^{1}$
Carbonate	$(-0.5 \pm 0.4) \times 10^{1}$	$(7.2 \pm 0.8) \times 10^{4}$

(a) k_{HB} is the general acid catalytic constant; k_B, the general base catalytic constant. It should be noted that k_{HB} for carbonate buffer and k_B for acetate buffer are essentially zero within experimental errors.

(b) The pH range for acetate buffers was 4.44 to 5.15, and that for carbonate buffers was 9.26 to 9.86; (I^-) was 2.0 mM in acetate and 20 mM in carbonate buffer; μ was 0.40 M in both buffers.

general base catalysis at high pH. This is at variance with the conclusion from studies of the iodination of aniline at pH 5.5 to 7.59 and the iodination of phenol at pH 5.71 to 6.61, both in phosphate buffers (11,12). The mechanism derived from isotope effect studies of the iodination of phenol was also based on an assumption that the reaction is general base catalyzed (13,14). The results of a recent study on the tritium kinetic isotope effect was similarly interpreted (15). Even then, in the studies on the iodination of aniline and phenol (11,12) both $H_2PO_4^-$ and $HPO_4^=$ were observed to participate in the catalysis; but $H_2PO_4^-$ was considered as a conjugate base of H_3PO_4 rather than as a conjugate acid of $HPO_4^=$ in arriving at the conclusion that the reaction is general base catalysed. From this observation and our present results, it appears that the change from general acid catalysis to general base catalysis occurs at about pH 6 to 7 where both acid and base component of the buffer are participating in the catalysis. Our results at the low pH are in consonance with an earlier study on the iodination of phenol at pH 4.66 to 5.26 in acetate buffer (1).

Generally, for keto-enol equilibria the keto- form is thermodynamically favored (16). Therefore, if accumulation of the iodinated quinoid intermediate is to be prevented, either (i) a strong base is required to deprotonate the

carbon to which iodine is attached, or (ii) a very active proton donor is required to protonate the oxygen of the quinoid structure. The latter appears to occur at low pH where the buffer base is very weak and the hydroxyl concentration is very low. At high pH the buffer base is strong enough to abstract a proton from the α-carbon to which iodine is attached, and the strong base, ^{-}OH, is in sufficient concentration to participate in the catalysis.

The behavior in tris buffer is unique, with the plot of k_{app} vs. buffer concentration showing a maximum at 0.055 M buffer concentration. This appears to be due to an inhibition effect resulting from a reaction of the buffer base with molecular iodine at higher buffer concentrations. Acetate and phosphate buffers were also observed to react with iodine, but at buffer concentrations much higher than those employed in these studies.

For citrate buffer at pH 4.53 there is no effect of buffer concentration on k_{app}. A possible explanation is the ratio of buffer base to acid which is about 24.5 at this pH. Thus, the concentration of the acid form needed to protonate the oxygen of the quinoid structure, in line with general acid catalysis at low pH, is very low.

Mechanism of reaction.

As a result of the number of oxidation states available to iodine at ambient conditions the system of molecular iodine in aqueous solution is known to be very complex. However, from a study of the hydrolysis of iodine in aqueous solution, using a kinetic and equilibrium theoretical model approach based on known constants for nine different equilibria and four differential rate equations, it has been shown that within the pH range 5 to 10, the dominant species in solution are, I_2, I^- and HOI (17). Therefore, the important equilibria to consider in the mechanism of iodination of tyrosine by molecular iodine in aqueous solution are:

[1] $$I_3^- \underset{<}{\overset{K_1}{\rightleftharpoons}} I_2 + I^-$$

[2] $$I_2 + H_2O \underset{<}{\overset{K_2}{\rightleftharpoons}} I_2OH^- + H^+$$

[3] $$I_2 + OH^- \underset{<}{\overset{K_3}{\rightleftharpoons}} I_2OH^-$$

[4] $$I_2OH^- \underset{<}{\overset{K_4}{\rightleftharpoons}} HOI + I^-$$

[5] $$I_2 + H_2O \underset{<}{\overset{K_5}{\rightleftharpoons}} HOI + H^+ + I^-$$

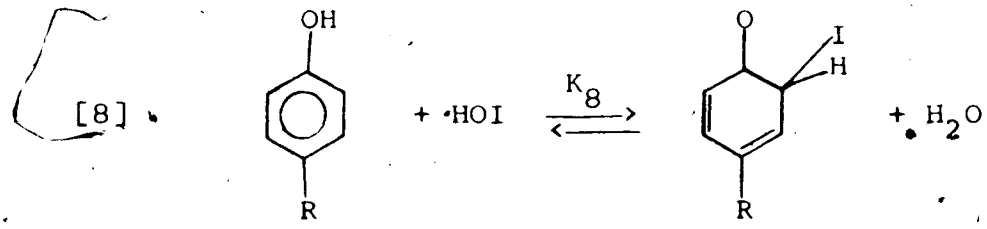
In the presence of initially added potassium iodide up to millimolar concentrations the formation of IO_3^- [6],

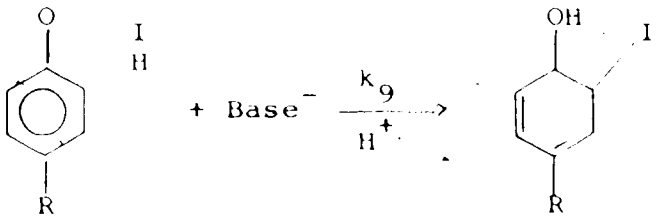
[6] $$3I_2 + 3H_2O \overset{K_6}{\rightleftharpoons} IO_3^- + 5I^- + 6H^+$$

is practically suppressed. Equation [1] is a rapid equilibrium for which $K_1 = 1.4 \times 10^{-3}$ M (9). Equations [2], [3] and [4] are steps in the proposed mechanism for the overall hydrolysis of molecular iodine in aqueous solution as shown in equation [5] (18,19). The ionization of the weak acid, tyrosine must also be considered.

