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Mechanism and kinetics of haloperoxidase-catalyzed bromination reaction

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MECHANISM AND KINETICS OF HALOPEROXIDASE-CATALYZED BROMINATION REACTION

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MECHANISM AND KINETICS OF HALOPEROXIDASE-CATALYZED BROMINATION REACTION

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ABSTRACT

MECHANISM AND KINETICS OF HALOPEROXIDASE-CATALYZED BROMINATION REACTION

ZHEN PING YANG

Haloperoxidase-catalyzed bromination of tyrosine was studied. Three haloperoxidases from different sources were used. When chloroperoxidase (CPO) was used as the enzyme, and when H_2O_2 was present, the conversion of Br^- to Br_3^-/Br_2 species was an enzyme-catalyzed reaction, and the enzyme, CPO, was irreversibly deactivated by the free molecular bromine formed in that reaction. It was observed that the above deactivation of CPO was associated with the irreversible destruction of CPO's heme structure. The minimum Br_2/CPO ratio to cause that deactivation was obtained. The actual bromination reaction was concluded to be a nonenzymatic reaction between Br_2 and tyrosine. Also observed was the reaction between H_2O_2 and Br_3^-/Br_2 species to produce O_2 and Br^-, if the Br^-/H_2O_2 ratio was not high enough. The minimum Br^-/H_2O_2 ratio required to inhibit the production of O_2 and Br^- was determined. Inhibition of this reaction would allow Br_3^-/Br_2 to accumulate. The maximum Br^-/H_2O_2 ratio that would allow the production of O_2 and Br^- was also determine. This would prevent the Br_3^-/Br_2 species from accumulating.

Zhen Ping Yang

In the horseradish peroxidase-catalyzed bromination system, horseradish peroxidase (HPO) would not be deactivated irreversibly by Br_2 under the condition of the highest Br_2 concentration used in our system. When HPO was mixed with Br_2 or when HPO was used as the enzyme in the bromination reaction system, the same shift of HPO's Soret band from its original position was observed, which indicated a similar change of HPO's heme structure. It was also observed that HPO could "recover" from the above structural change and shift back to its original position when HPO was exposed to Br_2 long enough or when the bromination reaction was close to equilibrium. A probable reaction mechanism of the HPO-catalyzed bromination was proposed, which suggested that the Br_2/Br_3^- species bound to HPO to form an enzyme intermediate, and that intermediate catalyzed the actual bromination reaction.

When lactoperoxidase (LPO) was used as the enzyme, it was observed that LPO was deactivated irreversibly by Br_2; but, the deactivation process was different from that of CPO.

ACKNOWLEDGEMENTS

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CHAPTER 1

INTRODUCTION

Haloperoxidases are enzymes which are capable of halogenating a variety of organic compounds using hydrogen peroxide and halide ions as substrates. These enzymes have been called chloro-, bromo-, or iodoperoxidases, depending on the smallest halide ion they can oxidize. Some 30 years ago, haloperoxidases were thought to be unique enzymes: their halogenating capabilities were unusual and they were considered to be rare. Nowadays, haloperoxidases are known from almost 100 sources, including mammals, birds, plants, algae, molds, and bacteria, clearly showing their wide occurrence.

Haloperoxidases are unique reagents, but their reaction mechanism is not fully understood, and the natural function of these enzymes is far from clear. They seem to be involved in the defense mechanism of their hosts; it is known that a lot of halogen-containing compounds are physiologically more active than their nonhalogenated counterparts (1).

Chloroperoxidase (E.C. 1.11.1.10, chloride:hydrogen-peroxide oxidoreductase) was first isolated in attempts to characterize an enzymic system which was involved in the biosynthesis of caldariomycin by the mold, Caldariomyces fumago (2). It is a monomeric glycoprotein with M_r = 42,000

involving 10 tyrosine residues and containing one ferriprotoporphyrin IX prosthetic group per enzyme molecule (3).

Chloroperoxidase (CPO) has an unusually diverse range of catalytic activities. It catalyzes the classical peroxidation reactions which are characteristic of peroxidases: hydrogen peroxide-supported oxidation (peroxidatic reaction) and iodination (halogenation reaction) of a variety of organic substrates. Unlike most other peroxidases, CPO can also utilize chloride and bromide in its halogenation reactions (4). In addition to peroxidase reactions, CPO also catalyzes the dismutation of hydrogen peroxide (catalatic reaction), a characteristic reaction of catalases (5,6), and the dismutation of organic hydroperoxides and peroxy acids, reactions not catalyzed by catalases or other peroxidases (7).

CPO catalyzes the chloride-dependent chlorination of nucleophilic substrates (8) and, unlike any other peroxidase, CPO also resembles other nonperoxidatic heme enzymes. Many of the spectral and magnetic properties of CPO closely parallel those of the cytochrome P-450 enzymes (9,10,11). Like cytochrome P-450, it has a cysteine thiolate as the fifth heme ligand (12,13). The H_2O_2-dependent substrate oxidation reactions catalyzed by CPO appear to be both typical peroxidase and cytochrome P-450 mechanisms. Thus, CPO oxidizes ascorbate, guaiacol, and

pyrogallol by simple one-electron abstractions (5,6), but catalyzes styrene epoxidation and *p*-methoxythioanisole sulf-oxidation by oxene-transfer mechanisms (14,15). Samokyszyn et al. (16) suggested a topological model for the CPO active site and indicated that the tertiary structure of the enzyme permitted substrates to interact with both the meso heme edge, like a peroxidase, and the catalytic ferryl ($Fe^{IV}=O$) species, like a monooxygenase, in agreement with the fact that CPO catalyzes both H_2O_2-dependent peroxidation and monooxygenation reactions.

The sixth ligand of the heme iron in CPO from C. fumago is still not known with certainty. In an attempt to shed more light on this matter, Blanke and Hager (17) studied the modification of the enzyme by the histidine-specific reagent diethyl pyro-carbonate. They found that the enzyme lost activity in a time-dependent manner during this process, which led them to the conclusion that a vital histidine residue was affected, presumably His-38. The authors suggest that this residue is the sixth axial ligand in CPO.

CPO from C. fumago is a mixture of three isoenzymes which differ in their sugar contents. The glycosylated positions are Asn-12 and Asn-216 (18). Attempts to crystallize the protein have so far been unsuccessful because of the heterogeneity in the sugar parts of the enzyme (19).

Knowledge of reaction mechanisms of CPO has been

gathered from various points of view. Models of the way in which the enzymes function have been developed, based on kinetic measurements and data obtained by spectroscopic techniques.

For CPO or other haloperoxidases, it is believed that the iron of the native enzyme (resting state, ENZ-Fe^{3+}) is in the ferric form, Fe(III) (4). When the native enzyme reacts with a hydroperoxide (ROOH) an enzyme intermediate, 2-equivalents oxidized above the native resting state, is formed (20)(reaction 1, when ROOH = H_2O_2). This intermediate, called Compound I, appears to be a ferryl porphyrin cation radical, wherein one of the oxidation equivalents is stored as the ferryl iron, Fe(IV), while the other equivalent is stored as a porphyrin-centered cation radical. Compound I is unstable (half-life of minutes or less) and has a characteristic green color.

The standard oxidation-reduction potentials of the halides, and hydrogen peroxide, are as follows (40):

$2I^- \rightarrow I_2 + 2e^-$ $\quad E^0 = -0.54$ volts

$2Br^- \rightarrow Br_2 + 2e^-$ $\quad E^0 = -1.07$ volts

$2Cl^- \rightarrow Cl_2 + 2e^-$ $\quad E^0 = -1.36$ volts

$2H_2O \rightarrow H_2O_2 + 2H^+ + 2e^-$ $\quad E^0 = -1.77$ volts

I^- is the most readily oxidizable of all the halides, whereas Cl^- requires a more powerful oxidant for its oxidation. Although H_2O_2 itself, in the presence of H^+, is a sufficiently powerful oxidant to oxidize all the halogens,

the oxidant in the peroxidase-catalyzed halogenation is not H_2O_2 itself but rather the reaction product of the peroxidase with H_2O_2, Compound I.

In the mechanistic studies of the hydrogen peroxide-dependent enzyme reactions, there is so far a general agreement about the formation of Compound I (reaction 1). However, there are some arguments about the further reactions (reactions between Compound I, halide ions, and halogenation substrates)(1,20). One possible reaction route is that Compound I may react with halide ions directly, and, as the result, Compound I is decomposed to native enzyme while X^- is oxidized to HOX (in equilibrium with X_2 and X_3^- in acidic solution, reaction 2), and the inorganic species (hypohalous acid/molecular halogen/trihalide ion) are the active halogenating agents in the reaction (reaction 5). Another reaction route, which is claimed more possible, is that the Compound I may bind with X^- to form another complex, named Compound EOX (reaction 3). Then, again, two alternative pathways are advanced concerning the reactions following the formation of EOX: pathway 1, in which EOX decomposes to native enzyme and a molecule of hypohalous acid/molecular halogen/trihalide ion (reaction 4), and the latter species will react with AH as the active halogenating agents (reaction 5); and/or pathway 2, suggesting that the organic substrate (AH) binds to the enzyme to form a ternary complex (reaction 6) which splits into the native enzyme,

the organic product (AX), and a molecule of water (reaction 7).

$$ENZ\text{-}Fe^{3+} + H_2O_2 \text{ ---> } ENZ\text{-}Fe^{4+}\text{-}O^{2-} \text{ (Compound I)} + H_2O \quad (1)$$

$$ENZ\text{-}Fe^{4+}\text{-}O^{2-} + X^- + H^+ \text{ --->}$$
$$ENZ\text{-}Fe^{3+} + HOX \; (\text{-- } X_2 \text{ -- } X_3^-) \quad (2)$$

$$ENZ\text{-}Fe^{4+}\text{-}O^{2-} + X^- \text{ ---> } ENZ\text{-}Fe^{3+}\text{-}O^{-}\text{-}X \text{ (Compound EOX)} \quad (3)$$

$$ENZ\text{-}Fe^{3+}\text{-}O^{-}\text{-}X + H^+ \text{ ---> } ENZ\text{-}Fe^{3+} + HOX \; (\text{-- } X_2 \text{ -- } X_3^-) \quad (4)$$

$$HOX \; (\text{-- } X_2 \text{ -- } X_3^-) + A\text{-}H \text{ ---> } A\text{-}X + H_2O \quad (5)$$

$$ENZ\text{-}Fe^{3+}\text{-}O^{-}\text{-}X + A\text{-}H \text{ ---> } \begin{matrix} ENZ\text{-}Fe^{3+}\text{-}O^{-} \\ | \; | \quad\quad | \\ H\text{-}A\text{-----}X \end{matrix} \quad (6)$$

$$\begin{matrix} ENZ\text{-}Fe^{3+}\text{-}O^{-} \\ | \; | \quad\quad | \\ H\text{-}A\text{-----}X \end{matrix} + H^+ \text{ ---> } ENZ\text{-}Fe^{3+} + A\text{-}X + H_2O \quad (7)$$

Analyzing the above reactions, it could be deduced that if $HOX/X_2/X_3^-$ is the halogenating species as shown in reaction 5, then the reaction rate, substrate specificity, and product selectivity of the enzyme reaction should be identical with that of chemical halogenation processes; on the other hand, if an enzyme-bound intermediate (Compound EOX) is the halogenating species as shown in reaction 6 and 7), then the reaction rate, substrate specificity, and product selectivity of the enzyme reaction should be quite different from that of the chemical halogenation processes.

In the above complicated reaction system, the halide species ($HOX/X_2/X_3^-$, especially the chloro- and bromo-species) and other reaction intermediates are unstable. As the result, the monitoring of the whole reaction processes

including the intermediates' formation/decomposition is difficult. For those reasons, studies on the reaction rate, substrate specificity, and product selectivity are not complete, and the reaction mechanisms are still the subject of some dispute. Those who have studied substrate halogenation rates favor the reaction 3, 4, 5, 6, and 7, while those who have studied the nature of the halogenated products prefer reaction 1, 2, and 5 (1,20).

For CPO from C. fumago, in the absence of a substrate (AH), the enzyme is believed to catalyze the oxidation of halide ion to hypohalous acid (1,20). Since it is impossible to obtain any Visible/UV spectrum of hypohalous acids directly, the species of HOX in equilibrium with X_2 and X_3^- may be considered here as an alternative expression of the product.

In the CPO-hydrogen peroxide-halide system (in the absence of a substrate), singlet oxygen production was studied by Kanofsky (21). With chloride or bromide ions, singlet oxygen is produced by the mechanism

$$H_2O_2 + X^- + H^+ \text{---> } H_2O + HOX \text{ (with CPO)} \quad (8)$$

$$H_2O_2 + HOX \text{---> } H_2O + X^- + H^+ + O_2 \quad (9)$$

Under conditions where there is high enzyme activity and when reaction 9 is fast relative to reaction 8, singlet oxygen is produced in near stoichiometric amounts. In contrast, when reaction 8 is fast relative to reaction 9, oxidized halogen species ($HOX/X_2/X_3^-$) are the principle

reaction products. With iodide ion, no singlet oxygen was detected.

Because of the reaction 9, attempts to compare the formation rate of oxidized halogen species with the reaction rate (formation rate) of halogenated substrate (AX), with or without enzyme, does not yield data that allows a conclusion as to whether $HOX/X_2/X_3^-$ or EOX is the halogenating species.

In the presence of a substrate, there are some disagreements about the reaction mechanism. For the enzyme catalyzed tyrosine iodination reaction, the main conflict centers upon the identity of the iodinating species. Although it was suggested that I_2 is an obligatory intermediate (22), there is evidence indicating that I_2 is not the active iodinating agent in thyroid peroxidase-catalyzed iodination (23). Other experimental reports support the conclusion that iodination does not occur by way of free I_2, but rather via an iodination species associated with the enzyme (24,25,26). It has been suggested that the iodinating species is enzyme-activated hypoiodous acid, HOI, on the basis of a kinetic analysis of tyrosine iodination catalyzed by horseradish peroxidase (HRP) at low pH (27). Magnusson et al. (28) claimed that free HOI is the active iodinating species in the lactoperoxidase (LPO) iodination system. A proposal has been made that both I_2 and HOI may play roles as iodinating agents (29). Huber et al. (30) concluded that a highly reactive iodinating intermediate is

produced on LPO, which diffuses from the enzyme before reacting. Sun & Dunford (31) compared the LPO and HRP catalyzed tyrosine iodination reactions, and from the initial and overall reaction rates they concluded that the role of HRP was to catalyze the formation of HOI, which reacted with excess I^- to form I_2. Thus, in the HRP system the actual iodination step was nonenzymatic. On the other hand, they concluded that an LPO-bound hypoiodite complex, LPO-O-I^-, was an efficient iodinating species and under physiological conditions was probably the only iodinating reagent.

The CPO (from C. fumago) and LPO catalyzed tyrosine iodination reactions have been studied in our lab by Shelton (32) using a diode array spectrophotometer. A reaction sequence was observed clearly: the formation of monoiodo-tyrosine (MIT) would not begin until the amount of triiodide ion was decreased to a minimum value while the concentration of iodine reached a maximum. A ping-pong type of mechanism was applied to both CPO and LPO catalyzed iodination reactions, and iodine was believed to serve as an obligatory intermediate in the above reactions.

Libby et al. (33) studied the CPO catalyzed bromination and chlorination reactions. The rate of enzymatic oxidation of chloride to its respective molecular species (i.e., HOCl) was found to be slow compared to the rate of chlorination of the substrate. Further, in a competitive substrate study,

it was observed that major differences in the substrate specificity occurred between enzymatic and chemical chlorination. In the bromide-dependent reaction, bromine is formed at a rate equivalent to that of the bromination of acceptor substrates. However, the substrate specificities of the bromide-dependent reactions were quite different from the reactions of these substrates with molecular bromine. As the result, they claimed that unlike the iodide-dependent CPO reaction, the chloride and bromide-dependent reactions did not involve the formation of free molecular halogen intermediate.

The above conclusion was doubted by Neidleman & Geigert (20): since HOCl can readily attack CPO thus both affecting the rate of HOCl formation and consuming HOCl, and since HOCl is decomposed in reaction with H_2O_2, it is not possible to accurately quantitate the true amount of HOCl formed in the CPO study. Also, since the specificity of a chemical reaction can vary, depending upon the experimental conditions, some further studies are still needed.

Dunford et al. (34) have used stopped-flow, rapid-scan techniques to study the reaction of Compound I and HOCl with chloride and monochlorodimedone, respectively. They had to apply these advanced methods because Compound I and all subsequent intermediates are unstable and have very short lifetimes. They could deduce from their experimental data that Compound EOX really exists, but the species was so

unstable that its specific ultraviolet absorption spectrum was not demonstrated. Their kinetic studies, combined with previous results (9,35) strongly favored reaction 3, 6, and 7, although the other reactions could not be completely excluded. Other routes involving molecular chlorine or radical species were insignificant.

Libby et al. (36) performed competition reactions with Compound I as the enzyme intermediate and catechol and chloride as substrates. The authors found that the two substrates competed for the same binding site on Compound I, again proving the (transient?) existence of Compound EOX, although the other reaction routes still can not be excluded.

On the other hand, Kollonitsch et al. (37) noted that CPO failed to form optically active chlorohydrins when reacted with propenylphosphonic acids. Subsequent enzymatic studies on the other alkenes have confirmed this finding. Ramakrishnan et al. (38) documented the absence of stereo-selectivity with CPO in the reaction of additional substrates -- two bicycloalkenes, methionine, and a diketone. This lack of stereoselective product formation is consistent with a chemical halogenating intermediate like HOX.

Libby & Rotberg (39) studied the kinetics of CPO-catalyzed bromination and chlorination reactions. They found that at very high halide concentrations, both chloride

and bromide were competitive substrate inhibitors *versus* hydrogen peroxide. Results at subinhibitory halide concentrations for bromination reactions and chlorination reactions clearly indicated that halide oxidation is rate-limiting in chlorination reactions. However, in bromination reactions, both Compound I formation and bromide oxidation are partially rate-limiting. This is the first documented case where Compound I formation participates in determining the overall rate of a peroxidase reaction.

Taurog & Dorris (40) compared thyroid peroxidase (TPO) with LPO and CPO for ability to catalyze bromination of tyrosine using ^{82}Br and HPLC. With TPO, formation of organically bound ^{82}Br was undetectable. LPO and CPO, on the other hand, displayed considerable brominating activity. LPO was more active at pH 5.4 than at pH 7.0. Although monobromotyrosine (MBT) and dibromotyrosine (DBT) together comprised the greatest part of the bound ^{82}Br, a surprisingly high percentage (20-25%) was present as monobromohistidine. Evidence was also obtained for the presence of a small percentage of the bound ^{82}Br as tetrabromothyronine.

Spectral studies of the enzyme itself during the enzyme-catalyzed reactions have been done by several workers. Spectral scans in both the visible (659-450 nm) and the Soret (450-380 nm) regions were recorded for the native enzyme, Compound II (an enzyme intermediate formed by

an one-equivalent reduction of Compound I), and Compound III (an enzyme intermediate called the oxy-ferrous complex, formed when the native enzyme reacts with molecular oxygen, or more correctly superoxide anion radical, O_2^-) of LPO and TPO (41). Compound II for both enzyme was prepared by adding a slight excess of H_2O_2, whereas Compound III was prepared by adding a large excess of H_2O_2. After these compounds had been formed it was observed that they were slowly reconverted to the native enzyme in the absence of exogenous donors. The pathway of Compound III back to the native enzyme involved Compound II as an intermediate, and this recoversion was accompanied by the disappearance of H_2O_2 and generation of O_2. They also found that iodide markedly affected the inter-conversions between native enzyme, Compound II, and Compound III. The effects of iodide were explained by (a) the two-electron oxidation of iodide to hypoiodite by Compound I, which bypasses Compound II as an intermediate, and (b) the rapid oxidation of H_2O_2 to O_2 by the hypoiodite formed in the reaction between Compound I and iodide.

Dunford et al. (34) also observed the spectral changes of CPO in the reaction of CPO with peracetic acid and chloride ion in both the presence and the absence of monochlorodimedone. The results showed that the only detectable enzyme intermediate species is Compound I. The disappearance of Compound I was accelerated by the presence

of chloride ion, and was further accelerated if both chloride and monochlorodimedone were present. It was concluded that Compound I was an obligate intermediate species in the reaction.

Summarizing, it is evident that the mechanism of halogenation by haloperoxidases remains unsolved, and the controversy as to whether HOX or Compound EOX is the key halogenating intermediate continues.

Chapter II

SCOPE OF THE RESEARCH

Compared to the haloperoxidase-catalyzed iodination reactions, the $Br_3^-/Br_2/HOBr$ species in the haloperoxidase-catalyzed bromination system are more unstable and their relative reaction rates are higher. As a result, it is difficult to monitor the whole reaction process involving the $Br_3^-/Br_2/HOBr$ species (especially the reactions involving Br_2 and HOBr). Also, the oxidizing ability of Br_2 is much stronger, and, so, the $Br_3^-/Br_2/HOBr$ species will not only brominate the organic substrates, but may also deactivate the enzyme in the reaction system. Moreover, unlike $I_3^-/I_2/HOI$ species, $Br_3^-/Br_2/HOBr$ can react with H_2O_2 to form Br^- and O_2, which will make the reaction system more complicated. For the above reasons, the mechanism and kinetics studies on the haloperoxidase-catalyzed bromination reactions are far from being elucidated, and further studies on the haloperoxidase-catalyzed bromination reactions are necessary and exciting.

With the rapid-scanning and the stopped-flow techniques, now it is possible to achieve the above studies. This research will be focused on studies of the mechanism and kinetics of the chloroperoxidase-catalyzed bromination of tyrosine. Lactoperoxidase and horseradish peroxidase will also be used in the bromination system, and the results

will be compared with that of the chloroperoxidase-catalyzed reactions.

Chapter III

MATERIALS AND METHODS

INSTRUMENTATION AND EQUIPMENT

A Hewlett-Packard 8452A Diode Array Spectrophotometer (Hewlett-Packard Corporation) was used for optical absorption measurements including rapid-scan experiments.

Stopped-flow experiments were performed using a SFA-II Rapid Kinetics Accessory (Hi-Tech Scientific).

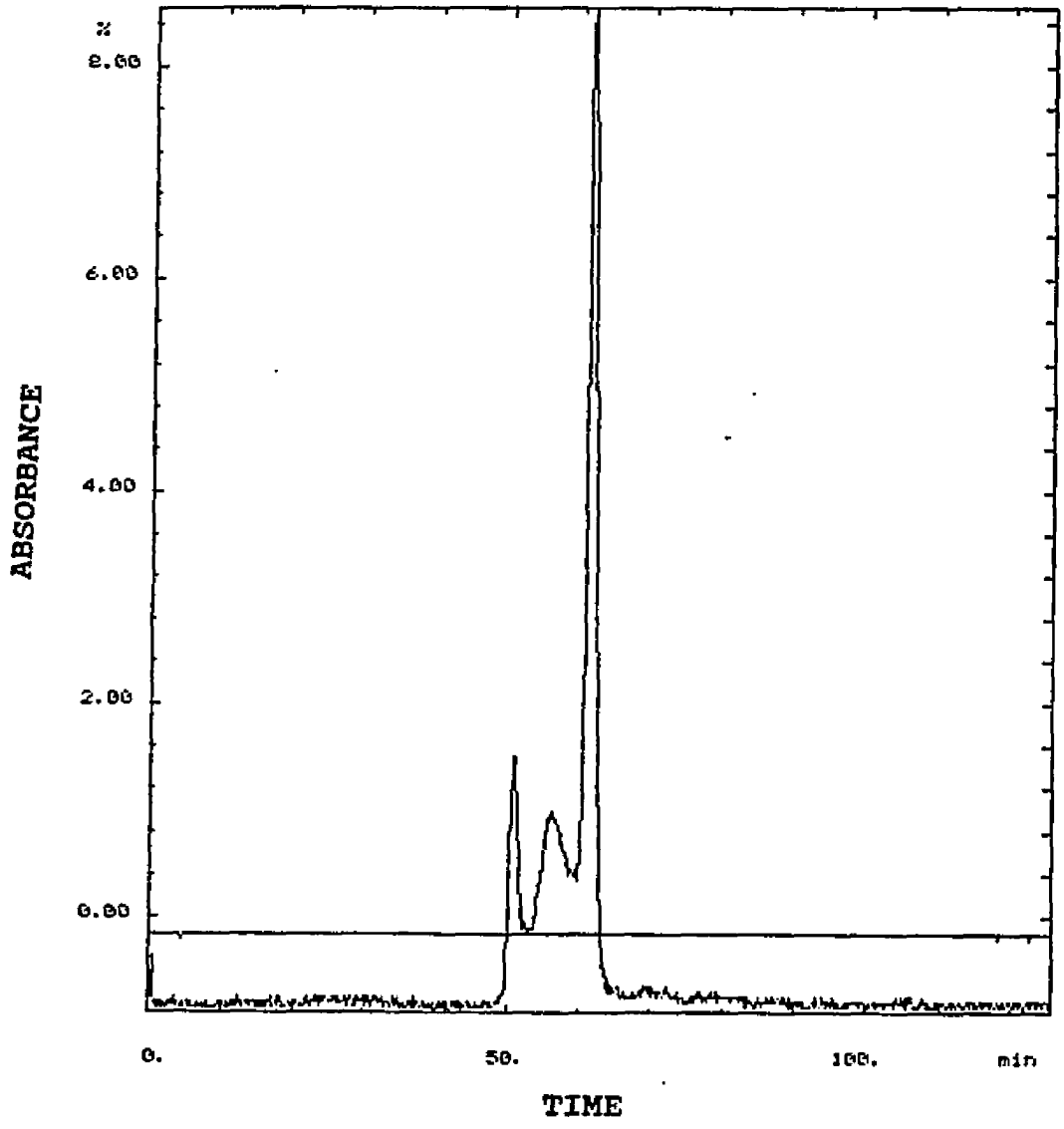
A FPLC® (Fast Protein Liquid Chromatography) system (Controller LCC-500 Plus, Pharmacia LKB Biotechnology AB) was used for further purifications of the enzymes including CPO, LPO, and HPO.

MATERIALS AND REAGENTS

CPO (E.C. 1.11.1.10, chloride:hydrogen-peroxide oxidoreductase, from Caldariomyces fumago) was obtained from Sigma Chemical Company as purified lyophilized powder with a specific activity of 1200 units/mg protein and an R_z value of 0.8. The lyophilized enzyme was further separated on FPLC using a Superose 12 HR 10/30 column (eluted with 0.1 M phosphate buffer, pH 6.0). CPO was isolated as a single peak with an R_z value of 0.92. Two other small proteins were observed and comprised only 14% of the mixture (Fig. 1). The FPLC purified CPO was used in the experiments.

Lactoperoxidase (E.C. 1.11.1.7, hydrogen-peroxide

Figure 1. FPLC chromatogram of CPO. 0.2 ml of 0.03 g/ml CPO solution (prepared by dissolving lyophilized CPO with 0.1 M phosphate buffer, pH 6.0) was eluted with the same buffer on a Superose 12 HR 10/30 column. Eluting speed: 0.5 ml/min.



oxidoreductase, from bovine milk) and horseradish peroxidase (E.C. 1.11.1.7, hydrogen-peroxide oxidoreductase, type VI-A, from horseradish) were also obtained from Sigma Chemical Company as purified lyophilized powder. The specific activity of lactoperoxidase (LPO) was 114 units/mg protein (pyrogallol substrate) and the A_{412nm}/A_{280nm} ratio was 0.91. The purity of the lyophilized LPO was examined on FPLC using a Superose 12 HR 10/30 column (eluted with 0.1 M sodium acetate buffer, pH 6.0). Only a single peak was detected (Fig. 2) and the same A_{412nm}/A_{280nm} ratio of 0.91 was obtained. Horseradish peroxidase (HPO) from Sigma has a specific activity of 280 units/mg solid (pyrogallol substrate) and a A_{430nm}/A_{275nm} ratio of 3.2. HPO was also loaded on a Superose 12 HR 10/30 column on FPLC (eluted with 0.1 M sodium acetate buffer, pH 6.0), and only a single peak was observed (Fig. 3) with the same A_{430nm}/A_{275nm} ratio of 3.2. The FPLC purified LPO and HPO were used in this experiments.

L-tyrosine, D-tyrosine and hydrogen peroxide (30% solution) were purchased from Sigma Chemical Co.

3-Bromotyrosine was synthesized in our laboratory by the method of Chitwood (42) and separated from the reaction mixture, followed by solvent evaporation and gel chromatography as described below (see *Methods*).

Tribromide (Br_3^-) solutions were prepared by mixing a very diluted Br_2/water solution with a relatively large amount of KBr in pH 4.0 acetate buffer.

Figure 2. FPLC chromatogram of LPO. 0.2 ml of 0.008 g/ml LPO solution (prepared by dissolving lyophilized LPO with 0.1 M sodium acetate buffer, pH 6.0) was eluted with the same buffer on a Superose 12 HR 10/30 column. Eluting speed: 0.5 ml/min.