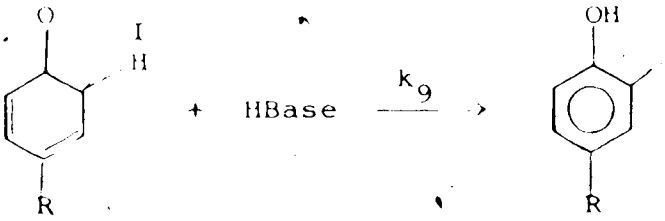
[7] $$HTOH \overset{K_7}{\rightleftharpoons} HTO^- + H^+$$

A mechanism which best fits the results of the present study is one in which the iodination step involves a reversible attack of unionized tyrosine by hypoiodous acid; the rate determining step is then the deprotonation of the iodinated quinoid intermediate.

[8] 

[9A]  + HBase

or

[9B]  + HBase

giving an apparent second order rate constant,

$$[10] \qquad k_{app} = \frac{k_9' K_1 K_5 K_8}{[K_7+(H^+)][K_1+(I^-)](I^-)}$$

where $k_9' = k_9(Base^-)$ at high pH

$k_9' = k_9(HBase)$ at low pH

i.e.,

$$[11] \qquad [K_1 + (I^-)](I^-)k_{app} = \frac{k_9' K_1 K_5 K_8}{K_7 + (H^+)}$$

A slope of 0.81±0.03 obtained for the plot of $[K_1+(I^-)](I^-)k_{app}$ against pH (Fig. III.8) is reasonably close to a value of 1 expected from the model.

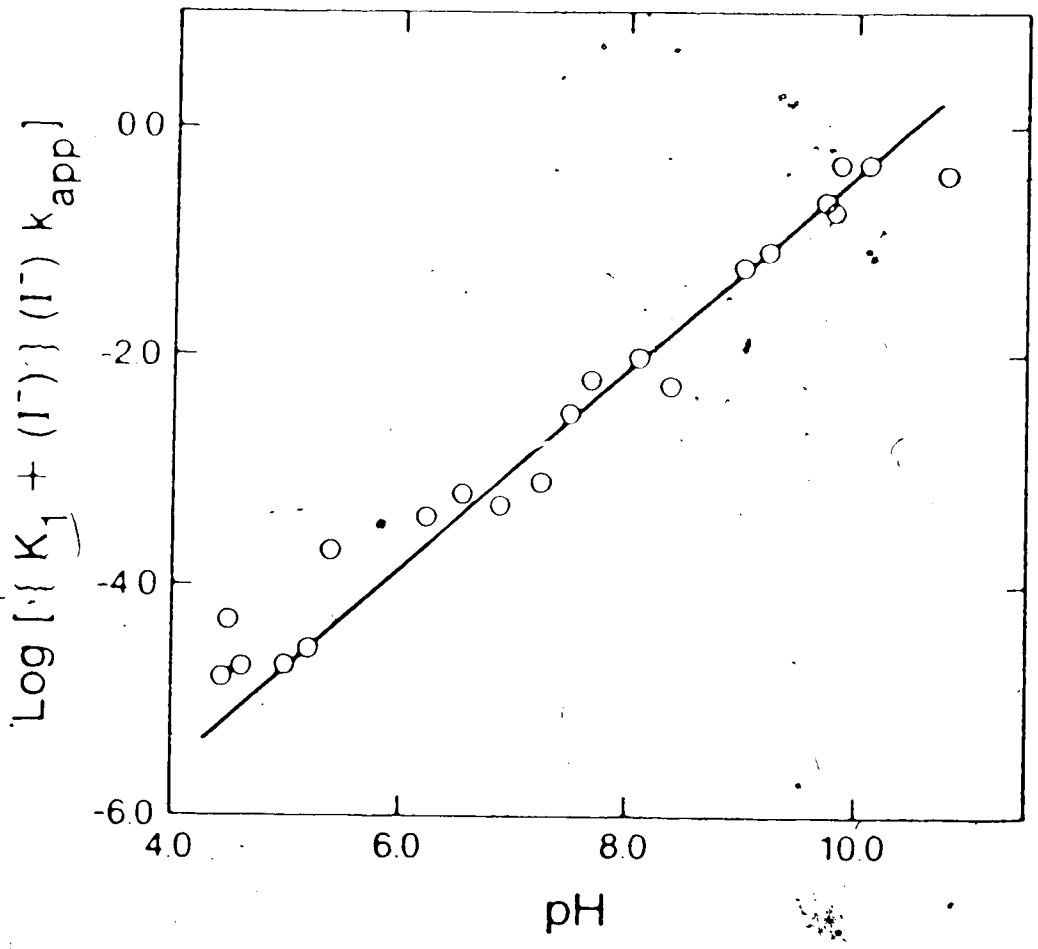
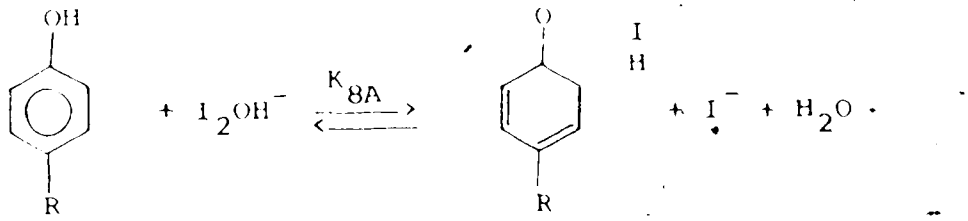
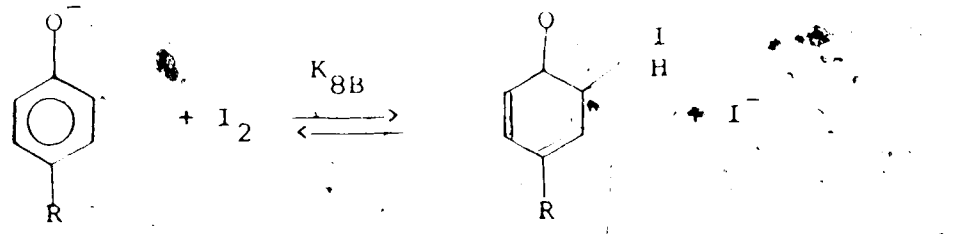


Figure III.8. Log plot of $\{K_1+(I^-)\}(I^-)k_{app}$ against pH. k_{app} is the apparent second order rate constant for the reaction of iodine with tyrosine in the presence of initially added potassium iodide.

However, two other possible mechanisms are: (i) one in which the iodination step is replaced by [8A];

[8A]  $+ I_2OH^- \rightleftharpoons$ $+ I^- + H_2O$.

and (ii) one in which the iodination step is replaced by [8B]:

[8B]  $+ I_2 \rightleftharpoons$ $+ I^-$

The aromatic ring of tyrosine exists as a nucleophile in aqueous solution which would tend to discourage reaction with the negatively charged species, I_2OH^-, as the active iodinating species. Thus, I_2OH^- could not possibly be the active iodinating species. We therefore reject the mechanism involving [8A] as the iodination step.

The other alternative mechanism involving the attack of molecular iodine on ionized tyrosine gives an apparent second order rate constant, k_{app} of the form:

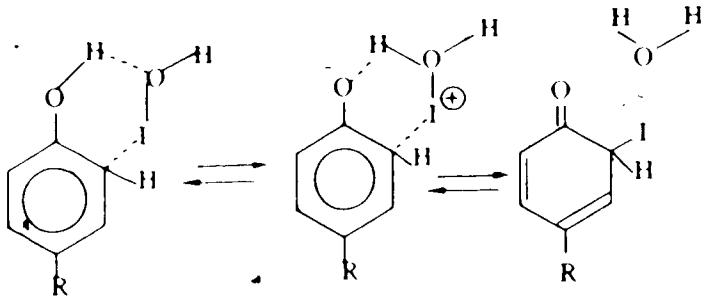
$$[12] \qquad k_{app} = \frac{k_9 K_1 K_7 K_{8B}}{[K_7+(H^+)][K_1+(I^-)](I^-)} \quad M^{-1}\ s^{-1}$$

which is similar to [10].

$$[13] \qquad [K_1+(I^-)](I^-)k_{app} = \frac{k_9 K_1 K_7 K_{8B}}{K_7+(H^+)}$$

According to equation [13], analogous to equation [11] the log plot of $[K_1+(I^-)](I^-)k_{app}$ against pH gives an intercept of -8.6±0.2: thus, for (H^+) = 1.0 M $k_9K_1K_7K_{8B} = 2.51\times10^{-9}$ M s^{-1}. Substitution for known values of $K_1 = 1.4\times10^{-3}$ M (19) and $K_7 = 8.51\times10^{-11}$ M (20) gives $k_9K_{8B} = 2.11\times10^4$ M^{-1} s^{-1}. For equation [9A] to be the rate determining step as has been established for the iodination of phenol (14), $k_{-8B}(I^-) >> k_9(Base^-)$; i.e., $(k_9/k_{-8B}) << (I^-)/Base \cong 10^{-1}$. Since $k_9k_{8B}/k_{-8B} = 2.11\times10^4$, $k_{8B} >> 2.11\times10^5$ M^{-1} s^{-1}. This value would not be impossible for a reaction between a neutral molecule and an aromatic anion. In fact, this reaction step has been considered to be the iodination step in earlier studies on the iodination of different substituted tyrosine (12), the major argument in favor of this mechanism being that O-methyl-L-tyrosine did not undergo any reaction with molecular iodine. However, from the present study an alternative explanation for non-reactivity observed with O-methyl-L-tyrosine is that the

species,



[14]

might occur along the reaction coordinate for the attack of unionized tyrosine by hypoiodous acid. Therefore when the phenolic hydrogen is replaced by a methyl group, the formation of H_2OI^+ is blocked.

The mechanism shown in equation [14] involves a concerted process in which proton transfer to HOI simultaneously creates a negatively charged tyrosinate (nucleophile) and a positively charged H_2OI^+ (electrophile) greatly enhancing the rate of the iodination process.

Absence of initially added iodide ions.

In the absence of initially added potassium iodide, the formation of IO_3^- is not suppressed [6]. This equilibrium produces iodide ions in solution but the concentration is very small compared to the magnitude of K_1 in equation [10]. Also, within the pH range 3.55 to 7.77 the hydrogen ion concentration is much higher than the value of K_7 in

equation [10]. Therefore the apparent second order rate constant, k_{app} becomes:

$$[15] \qquad k_{app} = \frac{k_9' K_5 K_8}{(H^+)(I^-)}$$

and a log plot of $(I^-)k_{app}$ against pH is expected to give a slope of 1.0. However a slope of 1.70±0.05 was obtained from the results (Fig. III.9). This is an indication that there is hydrogen ion inhibition greater than that predicted by the model. Thus, the reaction appears to be more complicated than in the presence of added potassium iodide. Table III.4 shows the log of calculated values of $(H^+)^2(I^-)k_{app}$. Two different constant values are obtained: -11.8±0.1 in the pH range 3.55 to 5.15 where the correction for IO_3^- formation is minimal, and -12.56±0.07 in the pH range 5.44 to 7.77 where the correction for IO_3^- formation is considerable. The observed discrepancies might have arisen from errors in the huge correction for iodate formation at higher pH. However an alternative explanation is possible.