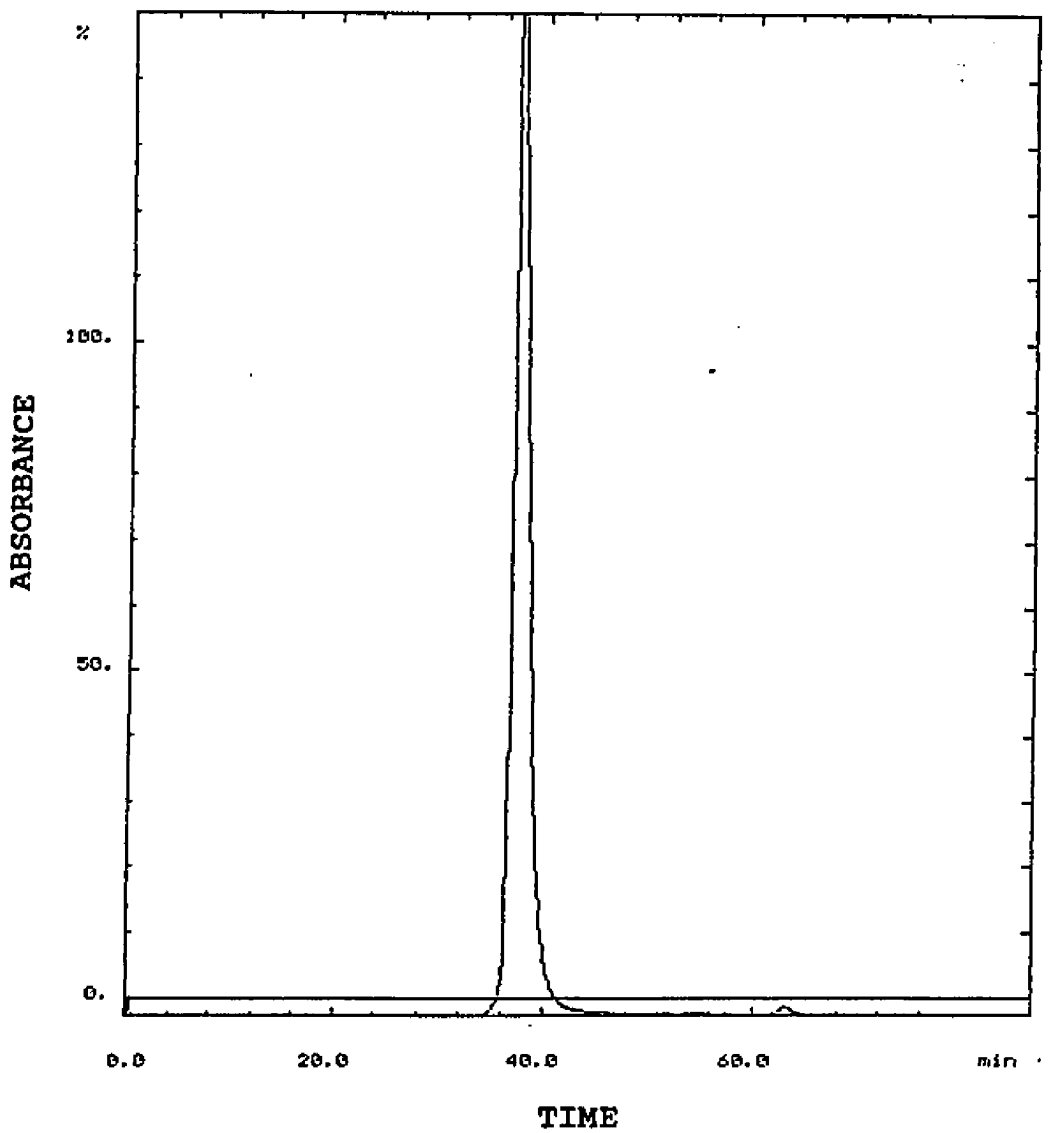
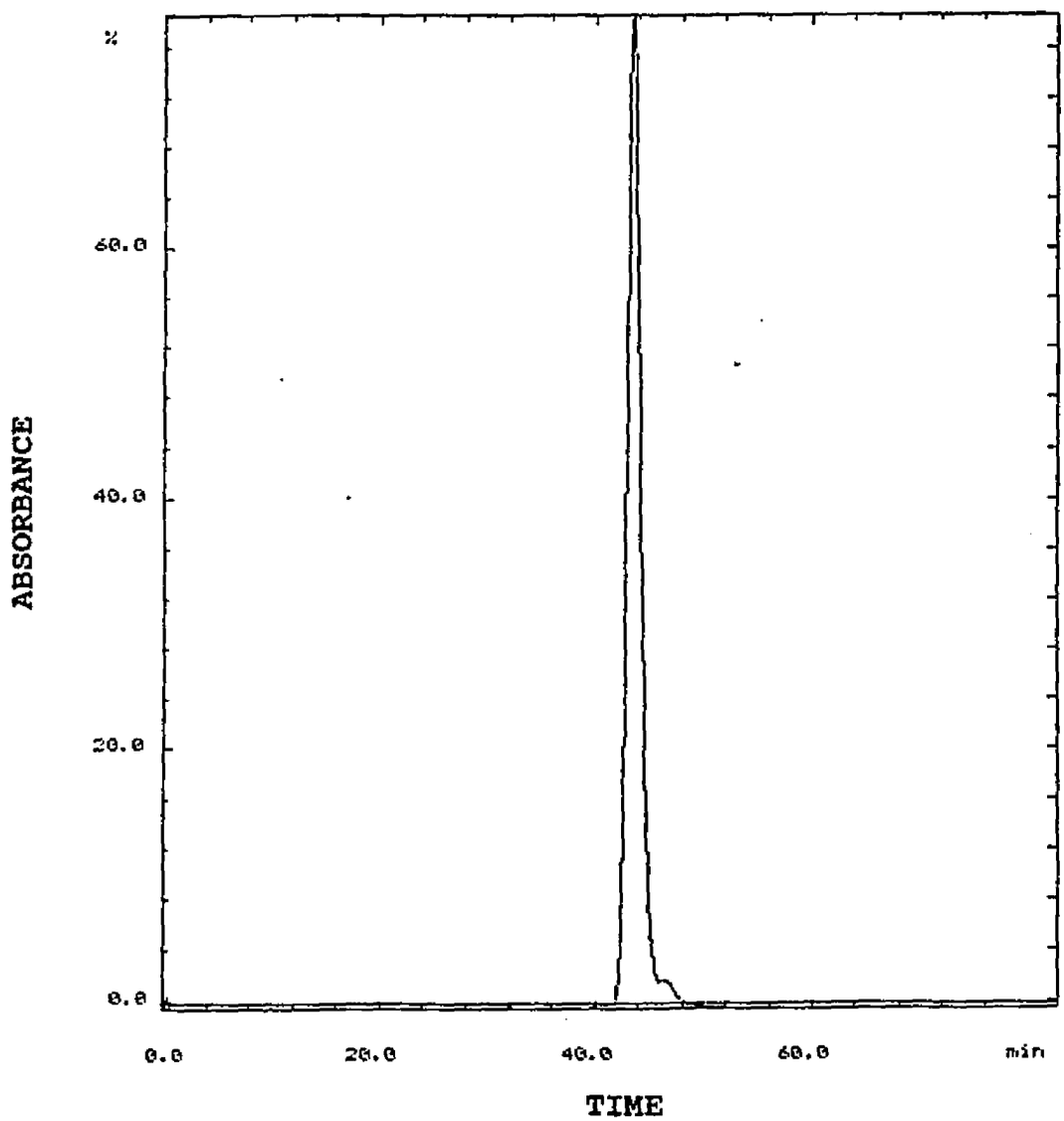


Figure 3. FPLC chromatogram of HPO. 0.2 ml of 0.01 g/ml HPO solution (prepared by dissolving lyophilized HPO with 0.1 M sodium acetate buffer, pH 6.0) was eluted with the same buffer on a Superose 12 HR 10/30 column. Eluting speed: 0.5 ml/min.



50 mM acetate buffer, pH 4.0, was prepared and used throughout the haloperoxidation reactions. Chemicals for buffers were reagent grade and were used without further purification. KBr and Br_2 were reagent grade obtained from Fisher Scientific.

All solutions were prepared using water from a reagent grade water system (Modulab Polisher I HPLC, Continental Water Systems Corporation).

METHODS

According to the method developed by Chitwood (42) and Dalton (43), gel chromatography of L-tyrosine and its halogenated analogues was carried out on a 2.5 X 90 cm column of Sephadex G-15 with particle size of 40-120 μm. The column was eluted with 20 mM acetic acid on a FPLC system. L-tyrosine, 3-bromotyrosine, and 3,5-dibromotyrosine were separated completely.

MBT has a maximum absorption at 286 nm and tyrosine has a maximum absorption at 276 nm. The difference spectrum of MBT and tyrosine was measured, and two absorption peaks at 238 and 290 nm were found. From these spectra, a difference molar absorptivity of 1.03 X 10^3 $M^{-1}cm^{-1}$ at 290 nm was determined for MBT. The formation of MBT was measured at 290 nm using that difference molar absorptivity.

The formation of Br_2/Br_3^- species was measured at 267 nm as the maximum absorption of tribromide (Br_3^-) using the molar absorptivity of 3.64 X 10^4 $M^{-1}cm^{-1}$ (33).

Concentrations of CPO were determined by measuring the absorbance at 398 nm, using a molar absorptivity of 8.5 X 10^4 $M^{-1}cm^{-1}$ (44). Concentrations of LPO were measured at 412 nm using a molar absorptivity of 1.12 X 10^5 $M^{-1}cm^{-1}$ (45). Concentrations of HPO were measured at 403 nm, where the molar absorptivity is 1.02 X 10^5 $M^{-1}cm^{-1}$ (46).

Hydrogen peroxide was standardized by measuring the absorbance at 230 nm, with a molar absorptivity of 72.4 $M^{-1}cm^{-1}$ (47).

The initial formation rates of the reaction products (MBT or Br_2/Br_3^- species) were calculated using the HP 8953K Multicell Kinetics MS-DOS Software (Rev. A.00.00) provided with the spectrophotometer, and the K_m (Michaelis-Menten Constant) was obtained using Enzfitter© Software (version 1.05, CGA, Elsevier-Biosoft Ltd).

Chapter IV

RESULTS AND DISCUSSIONS

RESULTS

CPO/HYDROGEN PEROXIDE/BROMIDE SYSTEM

The formation of Br_2/Br_3^- at various H_2O_2/Br^- ratios and a fixed CPO concentration was examined. When the enzyme was saturated by Br^- ($[Br^-]$ = 0.05 M, and [CPO] = 2.12 nM), the Lineweaver-Burk plot of $1/v_0$ (Br_3^- formation) *vs* $1/[H_2O_2]$ was linear (Fig. 4), and a K_m of 4.4×10^{-4} M for H_2O_2 was obtained. If the enzyme was saturated by H_2O_2 ($[H_2O_2] = 1 \times 10^{-5}$ M, and [CPO] = 2.12 nM), $1/v_0$ (Br_3^- formation) *vs* $1/[Br^-]$ also showed linearity (Fig. 5), and a K_m of 3.2×10^{-2} M was obtained for Br^-.

When $[Br^-] >> [H_2O_2]$, the accumulation of Br_2/Br_3^- species could be observed. However, if $[H_2O_2]$ was high enough, the increase of the Br_3^- peak (267 nm) was observed followed by a decrease until it totally disappeared. The minimum $[Br^-]/[H_2O_2]$ ratio (M/M) to maintain the accumulation of the Br_2/Br_3^- species was determined to be $(6.2 \pm 0.2) \times 10^3$. Fig. 6 showed that when $[Br^-]/[H_2O_2]$ ratio (M/M) was higher than $(6.2 \pm 0.2) \times 10^3$, no decrease of A_{267} can be observed (Fig. 6, a and b); when $[Br^-]/[H_2O_2]$ ratio (M/M) was slightly less than $(6.2 \pm 0.2) \times 10^3$ (Fig. 6, c), the decrease of A_{267} was observed.

Figure 4. Lineweaver-Burk plot of $1/v_0$ (initial Br_3^- formation rate) *vs* $1/[H_2O_2]$. Stopped-flow accessory was used. Syringe 1 contained 2 X 10^{-5}, 2 X 10^{-4}, 1 X 10^{-3}, or 2 X 10^{-3} M of H_2O_2. Syringe 2 (as blank) contained 2.12 nM CPO. Both of the two syringes contained 0.05 M KBr and 8 mM acetate buffer (pH 4.0).

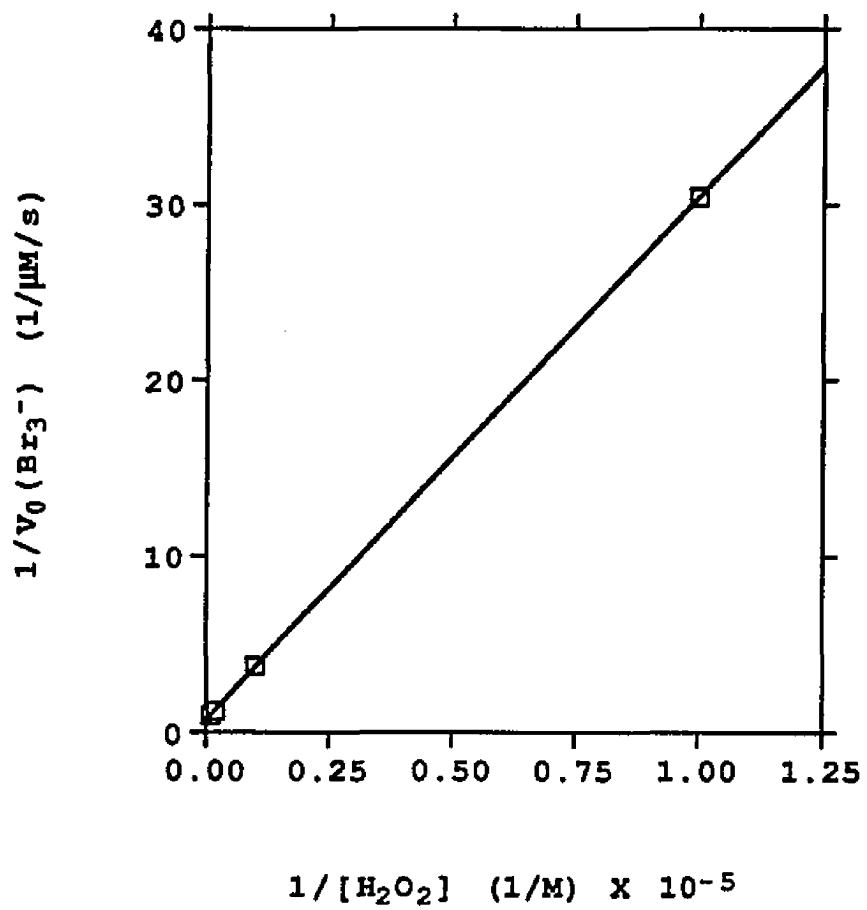


Figure 5. Lineweaver-Burk plot of $1/v_0$ (initial Br_3^- formation rate) *vs* $1/[Br^-]$. Stopped-flow accessory was used. Syringe 1 contained 2 X 10^{-5} M of H_2O_2 and 8 mM acetate buffer, pH 4.0. Syringe 2 (as blank) contained 2.12 nM CPO and 8 mM acetate buffer, pH 4.0. Both of the two syringes contained 0.04, 0.06, 0.064, 0.08, or 0.16 M of KBr.

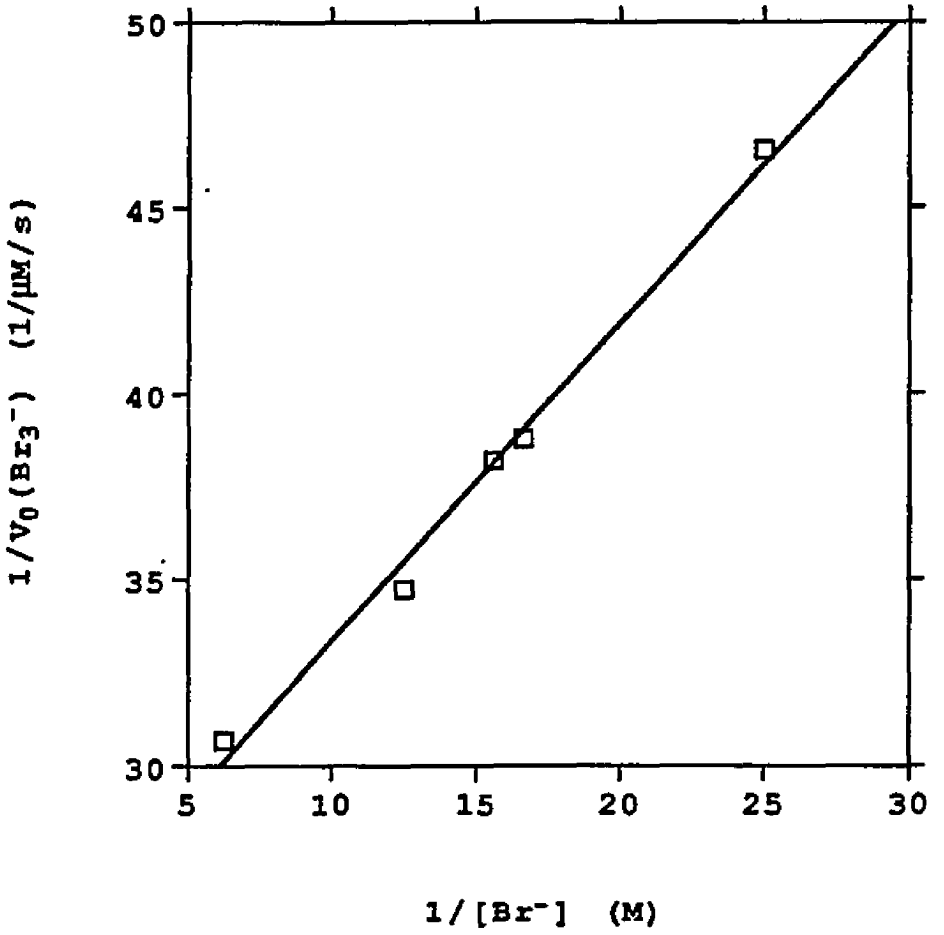
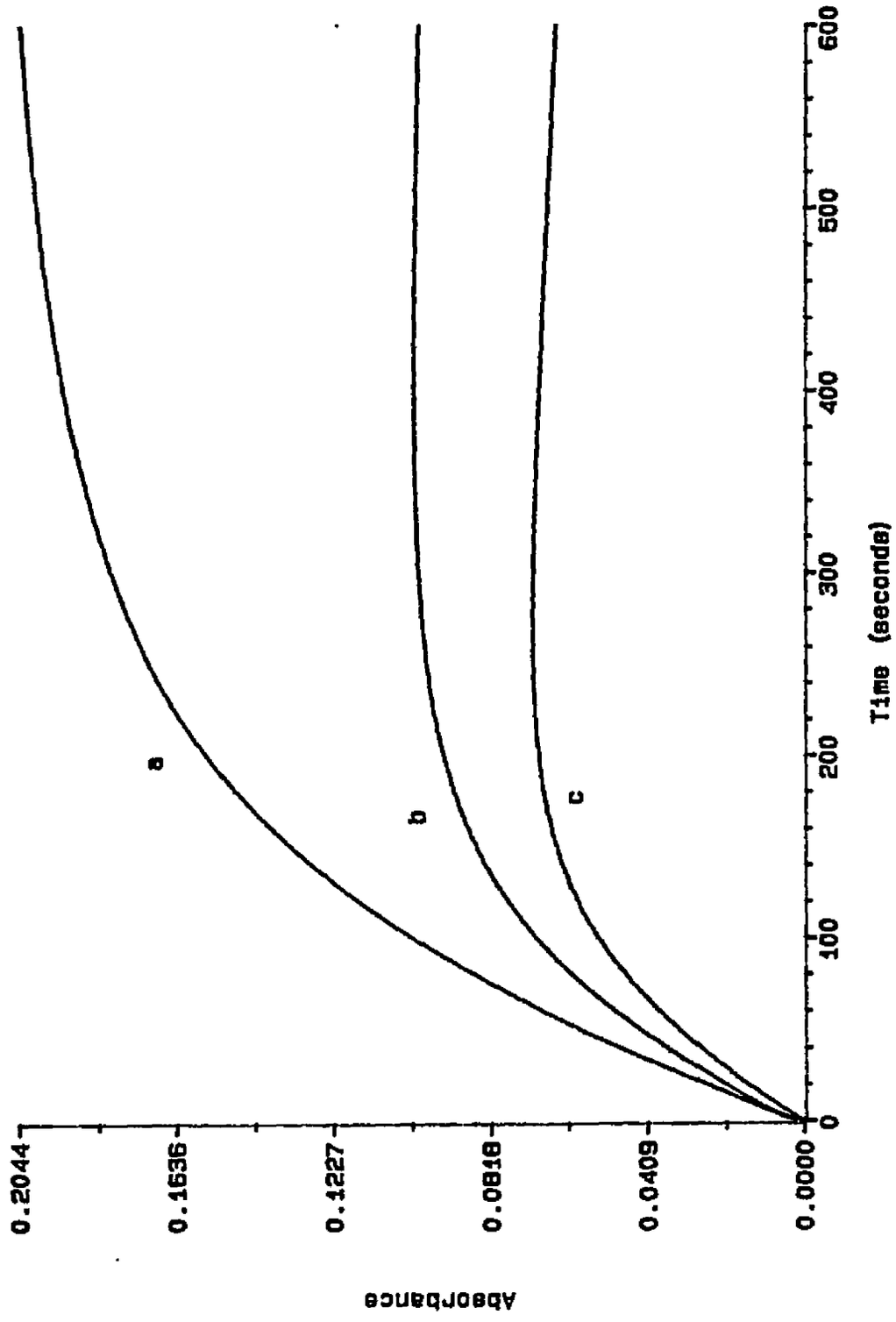


Figure 6. Formation of Br_3^- monitored at 267 nm in the CPO-catalyzed hydrogen peroxide/bromide system. Stopped-flow accessory was used. Both syringe 1 and 2 contained (a) 0.08 M, (b) 0.064 M, or (c) 0.04 M KBr. Other conditions were the same as Fig. 5.