It has been suggested that a decrease in iodide ion concentration causes the iodination step to become partially rate-determining (14): the rate of the reverse reaction at the iodination step becomes slower compared with the step for the deprotonation of the quinoid intermediate. That is,

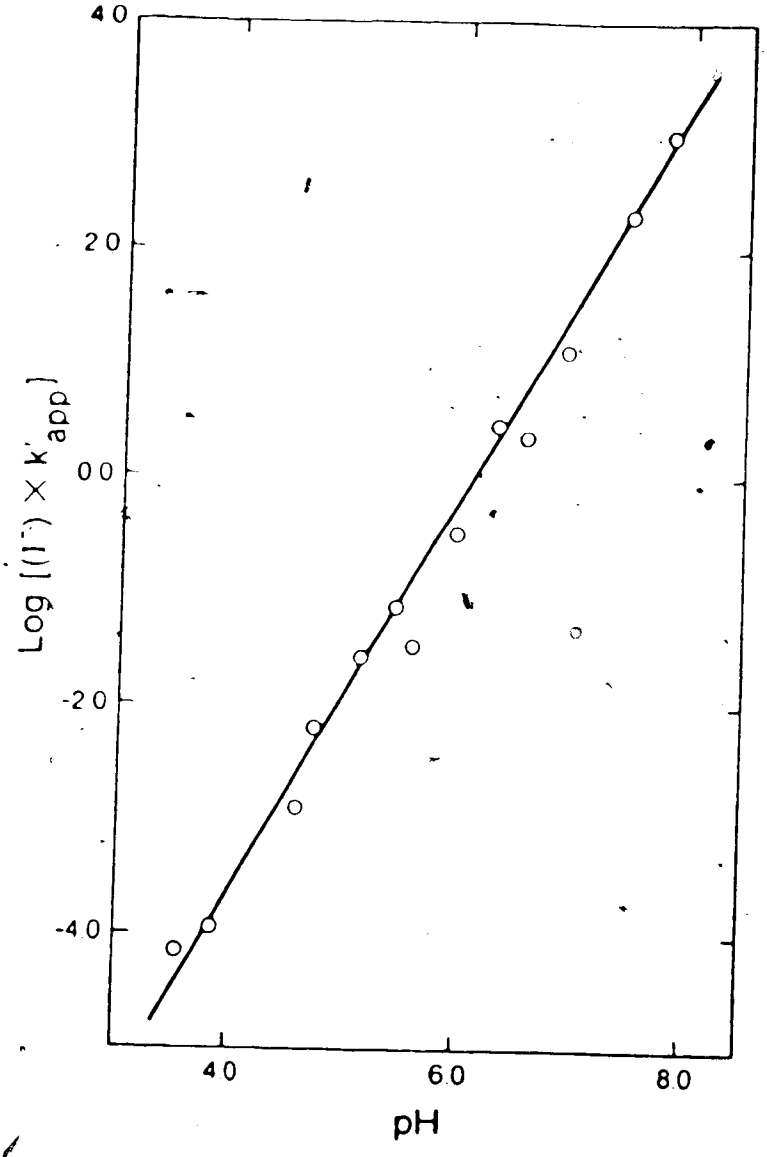


Figure III.9 Log plot of $(I^-)k'_{app}$ against pH. k'_{app} is the apparent second order rate constant for the reaction of I_2 with tyrosine in the absence of initially added iodide ions, after correction for the I_2/IO_3^- equilibrium.

Table III.4. Log of calculated values of $(H^+)^2(I^-)k'_{app}$ for the reaction of tyrosine with iodine in the absence of initially added iodide ions.

pH	[a]Log$[(H^+)^2(I^-)k'_{app}]$
3.55	-11.25
3.76	-11.69
3.85	-11.64
4.59	-12.10
4.75	-11.71
5.15	-11.92
5.44	-12.69
5.61	-12.69
5.94	-12.36
6.31	-12.18
6.56	-12.77
6.93	-12.74
7.44	-12.56
7.77	-12.51

(a) k'_{app} is the apparent second order rate constant after correction for the I_2/IO_3^- equilibrium.

from [8B], $k_{-8B}(I^-) << k_9(Base^-)$. However, even though the present results in the absence of initially added iodide ions could be explained in terms of a partial shift in the rate-determining step, it does not eliminate the possibility of hypoiodous acid being the active iodinating species. Since iodide ions are not present in sufficient concentration to suppress the I_2/IO_3^- equilibrium, the mechanism of which is known to involve hypoidous acid, the concentration of HOI becomes very low. The attack of unionized tyrosine by HOI, reaction [8], becomes very slow compared to reaction [9], and then the iodination step becomes partially rate-determining.

Conclusion

In this study it has been possible to thin down the number of possible schemes for the mechanism of iodination of tyrosine to two; viz., a scheme in which hypoiodous acid attacks unionized tyrosine (i.e., unionized with respect to the OH of the phenolic group) at the iodination step, and one in which molecular iodine reacts with the ionized form (6,7). It has been shown in a recent study that hypoiodous acid solution which is free of I_2, at least for some minutes, can be prepared by rapidly stirring a slurry of the mercuric carbide polymer, $[Hg_3CO(ClO_4)]_n$ in 1 mM $HClO_4$ solution of I_2 (19). By using a solution of HOI prepared in

this way as a starting reagent at low pH, and following the reaction with tyrosine at 278 nm, where HOI has its maximum absorptivity (19), it should be possible to distinguish between the two possible mechanisms.

Contrary to earlier contention that the iodination of tyrosine (7), and of phenols (6,10-13) generally is general base catalyzed, it has been possible in this study to show that general acid catalysis is operative in the lower pH, while reaction is general base catalyzed at high pH.

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CHAPTER FOUR

GENERAL DISCUSSION AND CONCLUSIONS

The pH dependence of the reaction of HRP-II with p-methoxyphenol has been shown to be indicative of a mechanism in which the protonated form of the enzyme and the unionized phenol are the reactive species. This conclusion is supported by the results of a Hammet $\rho\sigma$ correlation for the reaction of HRP-II with fourteen different phenols. That the apparent second order rate constants correlate well with σ, but not with σ^+, or with σ^- is indicative of a concerted process in which a substrate loses a proton and one electron simultaneously without going through an intermediate with a partial positive or negative charge (1,2).

The results of some earlier studies have also been interpreted to indicate that the undissociated substrates are the kinetically significant species in the reaction of peroxidase with ligands and oxidants (3-7).

The pH dependence study has also confirmed the presence of a heme-linked ionizable group in HRP-II with a pK_a 8.7, in agreement with conclusions from some earlier studies (8,9). These results can be explained in terms of the known structure at the heme active centre of the enzyme and its intermediates. It has been postulated that the heme-linked ionization observed from proton balance studies (9) arises from deprotonation of the distal histidine. Some investigators suggested that the ionization of the distal

histidine must be communicated to the Fe-His (proximal) bond without passing through the heme iron (10,11): it was claimed that the Raman spectrum of ferrous HRP in the 1200 to 1700 cm^{-1} region did not indicate the presence of any ligand in the sixth coordination position of the heme iron (10). A communication of the ionization via a conformational change (Fig. IV.1) was suggested. However, it has bee widely suggested that the iron of the heme in compound II of HRP exists as Fe(IV)=O (12-15) which has now been confirmed from identification of the Fe(IV)=O stretching frequency for HRP-II using resonance Raman spectroscopy. From the same technique a pK_a of 8.5 is observed which is due to hydrogen bonding to the oxygen of HRP-II Fe(IV)=O group from an amine acid group, which is suggested to be a histidine (17) (see Fig. IV.2). Thus the resonance Raman results reinforce the conclusion from kinetic studies. These results can be summarized in the mechanism shown in equation [IV.1]. Protonation of the distal base is required for compound II to react. Thus it becomes unreactive above pH 8.6. In its reactive form which exists below pH 8.6, it accepts both a proton and an electron from the reducing substrate. Iron(IV) is reduced to iron(III), the proton from substrate is added to the ferryl oxygen atom as is the proton from the distal acid group and water is the leaving group from the sixth coordination position of iron(III).

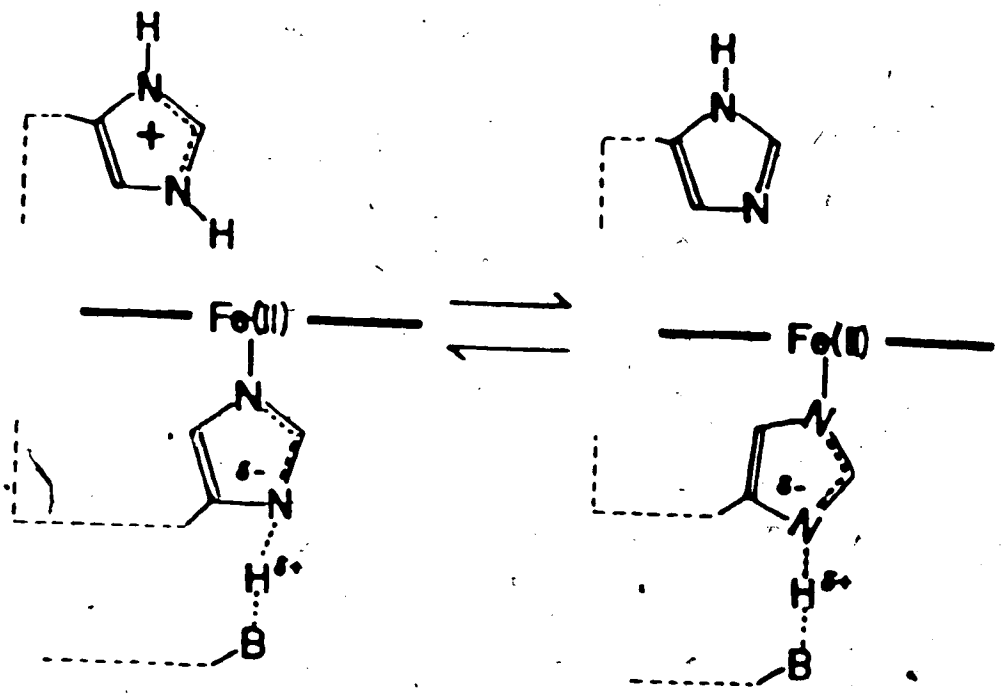


Figure IV.1 Suggested communication of heme-linked ionization via a conformational change (Ref. 11). This scheme was proposed because the authors claimed that no ligand was detectable in the sixth coordination position from their Raman spectroscopic study of ferrous HRP (Ref. 10).