The amount of CPO was directly related to the formation of Br_2/Br_3^- species. At constant concentrations of Br^- and H_2O_2, a linear relationship between the enzyme concentration and the initial formation rate of Br_3^- was observed (Fig. 7a). Moreover, a plot of $1/[Br_3^-]_{max}$(maximum concentration of Br_3^- obtained) *vs* 1/[CPO] was linear (Fig. 7b). If the enzyme was added twice (the first portion was added at the beginning to start the reaction, and the second portion was added when the reaction was close to equilibrium), a rapid increase in the Br_3^- 267 nm absorption (a Br_3^- reformation) was obtained, as shown on Figs. 8 & 9.

HYDROGEN PEROXIDE/BROMIDE SYSTEM

No reaction to produce Br_3^- occurred without the addition of CPO (data not shown).

CPO/L-TYROSINE/HYDROGEN PEROXIDE/BROMIDE SYSTEM

MBT was formed at different L-tyrosine, bromide, and hydrogen peroxide concentrations. At a fixed CPO concentration and when $[Br^-] >> [H_2O_2] >> [TYR]$, the maximum amount of MBT was formed and the highest MBT initial formation rate could be obtained. When $[Br^-] = [H_2O_2] >> [TYR]$, the amount of MBT formed was less and the MBT initial formation rate was lower. When $[Br^-] = [H_2O_2] = [TYR]$, the formation of MBT was minimum and the MBT initial formation rate was lowest (see Table 1, a -- c). Again, these data were related to the amount of CPO: when less CPO was added,

Figure 7. A) Plot of v_0 (initial Br_3^- formation rate) *vs* [CPO]. Stopped-flow accessory was used. Syringe 1 contained 2 X 10^{-3} M H_2O_2. Syringe 2 (as blank) contained 2.12, 4.24, 6.36, or 8.48 nM CPO. Both of the two syringes contained 0.05 M KBr and 8 mM acetate buffer (pH 4.0).

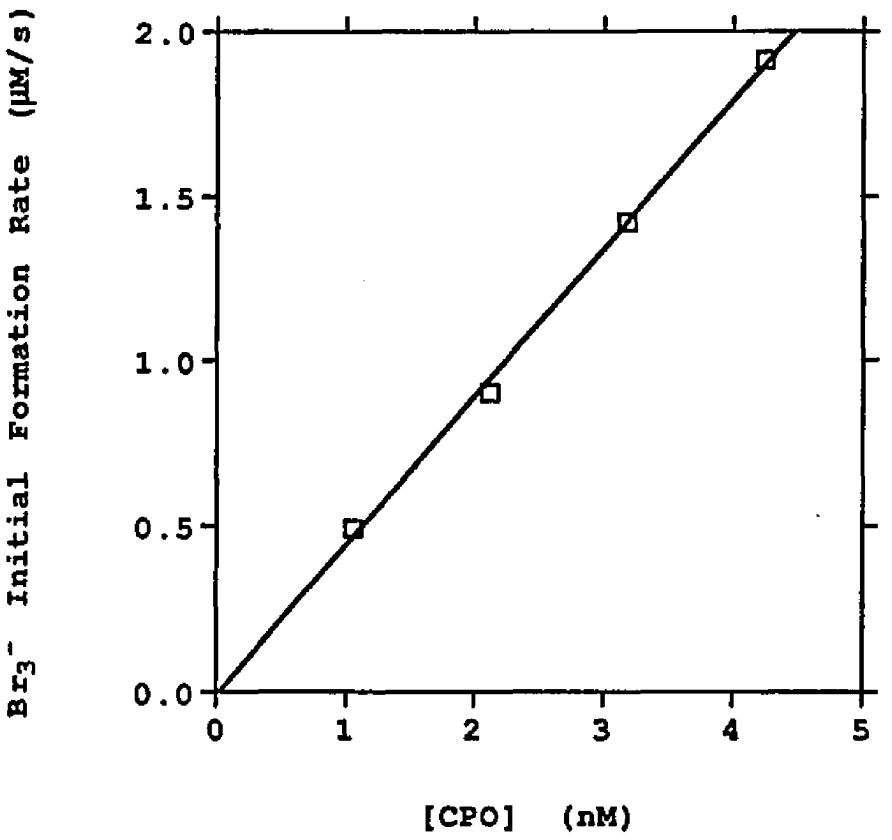


Figure 7. B) Plot $1/[Br_3^-]_{max}$(maximum Br_3^- concentration) *vs* 1/[CPO]. Reaction conditions were the same as Fig. 7A.

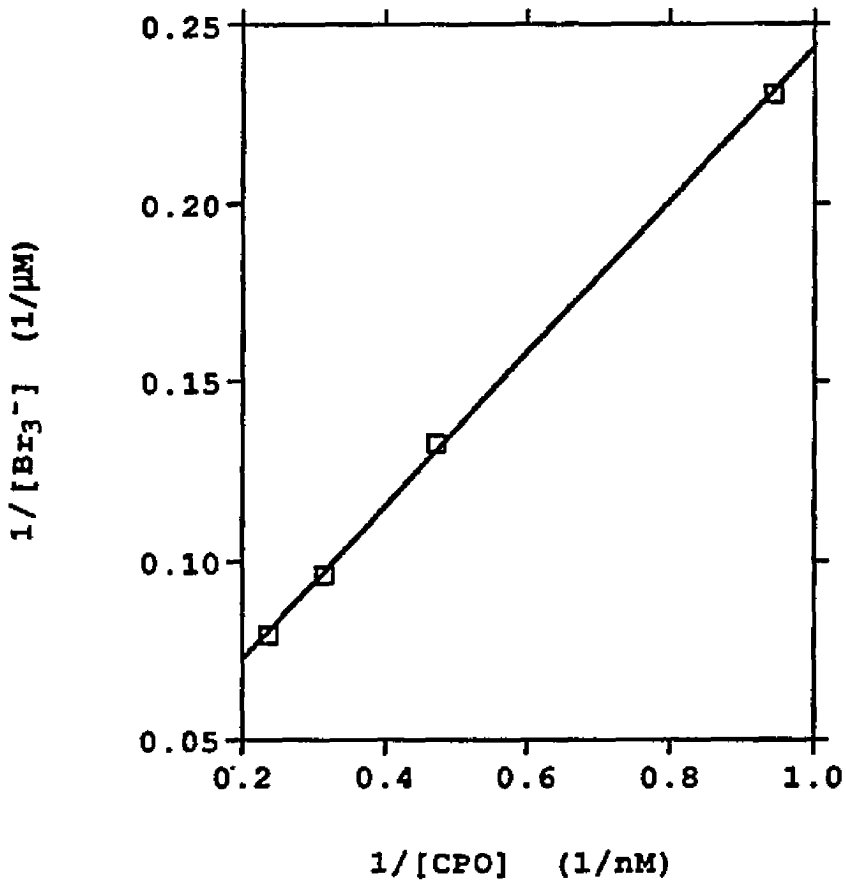


Figure 8. Formation of Br_3^- monitored at 267 nm in the CPO-catalyzed hydrogen peroxide/bromide system. 1 ml solution containing 0.1 M KBr, 8 mM acetate buffer, pH 4.0, and 1 X 10^{-5} M H_2O_2 was added into a microcuvette as blank. 0.02 ml 0.1 μM CPO was added two times into the microcuvette: at 0 second and at the 450th second.

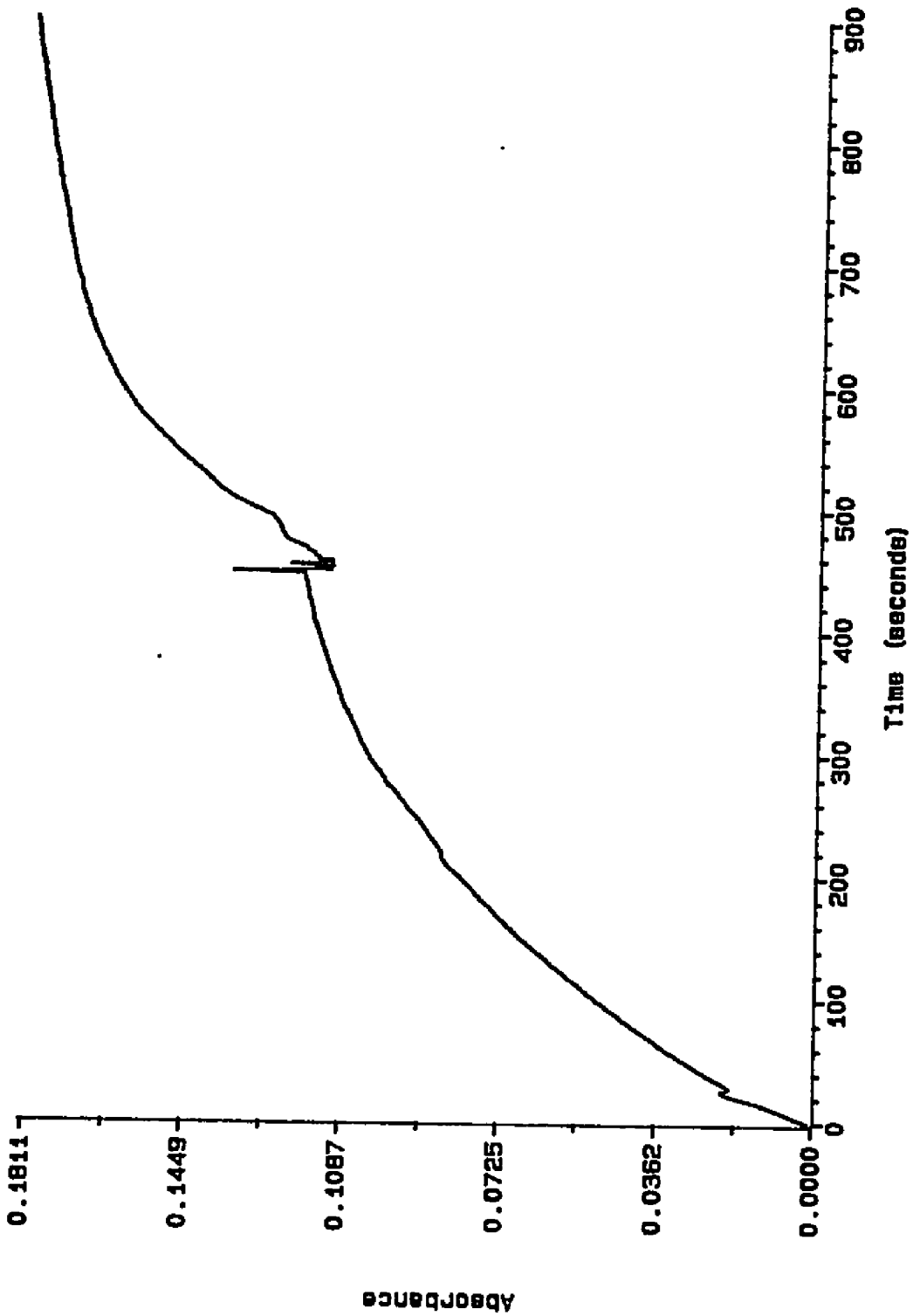


Figure 9. Formation of Br_3^- monitored at 267 nm in the CPO-catalyzed hydrogen peroxide/bromide system. 1 ml solution containing 0.05 M KBr, 8 mM acetate buffer, pH 4.0, and 5 X 10^{-4} M H_2O_2 was added into a microcuvette as blank. 0.02 ml 0.1 μM CPO was added two times into the microcuvette: at 0 second and at the 525th second.

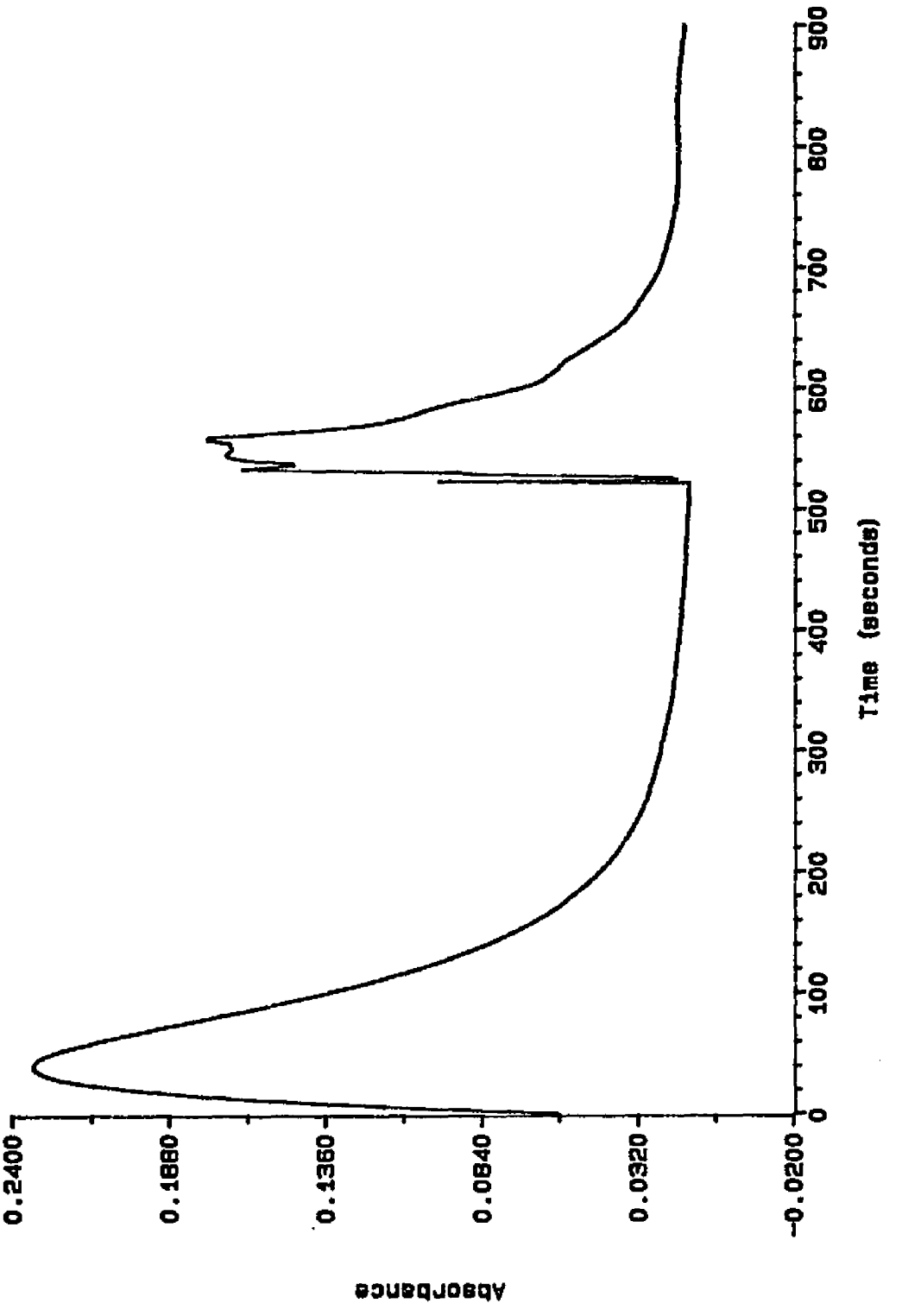


Table 1. Formation of MBT in CPO/L-Tyrosine/ Hydrogen Peroxide/Bromide System*

	MBT Concentration (mM)	MBT Initial Formation Rate (μM/s)
Reaction a	0.3711	1.774
Reaction b	0.2914	0.8355
Reaction c	0.1591	0.2970
Reaction d	0.2714	0.8570
Reaction e	0.3711	1.774
Reaction f	0.3832	3.763