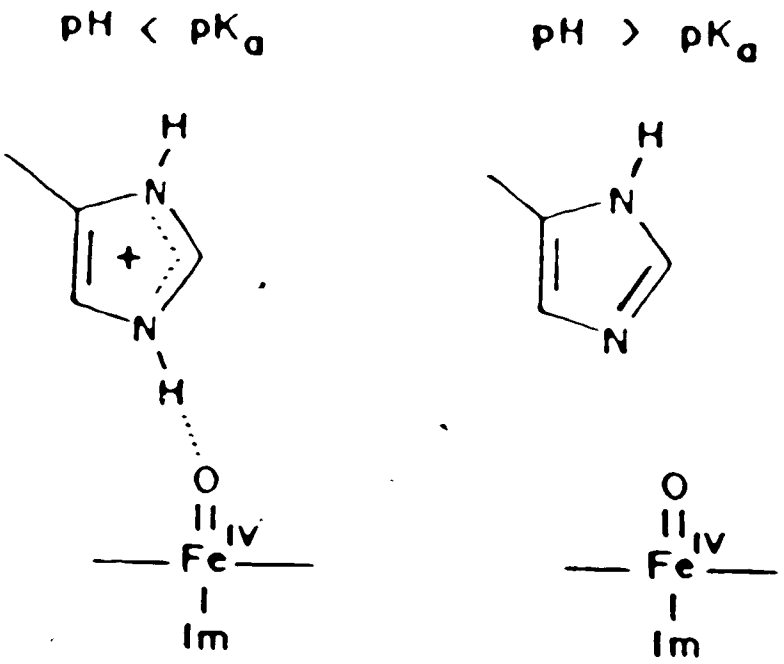
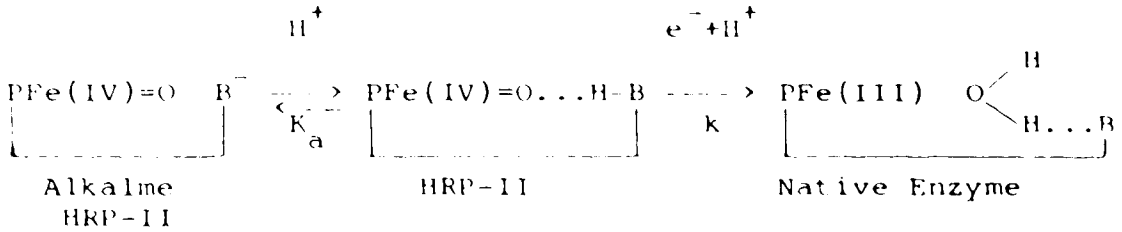


Figure IV.2 The heme ferryl group of HRP-II showing the presence of hydrogen bonding to the distal histidine below the pK_a, and the absence of it above the pK_a. For HRP-II a pK_a of 8.7 has been obtained from the present study compared with 8.6 obtained from an earlier kinetic study (8), and a value of 8.5 from proton balance studies (9).

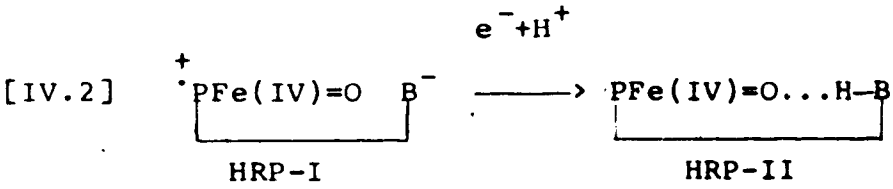


[IV.1]

where K_a and k are ionization and rate constants respectively.

Equation [IV.1] adequately explains the observations from the present pH dependence study. From the results of Hammet $\rho\sigma$ correlation studies, it appears that the electron and proton involved in the second step of equation [IV.1] could both have come from the substrate in a concerted process.

Since the conversion of HRP-I to HRP-II is also known to be accompanied by a proton uptake (18-20), and the proton adds to the protein moiety (15) and not to the ferryl oxygen, a scheme similar to equation [IV.1] can be written for the conversion of HRP-I to HRP-II.



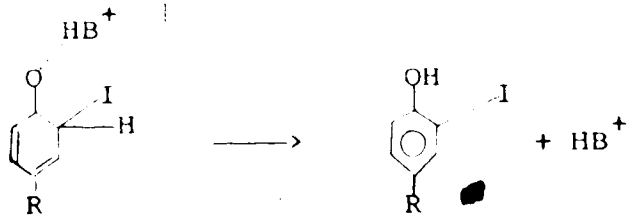
A comparison of equations [IV.1] and [IV.2] shows that the reduction of compound II to the native enzyme is a more complex process than the reduction of compound I to compound II. The former involves addition of two protons to the oxygen of the ferryl group and removing a water molecule from the inner coordination sphere of Fe(III) of the heme. The conversion of compound I to compound II is not very much different from a simple electron transfer from the substrate to the heme. This explains the observed difference in the ρ values for the oxidation of phenols by HRP-I (21), and by HRP-II in the present study. Thus the reaction of HRP-I with phenols is more sensitive to substituent effects as expected.

The nonenzymatic reaction of tyrosine with iodine has been shown to be general acid catalyzed at low pH in acetate buffer and general base catalyzed at high pH in carbonate buffer. The results of one of the earliest studies on iodination of phenol in acetate and phosphate buffers were interpreted to indicate general acid catalysis (22). However, results of subsequent studies have been interpreted to indicate general base catalysis (23-26). All of these studies have been done at pH values above 5.5, and most of them within narrow pH ranges. The results of some of the studies are relevant to the conclusion of the present study. In the studies of the effect of buffers on the

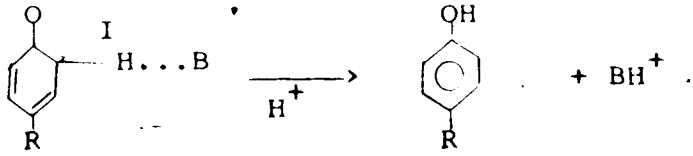
iodination of aniline (23), the iodination of phenol (24), and the iodination of N-acetyl-L-tyrosine and N-acetyl-3-iodo-L-tyrosine (25), both $H_2PO_4^-$ and $HPO_4^=$ were observed to take part in the general acid-base catalysis, even though the catalytic effect of the latter was much stronger than for the former in all cases. However, $H_2PO_4^-$ has been considered as the conjugate base of H_3PO_4 rather than as the conjugate acid of $HPO_4^=$ in arriving at the conclusion about the nature of the catalysis. From our present results it appears that the shift in catalysis from general acid to general base catalysis occurs in the neutral pH region and that the observed participation of $H_2PO_4^-$ (23-25) could be regarded as general acid catalysis.