*MBT concentrations were measured at the 900th second of the reactions. Reactions (a) -- (c): CPO concentration was 2.12 nM, and 8 mM acetate buffer, pH 4.0, was added. Other reaction conditions were: reaction (a). $[Br^-]$ = 0.05 M, $[H_2O_2]$ = 1 mM, and [TYR] = 0.3840 mM; (b). $[Br^-]$ = $[H_2O_2]$ = 2.5 mM, and [TYR] = 0.3840 mM; and (c). $[Br^-]$ = $[H_2O_2]$ = [TYR] = 0.3840 mM. Reaction (d) -- (f): $[Br^-]$ = 0.05 M, $[H_2O_2]$ = 1 mM, [TYR] = 0.3840 mM, and 8 mM acetate buffer, pH 4.0, was added. CPO concentration was (d) 1.06 nM, (e) 2.12 nM, and (f) 4.24 nM.

the formation of MBT was less and the MBT initial rate was lower (Table 1, d -- f). If a second portion of CPO was added when the reaction was close to equilibrium, a rapid increase of the absorption at 290 nm could be observed (Fig. 10).

In this reaction system, two unidentified products were also formed, which showed maximum absorptions at 330 - 340 nm and 430 - 440 nm, respectively. A formation sequence for the above products was observed: MBT formed first, the unknown at 330 - 340 nm second, and the unknown at 430 - 440 nm last (Fig. 11). When the unknown at 430 - 440 nm was formed, the color of the reaction liquid changed from colorless to brown. The concentration of the unknown at 430 - 440 nm was estimated to be at least at the same level as the MBT concentration.

L-TYROSINE/HYDROGEN PEROXIDE/BROMIDE SYSTEM

No reaction to produce MBT or Br_3^- occurred without the addition of CPO (data not shown).

L-TYROSINE/BROMINE SYSTEM

MBT was also formed in the nonenzymic bromination system. Different [TYR]/[Br_2] ratios were examined, and the results are listed as Table 2 (g -- i).

Two unidentified products, at 330 - 340 nm and 430 - 440 nm, respectively, were also detected. The same formation sequence as the CPO/tyrosine/hydrogen

Figure 10. Formation of MBT monitored at 290 nm in the CPO-catalyzed L-tyrosine/hydrogen peroxide/bromide system. 1 ml solution containing 0.385 mM L-tyrosine, 2.5 X 10^{-3} M KBr, 8 mM acetate buffer, pH 4.0, and 2.5 X 10^{-3} M H_2O_2 was added into a microcuvette as blank. 0.02 ml 0.1 μM CPO was added two times into the microcuvette: at 0 second and at the 600th second.

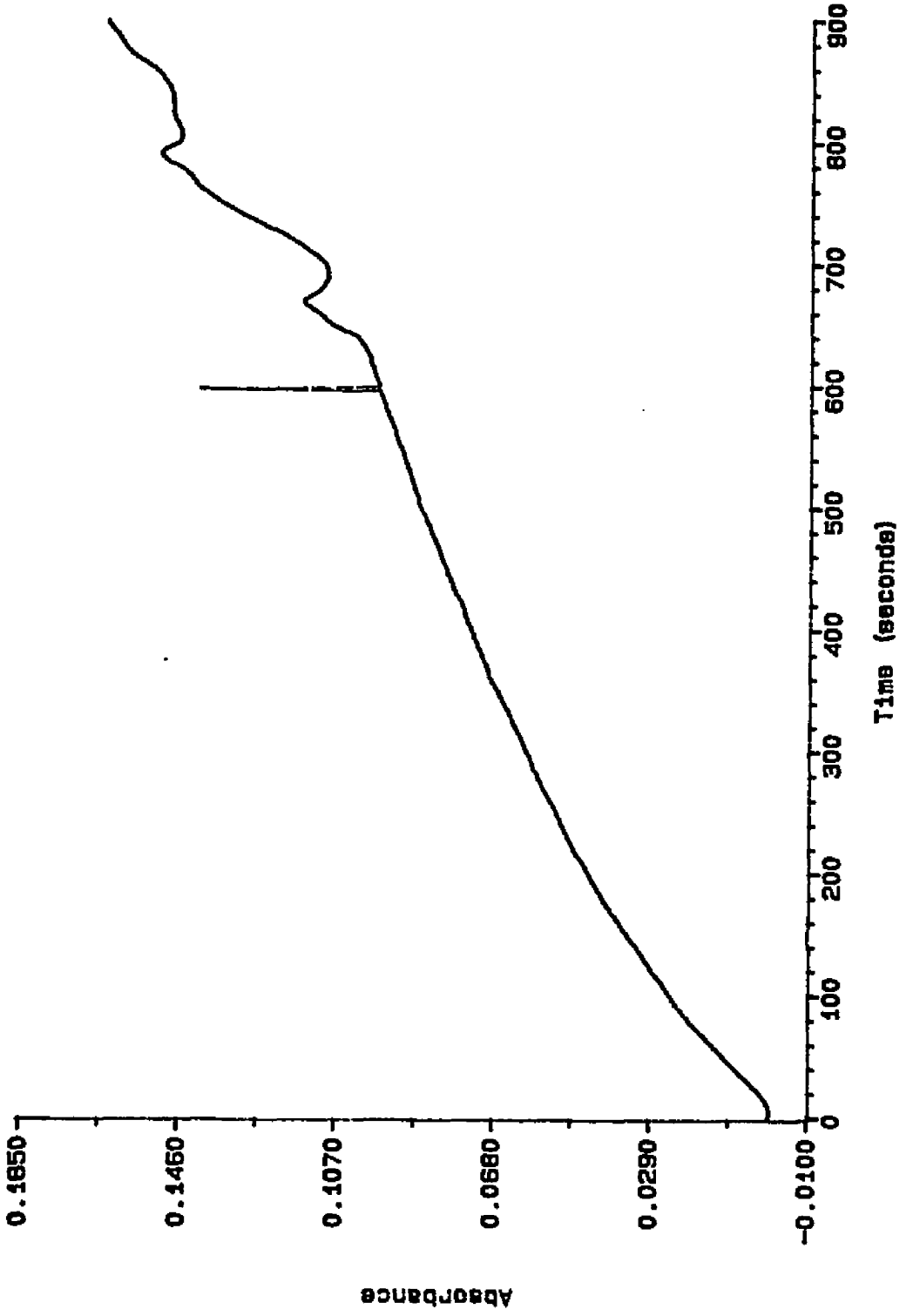


Figure 11. Products formation sequence in the CPO-catalyzed L-tyrosine/hydrogen peroxide/bromide system. Stopped-flow accessory was used. Syringe 1 contained 1 X 10^{-3} M H_2O_2. Syringe 2 (as blank) contained 2.12 nM CPO and 0.385 mM L-tyrosine. Both of the two syringes contained 0.05 M KBr and 8 mM acetate buffer (pH 4.0). Curve (a): monitored at 290 nm; curve (b): at 334 nm; and curve (c): at 434 nm.

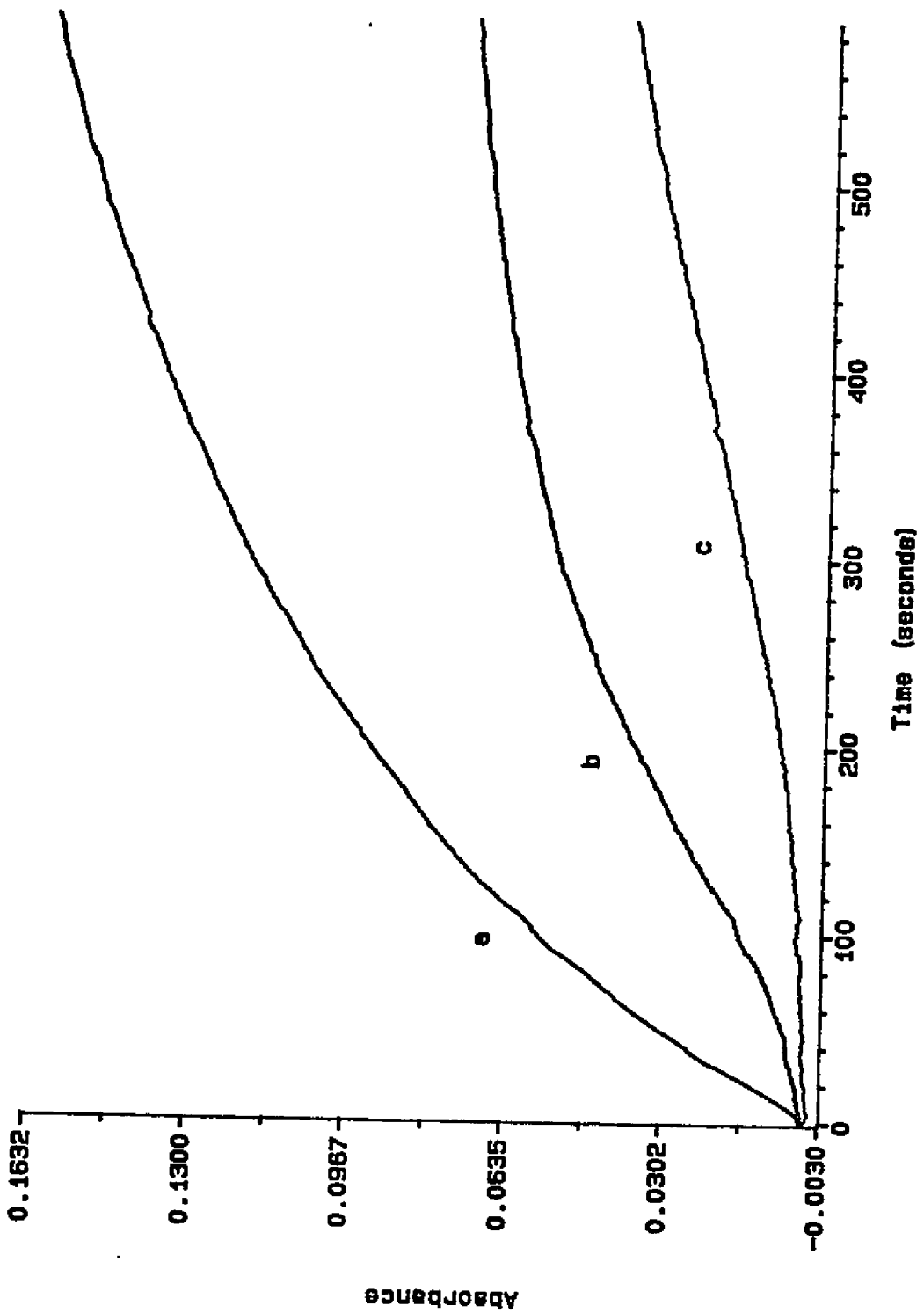


Table 2. Formation of MBT in L-Tyrosine/Bromine System and L-Tyrosine/Bromine/Hydrogen Peroxide System*

	MBT Concentration (mM)	MBT Initial Formation Rate (μM/s)
Reaction g	0.3825	17.38
Reaction h	0.1704	0.1564
Reaction i	0.2381	0.2610
Reaction j	0.2029	0.1817
Reaction k	0.2099	0.2221
Reaction l	0.2264	0.2312

*In L-tyrosine/bromine system (reaction g -- i), [TYR] = 0.3840 mM, and 8 mM acetate buffer, pH 4.0, was added. Br_2 concentration was (g) 1.553 mM, (h) 0.3840 mM, and (i) 0.768 mM. In L-tyrosine/bromine/hydrogen peroxide system (reaction j -- l), [TYR] = [Br_2] = 0.3840 mM, and 8 mM acetate buffer, pH 4.0, was added. H_2O_2 concentration was (j) 1.000 mM, (k) 0.3840 mM, and (l) 0.1920 mM. MBT concentration result was measured at the 30th second for reaction (g) since MBT reacted with Br_2 to form DBT very quickly, otherwise it was measured at the 900th second.

peroxide/bromide system for MBT and the two unknowns was found.

L-TYROSINE/BROMINE/HYDROGEN PEROXIDE SYSTEM

At fixed tyrosine and bromine concentrations, formation of MBT was examined with various H_2O_2 concentrations. The results are listed as Table 2 (j -- 1).

CPO/L-TYROSINE/BROMINE/HYDROGEN PEROXIDE SYSTEM

The formation of MBT (amount and initial rate) was not affected by the addition of CPO. Data essentially identical to the tyrosine/bromine/hydrogen peroxide system were obtained (data not shown).

CPO/BROMINE SYSTEM

CPO's absorption spectrum was measured when CPO was mixed with bromine at various concentrations at pH 4.0 (Fig.12). The destruction of CPO was observed: the Soret (heme) absorption (385 - 415 nm, with the maximum absorption at 398 nm) decreased irreversibly with the addition of bromine, as well as a peak near 267 nm formed and increased (Fig. 13). If the bromine concentration was high enough, the Soret band would totally disappear.

It was determined that when the Br_2/CPO ratio was higher than 53±3 (M/M), the Soret band disappeared totally. However, if the Br_2/CPO ratio was less than 4.3±0.8 (M/M), the Soret band remained unaffected. When 4.3±0.8 (M/M) < Br_2/CPO < 53±3 (M/M), the Soret band decreased with the

Figure 12. Soret band of the bromine-treated CPO. Stopped-flow accessory was used. Spectra shown were measured at the 100th second after the starting of the reaction. Syringe 1 contained 0.1478 μM CPO. Syringe 2 (as blank) contained (a) 3.882×10^{-4}, (b) 1.553×10^{-3}, (c) 3.882×10^{-3}, or (d) 1.553×10^{-2} mM Br_2. Both of the two syringes contained 8 mM acetate buffer, pH 4.0. The Soret spectrum of untreated-CPO (same concentration) was the same as curve (a).

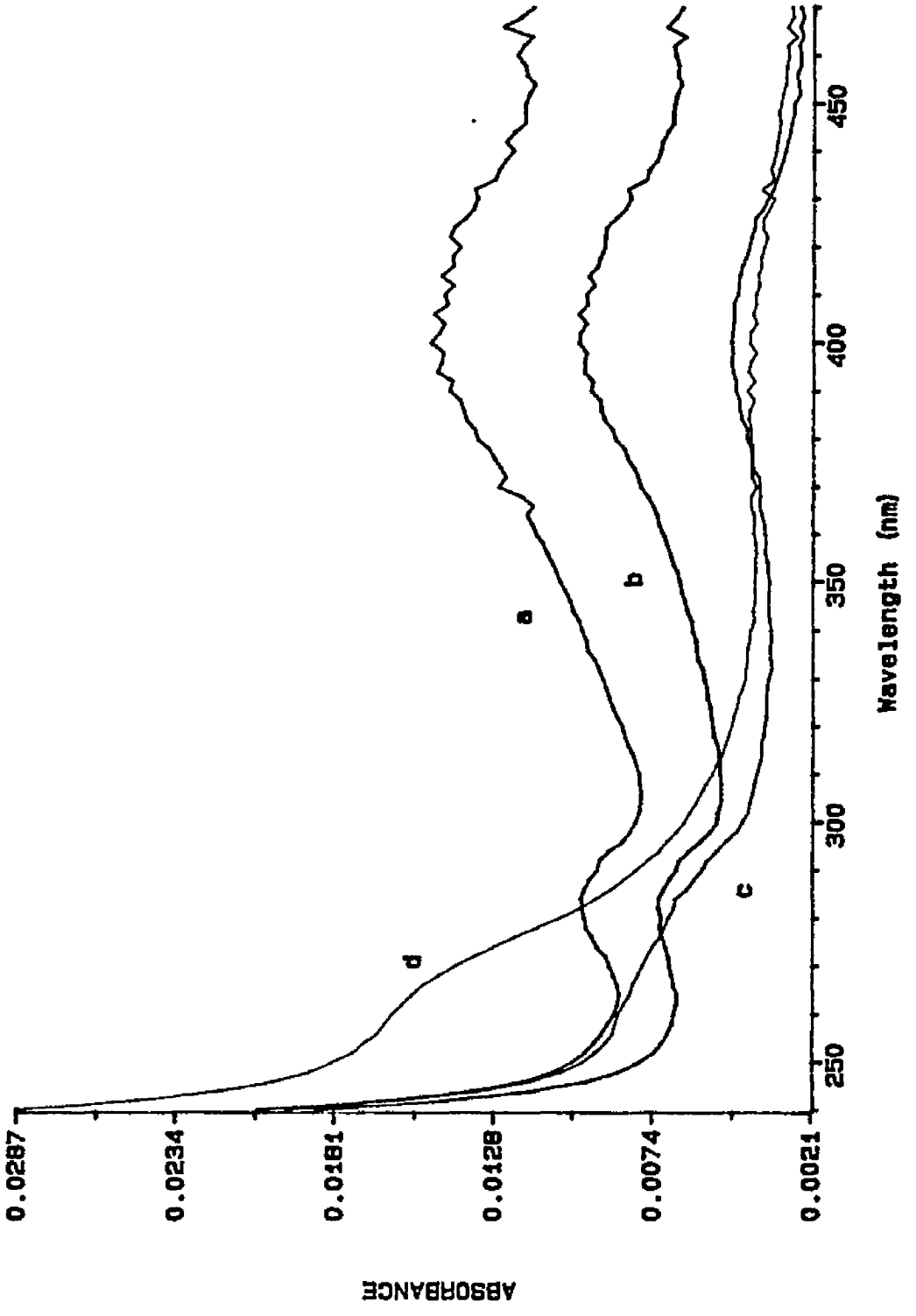
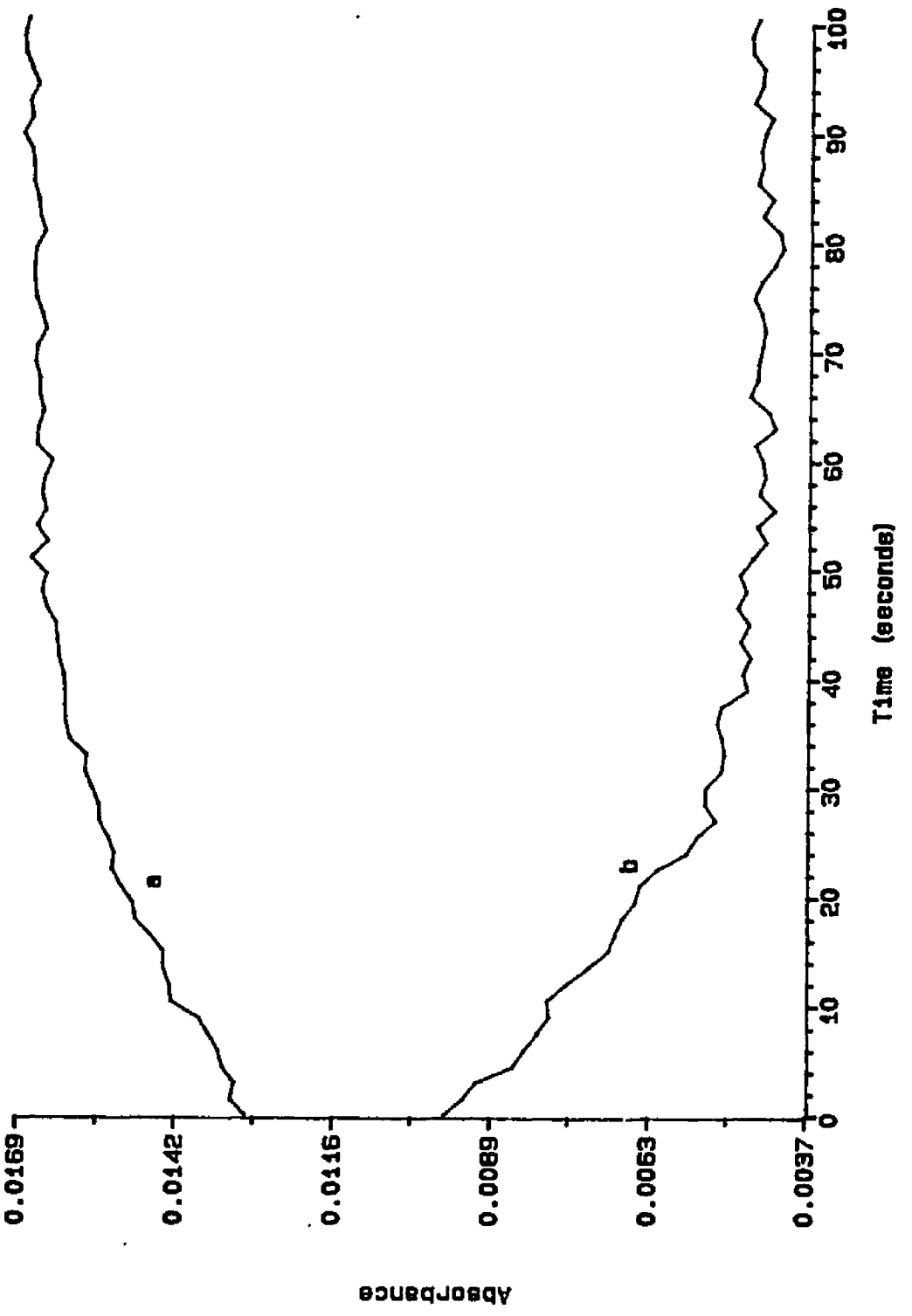


Figure 13. Bromine-treated CPO: absorbances at (a) 267 nm and (b) 398 nm. Same conditions as Fig. 12 curve (d).



increase of the Br_2/CPO ratio, and a first order relationship between them was observed.

HYDROGEN PEROXIDE/BROMIDE SYSTEM WITH BROMINE-TREATED CPO AS ENZYME

When Br^- and H_2O_2 concentrations were kept constant, a mixture of CPO and Br_2 with various Br_2/CPO ratios was added to the reaction solution to start the reaction, and the formation of tribromide (Br_2/Br_3^- species) was measured. The results were compared with that for the reaction catalyzed by CPO which was not treated with bromine. It was found that if the Br_2/CPO ratio was higher than 53±3 (M/M), the formation of Br_2/Br_3^- species was too slow to be observed; and if the Br_2/CPO ratio was less than 4.3±0.8 (M/M), the same result as for the untreated CPO-catalyzed reaction was obtained. When the Br_2/CPO ratio was less than 53±3 (M/M) but higher than 4.3±0.8 (M/M), the amount of Br_3^- formed was less and the initial Br_3^- formation rate was lower compared with that for the untreated CPO catalyzed reaction.(Fig. 14 & 15).

L-TYROSINE/HYDROGEN PEROXIDE/BROMIDE SYSTEM WITH BROMINE-TREATED CPO AS ENZYME

Results similar to the above system were obtained: at a Br_2/CPO ratio higher than 53±3 (M/M), no MBT formation could be observed, and at a Br_2/CPO ratio less than 4.3±0.8 (M/M), the MBT formation was not affected by the bromine treatment

Figure 14. Br_3^- formation monitored at 267 nm in the hydrogen peroxide/bromide system with bromine-treated CPO as the enzyme. 0.7 ml solution containing 0.05 M KBr, 1 X 10^{-3} M H_2O_2, and 8 mM acetate buffer, pH 4.0, was added into a microcuvette as blank. Reactions were started by adding 0.05 ml (a) 0.0739 μM untreated CPO, (b) solution containing 0.0739 μM CPO and 3.882 X 10^{-4} mM Br_2, (c) solution containing 0.0739 μM CPO and 3.882 X 10^{-3} mM Br_2, and (d) solution containing 0.0739 μM CPO and 1.553 X 10^{-2} mM Br_2.

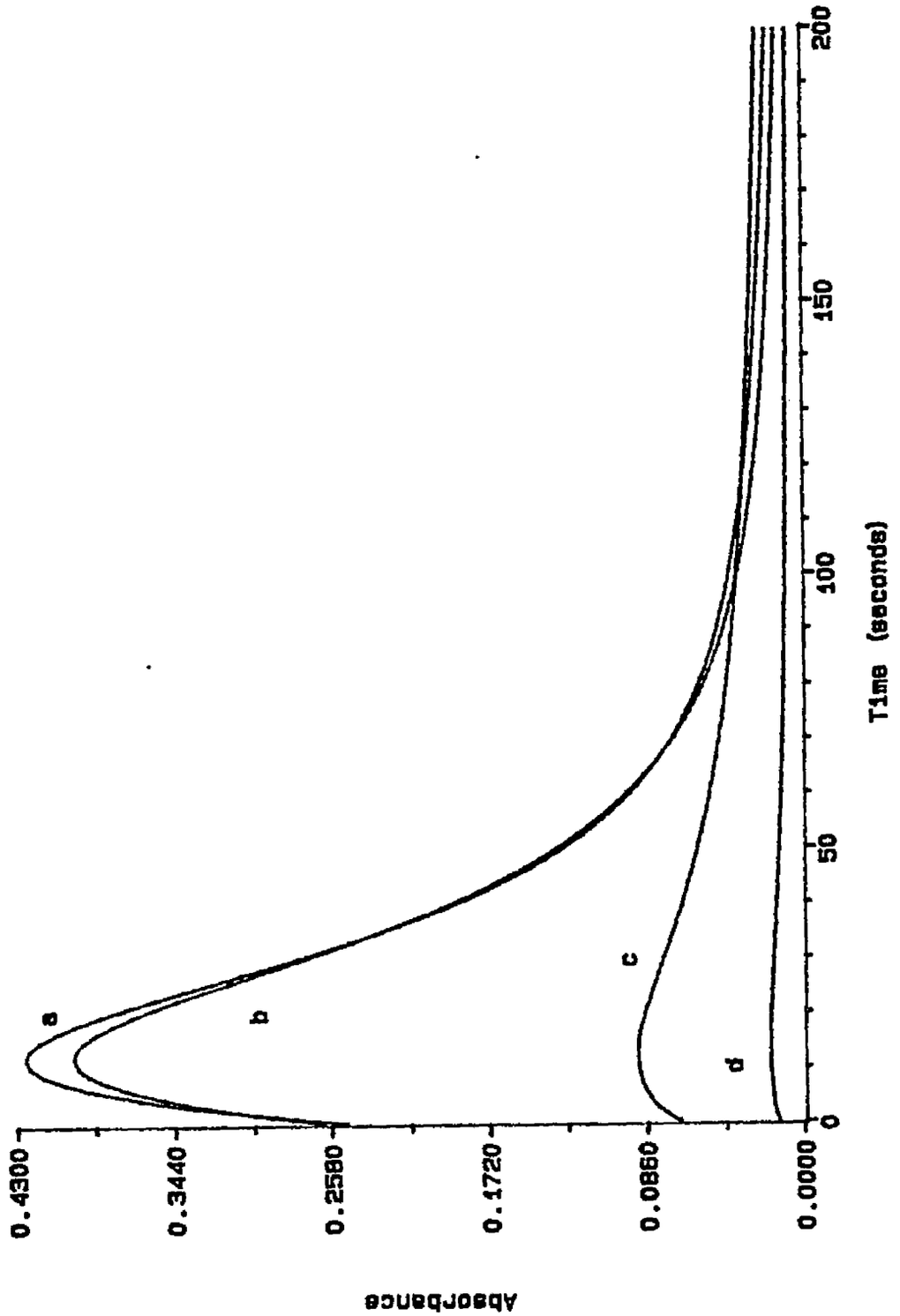
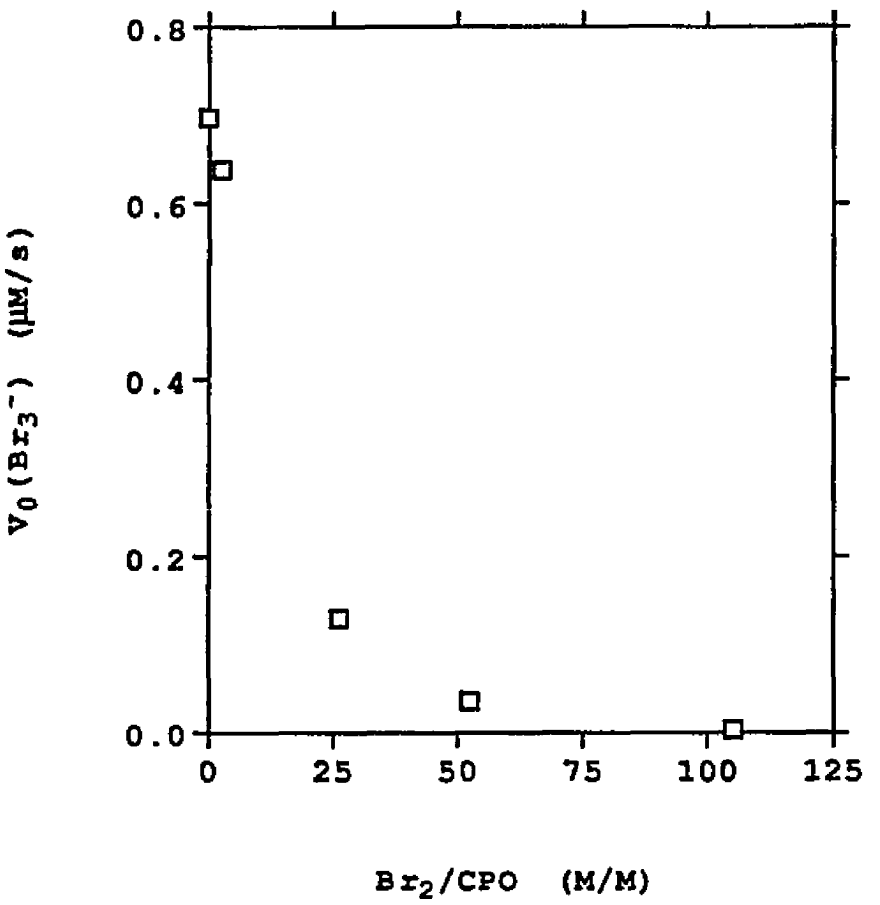


Figure 15. Plot of v_0 (initial Br_3^- formation rate) *vs* Br_2/CPO ratio (M/M). Reaction conditions were the same as Fig. 14.



(Fig. 16 & 17).

CPO/TRIBROMIDE SYSTEM

When CPO was mixed with Br_3^- solutions (different concentrations), no decrease of the Soret band was observed, and if the tribromide-treated CPO was used in the hydrogen peroxide/bromide system or tyrosine/hydrogen peroxide/ bromide system as the catalyst, the same results as for the untreated CPO-catalyzed reactions were obtained (data not shown).

L-TYROSINE/TRIBROMIDE SYSTEM

The formation of MBT and the two unknown products (at 330 - 340 and 430 - 440 nm, respectively) was not observed when mixing L-tyrosine with Br_3^- solutions (different concentrations) at pH 4.0 acetate buffer (data not shown).

CPO/D-TYROSINE/HYDROGEN PEROXIDE/BROMIDE SYSTEM

D-tyrosine was also used as the bromination substrate. The MBT initial formation rates and MBT concentrations were measured at different hydrogen peroxide and bromide concentrations (see Table 3). No stereospecific difference in the bromination rate was observed. The average initial bromination rate of D-TYR was 1.02 times of that of L-TYR (comparing Table 1, a - c with Table 3, m - o, respectively).