It was concluded from kinetic isotope effect studies that the rate-determining step for the iodination of phenol is the deprotonation of an iodinated quinoid intermediate (27). A similar conclusion has also been made from tritium isotope effect studies of the iodination of L-3-iodotyrosine (26). From the results of our present pH profile study it is concluded that the iodination step is the attack of unionized tyrosine by hypoiodous acid, HOI, giving an iodinated quinoid intermediate. The deprotonation of the intermediate, which occurs by general acid catalysis at low pH and by general base catalysis at high pH, is then the rate-determining step.

In general acid-base catalysis, the withdrawal of a proton, by a base, from an intermediate is thermodynamically favorable if the pK_a of the conjugate acid of the base is higher than that for the intermediate. Otherwise, enhancement of rate by general acid catalysis would be favored (28-30). This explains the shift of the nature of catalysis as observed. The rate determining step can then be represented by [IV.3].

[IV.3] 

at low pH, and by [IV.4]

[IV.4] 

at high pH.

However, an alternative iodination step in which ionized tyrosine is iodinated by molecular iodine (23,24,27) could not be excluded.

Suggestions for further studies.

Hypoiodous acid, HOI, unlike hypochlorous acid or hypobromous acid, is unstable. However, it has been shown in a recent study that a solution of HOI which contains no

I_2, at least for some minutes, can be prepared by rapidly stirring a slurry of mercuric carbido polymer, $[Hg_3CO(ClO_4)]_n$ in 1 mM $HClO_4$ solution of I_2 (31). By using a solution of HOI prepared in this way as starting reagent at low pH, and monitoring the reaction at 278 nm, the wavelength of maximum absorbance of HOI (31,32), it should be possible to distinguish between the alternative iodination steps in the mechanism for iodination of tyrosine.

The results of this study could also be extended to a study of enzymatic iodination of tyrosine. It should be possible to arrive at a conclusive mechanism by studying the pH profile of HRP catalyzed reaction; (i) starting with I_2 in the presence of I^-, (ii) starting with I_2 in the absence of I^- and (iii) starting with HOI.

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Appendix A (Chapter II)

From the Hammett relation

[A.1] $\log k_A - \log k_H = \rho\sigma_A$

[A.2] $\log k_B - \log k_H = \rho\sigma_B$

Therefore:

[A.3] $\log k_A - \log k_B = \rho(\sigma_A - \sigma_B) = \rho\Delta\sigma$

Equation (A.3) can be applied to data for both compound I and compound II.

For compound I:

$$-0.92 = -6.9\ \Delta\sigma$$

$$\Delta\sigma = 0.13$$

For compound II:

$$-0.60 = -4.6\ \Delta\sigma$$

$$\Delta\sigma = 0.13$$

Appendix A (Chapter III)

Correction for k_{app}, the apparent second order rate constant in the absence of initially added iodide ions.

In the absence of initially added iodide ions the equilibrium that has the greatest effect on the concentration of iodine in aqueous solution is reaction [6]:

[6] $$3I_2 + 3H_2O \underset{\leftarrow}{\xrightarrow{K_6}} IO_3^- + 5I^- + 6H^+$$

If, for an initial I_2 concentration of a mol L^{-1}, x mol L^{-1} of IO_3^- is produced at a given pH, the equilibrium concentrations will be as follows:

$(I_2) = (a-3x)$mol L^{-1}; $(IO_3^-) = x$mol L^{-1};

$(I^-) = 5x$ mol L^{-1}; and $(H^+) = h$mol L^{-1}

where $h = 10^{-pH}$.

[A.1] $$K_6 = \frac{(IO_3^-)(I^-)^5(H^+)^6}{(I_2)^3}$$

i.e.,

[A.2] $$K_6 = \frac{x(5x)^5h^6}{(a-3x)^3}$$

The value of K_6 is known to be 3×10^{-48} (9);

[A.3] $$\frac{X(5x)^5h^6}{(a-3x)^3} = 3\times10^{-48} .$$

Since a and h are constants, equation [A.3] can be solved by an iterative method.

The apparent second order rate constant, k_{app}, was obtained from k_{obs}, the pseudo-first order rate constant by the relation:

$$[A.4] \qquad k_{app} = \frac{k_{obs}}{a} \ (M^{-1} \ s^{-1}) \ ;$$

but the corrected value, k'_{app}, is given by:

$$[A.5] \qquad k'_{app} = \frac{k_{obs}}{a-3x_K} \ (M^{-1} \ s^{-1})$$

where x_K is the solution to equation [A.3], and $(a-3x_K)M$ is the equilibrium concentration of I_2. Therefore, the corrected rate constant, k'_{app} is related to the uncorrected constant by equation [A.6]:

$$[A.6] \qquad k'_{app} = \left(\frac{a}{a-3x_K}\right)k_{app} \ (M^{-1} \ s^{-1})$$

Appendix B (Chapter III)

Acid-base Catalysis

The expression relating the apparent rate constant, k_{app}, to the catalytic constants in terms of the buffer concentrations in an acid-base catalyzed reaction is derived as follows (21):

[B.1] $$k_{app} = k_O + k_H(H^+) + k_{OH}(OH^-) + k_{HB}(HB) + k_B(B^-)$$

where the catalytic constants are:

k_O, constant due to H_2O; k_H, constant due to H^+; k_{OH}, constant due to OH^-; k_{HB}, constant due to HB; k_B, constant due to B^-.