HPO/BROMINE SYSTEM

Figure 16. L-Tyrosine/hydrogen peroxide/bromide system with bromine-treated CPO as the enzyme. Spectra shown were measured at the 600th second of the reaction. 0.7 ml solution containing 0.1924 mM L-tyrosine, 2.5 X 10^{-3} M KBr, 2.5 X 10^{-3} M H_2O_2, and 8 mM acetate buffer, pH 4.0, was added into a microcuvette as blank. For curve (a) to (d), CPO was treated as same as Fig. 14, curve (a) to (d), respectively.

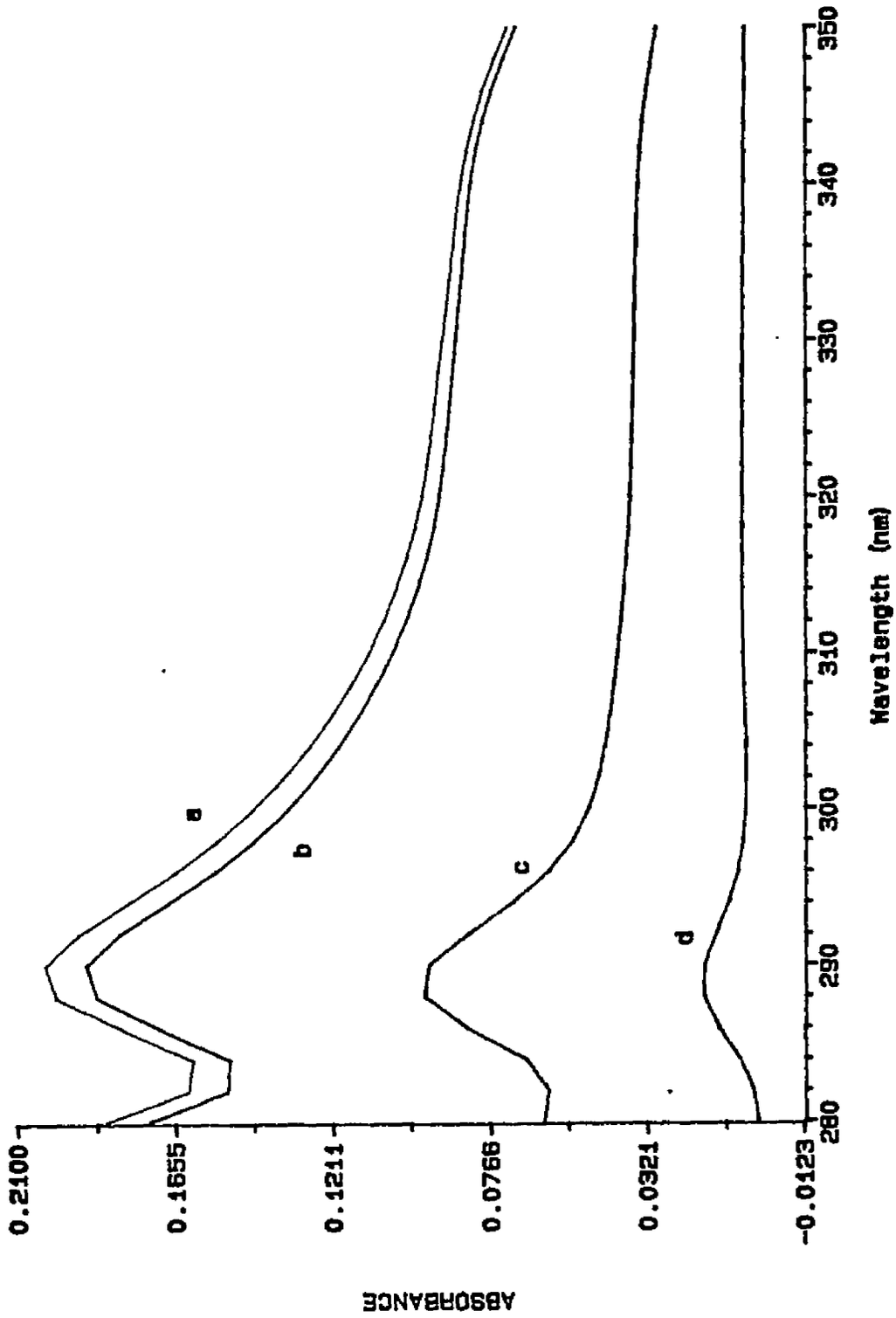


Figure 17. Plot of v_0 (initial MBT formation rate) *vs* Br_2/CPO ratio (M/M). Reaction conditions were the same as Fig. 16.

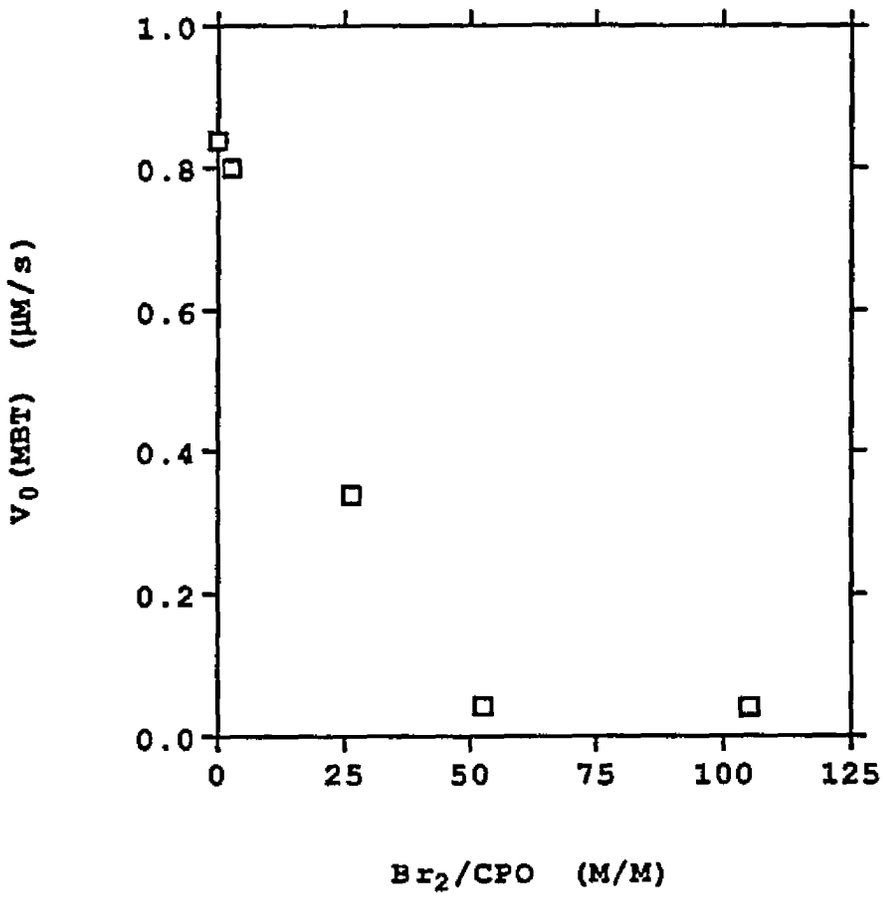


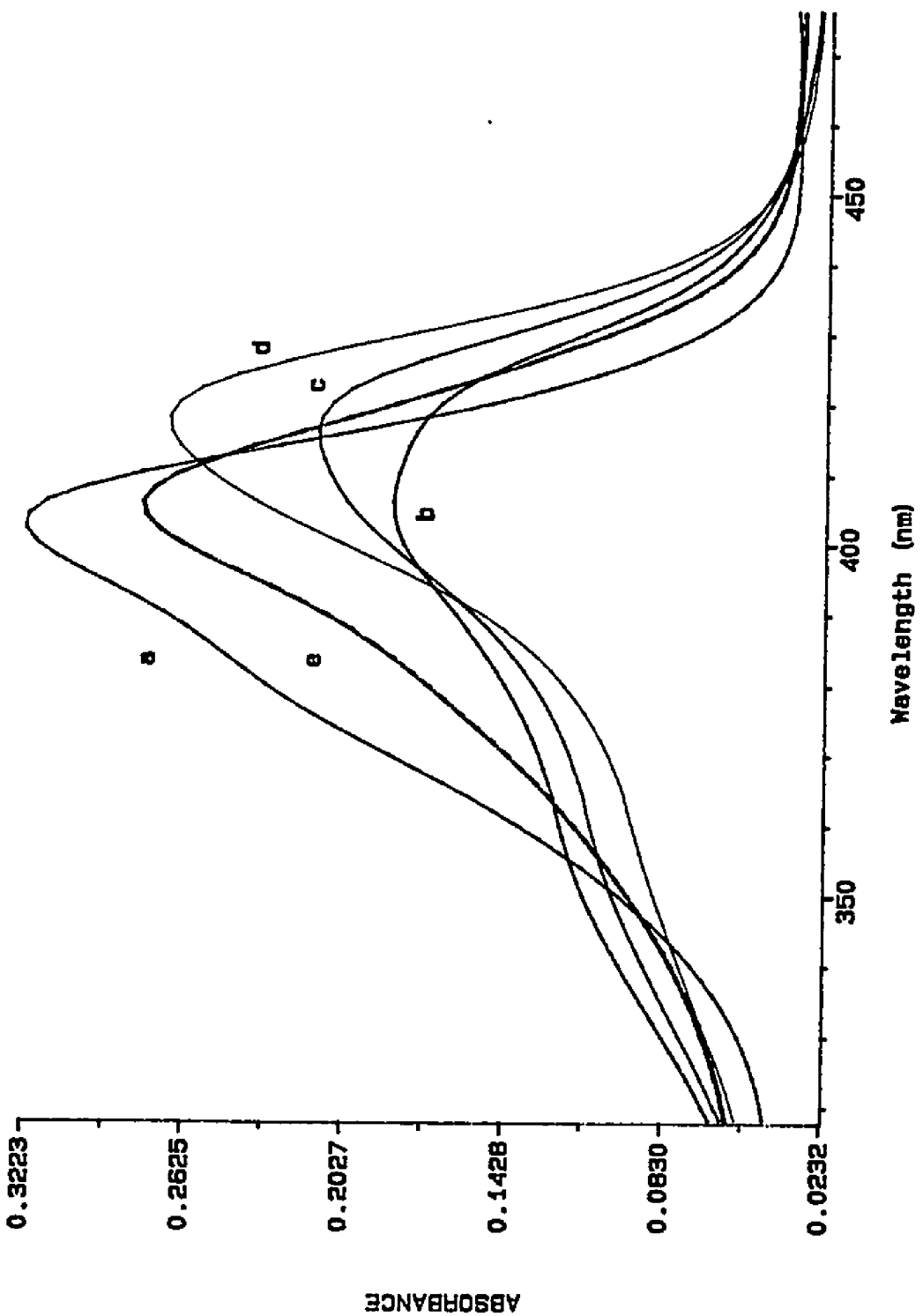
Table 3. Formation of MBT in CPO/D-Tyrosine/ Hydrogen Peroxide/Bromide System*

	MBT Concentration (mM)	MBT Initial Formation Rate (μM/s)
Reaction m	0.3786	1.813
Reaction n	0.3056	0.8642
Reaction o	0.1637	0.3310

*MBT concentrations were measured at the 900th second of the reactions. Reaction conditions for reaction (m) - (o) were the same as those for reaction (a) - (c), respectively, with the exemption that D-tyrosine were used instead of L-tyrosine.

HPO's absorption spectrum was also measured when HPO was mixed with bromine at various concentrations at pH 4.0. When HPO and Br_2 were mixed together, the following changes of HPO's Soret band (360 - 420 nm, with a maximum absorption at 402 nm) were observed: (1). the Soret band was decreased when HPO was mixed with Br_2 (Fig. 18, a: original Soret absorption; b: decrease of the Soret band); (2). when the Soret band was decreased, it was observed that this band shifted immediately from the original position (360 - 420 nm, with a maximum absorption at 402 nm) to a new wavelength range between 390 - 434 nm, with a maximum absorption at 418 nm (Fig. 18, b), and, meanwhile, the "shifted" band began to increase gradually (Fig. 18, b, c, and d); (3). when that band was increased and its maximum absorption was about 70 - 80% of the maximum absorption of the original Soret band, the "shifted" band began to shift again, but this time it shifted back to the original Soret band position (360 - 420 nm, with a maximum absorption at 402 nm, see Fig. 18, e). In our experiments, the decrease of the original Soret band (curve a to b, Fig. 18) happened as soon as HPO was mixed with Br_2, followed immediately by the shift of the original band (curve b, Fig. 18). It took 4 - 6 minutes (depending on the Br_2/HPO ratio) for the "shifted" band to increase (curve b to d, Fig. 18) before it started to shift back to the original Soret band position, and it would take another 2 - 4 minutes for the band to shift back to its original

Figure 18. Soret band of the bromine-treated HPO. 1 ml solution containing a mixture of (final concentration) 0.75 μM HPO, 0.025 mM Br_2, and 8 mM acetate buffer, pH 4.0, was added into a microcuvette. The absorption was measured immediately after the HPO and Br_2 was mixed, and also measured every 1 second after the mixing. Water was used as blank. Curve a: HPO original spectrum (without mixing with Br_2), b: 1 second after HPO was mixed with Br_2, c - e: 30, 60, 90 seconds after the mixing, respectively.



position (curve d to e, Fig. 18). The highest Br_2/HPO ratio (M/M) of 65 has been used in this system, and the decreased Soret band still could be recovered by the cycle of shift, increase, and shift back.

On the original HPO (untreated HPO) spectrum, another absorption band between 480 - 514 nm (with a maximum absorption at 500 nm) was also observed (Fig. 19, a). When HPO was mixed with Br_2, this band was decreased immediately, and, meanwhile, two new absorption bands (between 514 - 538 nm, maximum absorption at 526 nm; and between 546 - 562 nm, maximum absorption at 556 nm, respectively) were formed (Fig. 19, b). Again, it was observed that these two new bands increased gradually (took 4 - 6 minutes, see Fig. 19, c and d) and, then, shift back to the original band between 480 - 514 nm, with maximum absorption at 500 nm (took another 2 - 4 minutes, see Fig. 19, e).

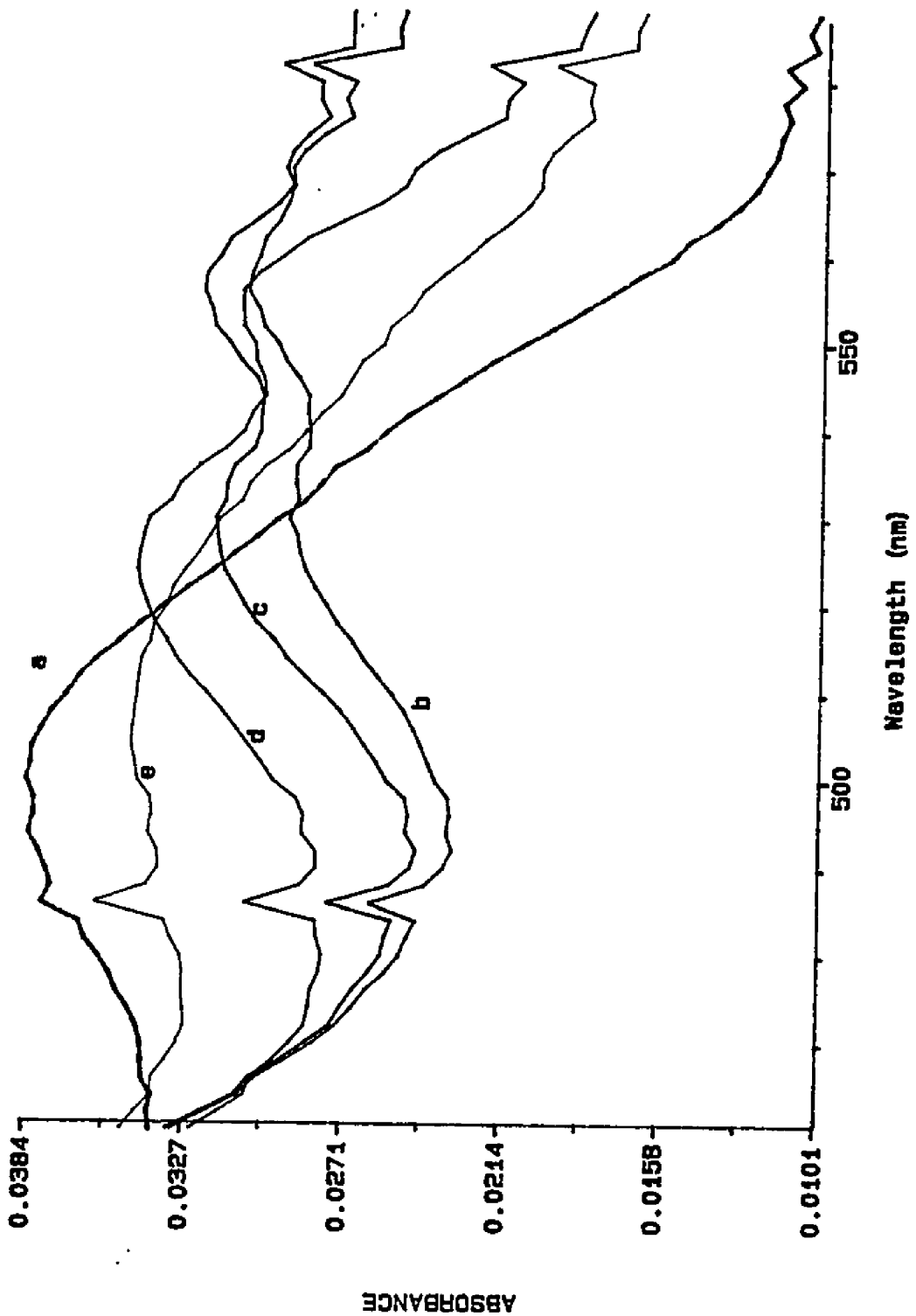
HYDROGEN PEROXIDE/BROMIDE SYSTEM WITH BROMINE-TREATED HPO AS ENZYME

Compared with the hydrogen peroxide/bromide system using untreated HPO as enzyme, the formation of Br_3^- monitored at 267 nm was not affected by using the bromine-treated HPO as enzyme (data not shown).