In a buffer solution, the equilibria

[B.2] $$H_2O \underset{<}{\overset{K_W}{\rightleftharpoons}} H^+ + OH^-$$

and

[B.3] $$HB \underset{<}{\overset{K_{HB}}{\rightleftharpoons}} H^+ + B^-$$

give:

$$K_W = (H^+)(OH^-) \text{ and } K_{HB} = \frac{(H^+)(B^-)}{(HB)}$$

Therefore,

$$(OH^-) = \frac{K_W(B^-)}{K_{HB}(HB)} \text{ and } (H^+) = K_{HB}\frac{(HB)}{(B)}.$$

$$[B.4] \qquad k_{app} = k_O + k_H K_{HB} \frac{(HB)}{(B)} + k_{OH} \frac{K_w}{K_{HB}} \frac{(B)}{(HB)} + k_{HB}(HB) + k_B(B^-)$$

For a single plot of k_{app} against (B) the first three terms are constant and (HB)/(B) = x, is constant.

$$[B.5] \qquad k_{app} = k_x + k_{HB}(HB) + k_B(B). \frac{(HB)}{(B)} \cdot \frac{1}{x}$$

$$[B.6] \qquad k_{app} = k_x + (HB)[k_{HB} + k_B \cdot \frac{1}{x}]$$

Thus, for a plot of k_{app} against (HB), slope = $k_{HB} + k_B \cdot 1/x$, and for a replot of slopes at different pH's against $1/x$, slope = k_B, and intercept = k_{HB}.

On the other hand, equation [B.4] can be expressed as:

$$[B.7] \qquad k_{app} = k_x + k_{HB}(HB). \frac{(B^-)}{(HB)} \cdot x + k_B(B^-)$$

$$[B.8] \qquad k_{app} = k_x + (B^-)[k_{HB} \cdot x + k_B] .$$

Then, for a plot of k_{app} against (B^-), slope = $k_{HB} \cdot x + k_B$, and a replot of slopes at different pH against x gives slope = k_{HB}, and intercept = k_B.

Appendix C (Chapter III)

Derivation of the rate law for the reaction of tyrosine with molceular iodine in the presence of initially added iodide ions.

The important equilibria have been represented in equations [1] to [7]; and the mechanism for the base catalyzed reaction is represented by equations [8] and [9A]. From this mechanism in which [9A] is the rate determining step (6), the rate of iodination is given by:

[C.1] $$V = k_9(HITO)(Base^-)\ M\ s^{-1}$$

At steady state,

[C.2] $$\frac{d(HITO)}{dt} = k_8(HTOH)(HOI) - [k_{-8}+k_9(Base^-)](HITO)$$

$$(HITO) = \frac{k_8(HTOH)(HOI)}{k_{-8} + k_9(Base^-)}$$

For reaction [9A] to be the rate-determining step, $k_{-8} >> k_9(Base^-)$. Therefore,

[C.3] $$(HITO) = \frac{k_8(HTOH)(HOI)}{k_{-8}}$$

[C.4] $$(HITO) = K_8(HTOH)(HOI)$$

where $K_8 = \frac{k_8}{k_{-8}}$

Substituting for (HITO) in equation [C.1]

$$[C.5] \qquad V = k_9 K_8 (HTOH)(HOI)(Base^-) \ .$$

From equation [5],

$$[C.6] \qquad [HOI] = \frac{K_5 (I_2)}{(H^+)(I^-)}$$

$$[C.7] \qquad V = \frac{k_9 K_5 K_8 (HTOH)(I_2)(Base^-)}{(H^+)(I^-)} \quad (M\ s^{-1})$$

Since $(Base^-) >> (HTOH)$ and $(Base^-) >> (I_2)$ $(Base^-)$ remains essentially constant. Therefofe,

$$[C.8] \qquad V = \frac{k_9' K_5 K_8 (HTOH)(I_2)}{(H^+)(I^-)} \quad (M\ s^{-1})$$

where $k_9' = k_9\ (Base^-)$, which is essentially constant at a given pH.

Generally, from the stoichiometry of the iodination reaction,

$$[C.9] \qquad V = k_{app}(HTOH)_0(I_2)_0 \quad (M\ s^{-1})$$

where $k_{app} = k_0 + k_B(Base^-)$,

$(HTOH)_0 = (HTOH) + (HTO^-)$

and $(I_2)_0 = (I_2) + (I_3^-)$

[C.10] $V = k_{app}[(HTOH) + (HTO^-)][(I_2) + (I_3^-)]$

Equating [C.8] and [C.10] gives:

[C.11] $$k_{app} = \frac{k_9' K_5 K_8 (HTOH)(I_2)}{[(HTOH) + (HTO^-)][(I_2) + (I_3^-)](H^+)(I^-)}$$

[C.12] $$k_{app} = \frac{k_9' K_5 K_8}{\left[1 + \frac{(HTO^-)}{(HTOH)}\right]\left[1 + \frac{(I_3^-)}{(I_2)}\right](H^+)(I^-)}$$

From equations [7] and [1]

[C.13] $$\frac{(HTO^-)}{(HTOH)} = \frac{K_7}{(H^+)}$$

and

[C.14] $$\frac{(I_3^-)}{(I_2)} = \frac{(I^-)}{K_1}$$

[C.15] $$k_{app} = \frac{k_9' K_5 K_8}{\left[1 + \frac{K_7}{(H^+)}\right]\left[1 + \frac{(I^-)}{K_1}\right](H^+)(I^-)}$$

$$[10] \qquad k_{app} = \frac{k_9' K_1 K_5 K_8}{[(H^+) + K_7][K_1 + (I^-)](I^-)}$$

$$[11] \qquad [K_1 + (I^-)](I^-)k_{app} = \frac{k_9' K_1 K_5 K_8}{K_7 + (H^+)}$$

Thus, a plot of $[K_1 + (I^-)](I^-)\ k_{app}$ vs. pH is expected to give a straight line with a slope of 1.

When the reaction is general acid catalysis the rate law is derived in a similar manner. However, k_9' is replaced by k_9'', where

$$[C.16] \qquad k_9'' = k_9\ (HBase)$$

and also, k_{app} is related to (HBase) by:

$$[C.17] \qquad k_{app} = k_0 + k_{HB}(HBase)$$

In the absence of initially added iodide ions, the derivation of the rate law follows the same procedure. The only modification in the expression for k_{app} which is necessitated by the low concentration of I^- is indicated in equation [14].