L-TYROSINE/HYDROGEN PEROXIDE/BROMIDE SYSTEM WITH BROMINE-TREATED HPO AS ENZYME

No enzyme deactivation was observed. The formation of

Figure 19. Absorption of the bromine-treated HPO at 450 - 600 nm. Same conditions as Fig. 18 were used. Curve a: HPO original spectrum (without mixing with Br_2), b: 1 second after HPO was mixed with Br_2, c - e: 30, 60, 90 seconds after the mixing, respectively.



MBT was not affected by the using of the bromine-treated HPO (data not shown).

HPO/HYDROGEN PEROXIDE/BROMIDE SYSTEM

Three portions of HPO were added to this system (the first portion was added at the beginning to start the reaction, and the other two portions were added when the reaction was close to equilibrium), and no rapid increase in the Br_3^- 267 nm absorption (i.e., Br_3^- reformation) was observed. The absorption at 267 nm was not affected by the second or third addition of HPO (Fig. 20).

HPO/L-TYROSINE/HYDROGEN PEROXIDE/BROMIDE SYSTEM

Three portions of HPO were added (the first portion was added at the beginning to start the reaction, and the other two portions were added when the reaction was close to equilibrium). The MBT 290 nm absorption was still very close to the original equilibrium after the second and third addition of HPO (Fig. 21). No rapid increase (jump) of the 290 nm absorption was observed.

The change of the HPO Soret band was observed directly in this reaction process: at the beginning of the reaction, the Soret band was observed to have already shifted (Fig. 22, a; between 390 - 434 nm, with a maximum absorption at 418 nm, refer to the HPO/bromine system). When the reaction was close to equilibrium, it was observed that the Soret band shifted to the original absorption range (Fig. 22, b;

Figure 20. Formation of Br_3^- monitored at 267 nm in the HPO-catalyzed hydrogen peroxide/bromide system. 1 ml solution containing 0.065 M KBr, 8 mM acetate buffer, pH 4.0, and 1 X 10^{-4} M H_2O_2 was added into a microcuvette as blank. 0.02 ml 0.1 μM HPO was added three times into the microcuvette: at 0 second, the 270th second, and the 400th second, respectively.

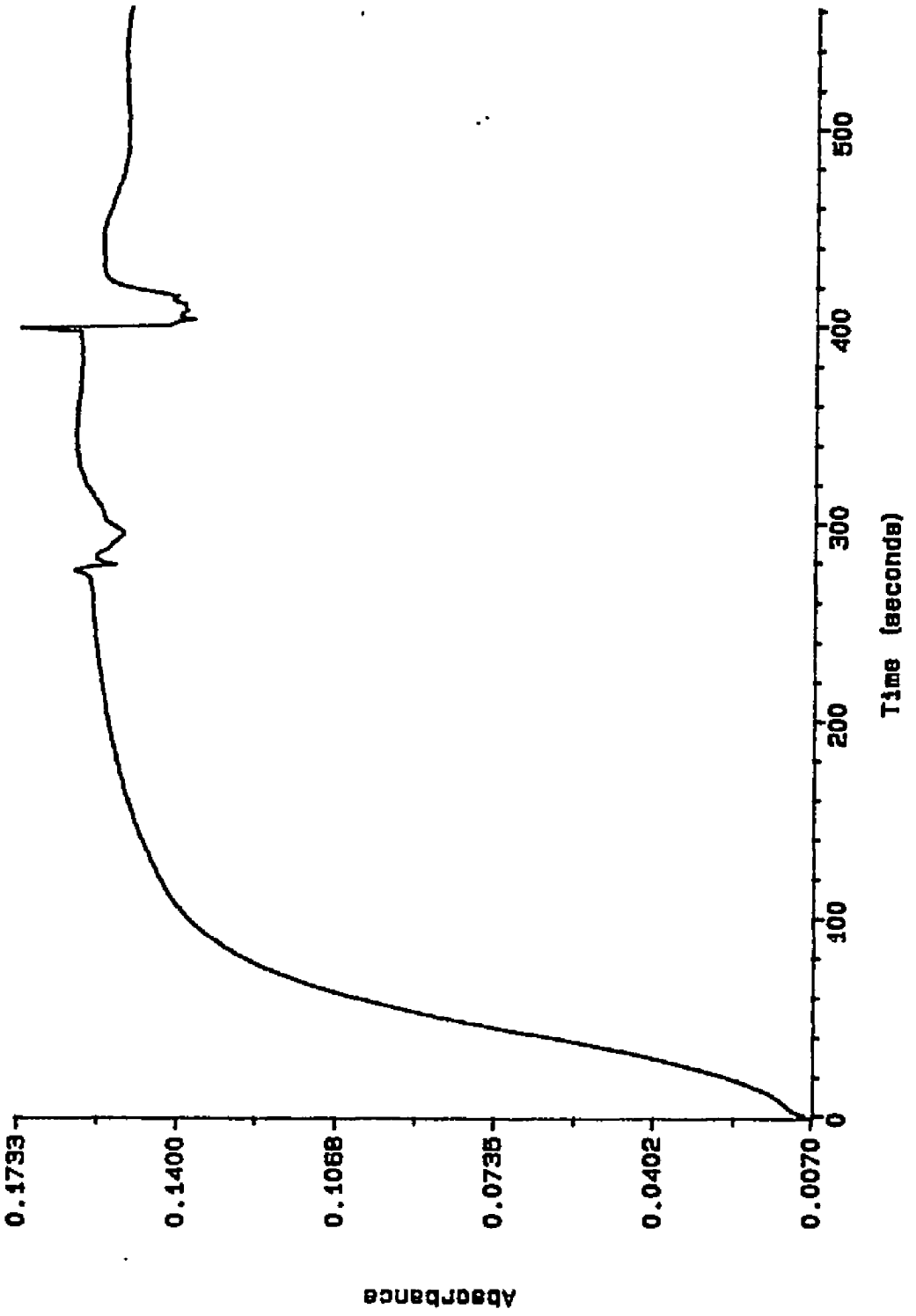


Figure 21. Formation of MBT monitored at 290 nm in the HPO-catalyzed L-tyrosine/hydrogen peroxide/bromide system. 1 ml solution containing 0.385 mM L-tyrosine, 0.065 M KBr, 8 mM acetate buffer, pH 4.0, and 1 X 10^{-3} M H_2O_2 was added into a microcuvette as blank. 0.02 ml 0.1 μM HPO was added three times into the microcuvette: at 0 second, the 990th second, and the 1310th second, respectively.

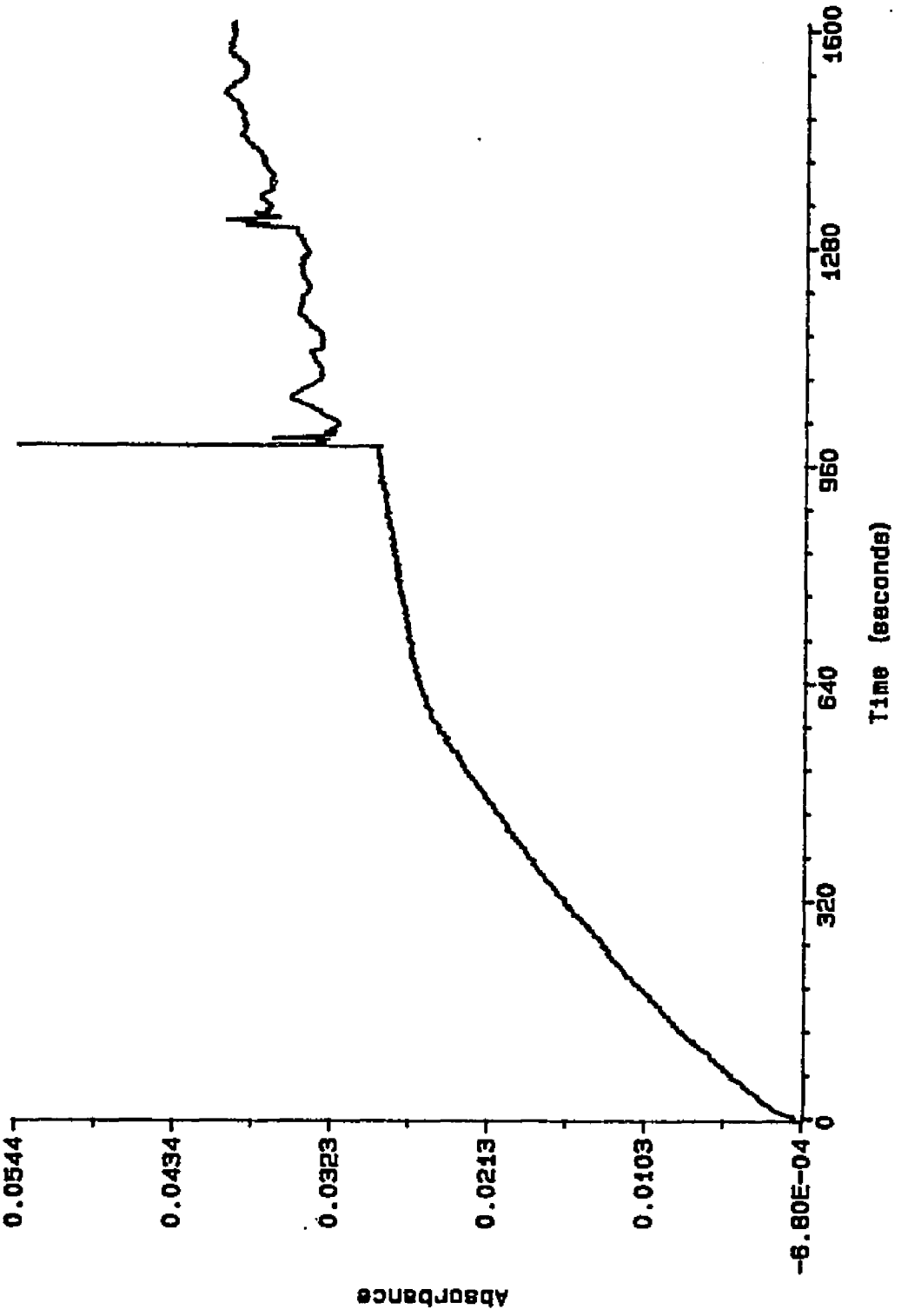
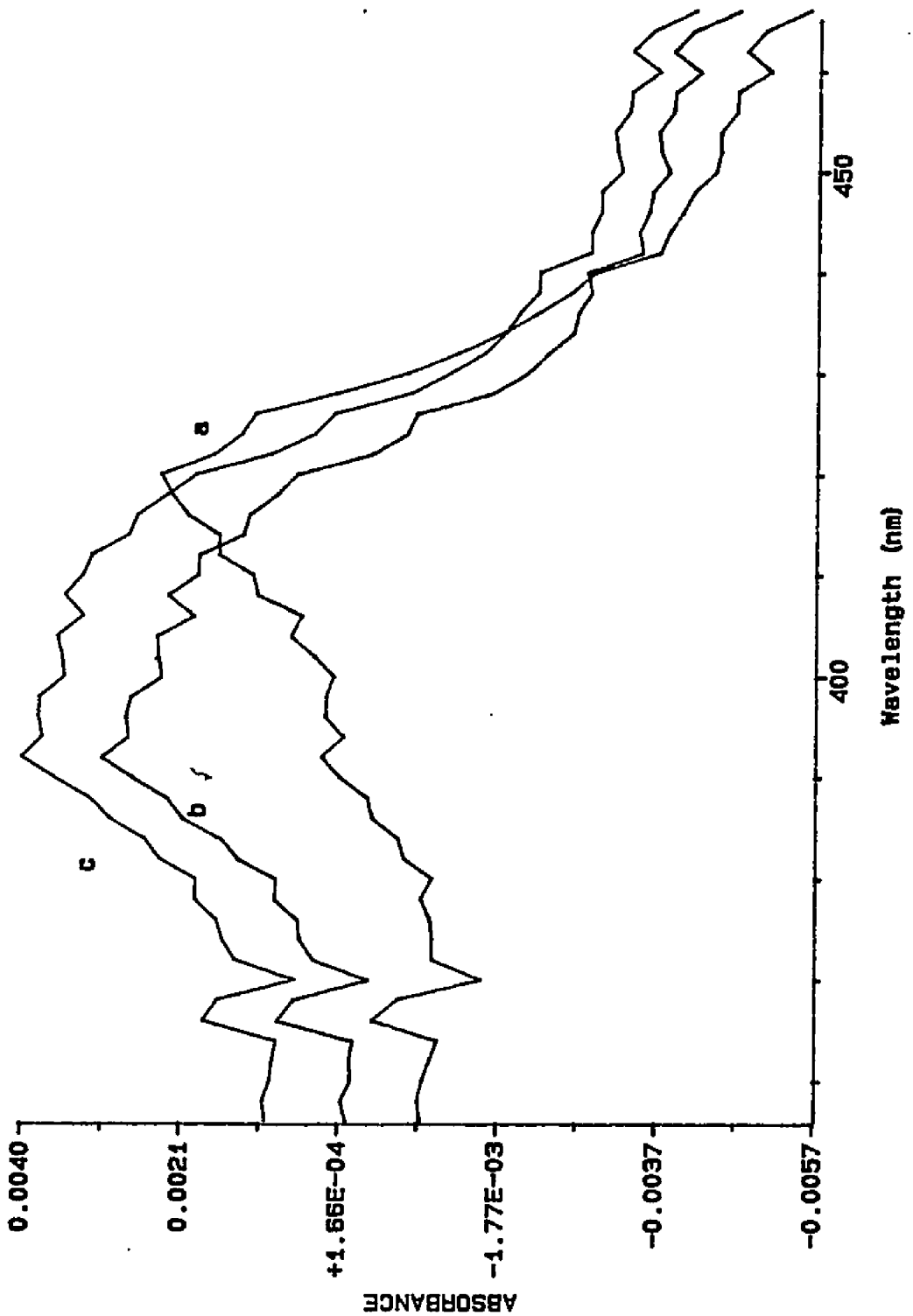


Figure 22. Soret band of HPO in the HPO-catalyzed L-tyrosine/hydrogen peroxide/bromide system. Reaction conditions were the same as Fig. 21. Curve a was obtained at the 400th second after the beginning of the reaction (i.e., after the addition the first portion of HPO), curve b was at the 900th second, and curve c at the 1150 second, respectively.



360 - 420 nm, with a maximum absorption at 402 nm, refer to the HPO/bromine system). The addition of the second portion of HPO caused an increase in the Soret band at the original absorption maximum (402 nm, Fig. 22, c), and no more band shift was observed.

LPO/BROMINE SYSTEM

LPO was mixed with bromine at different Br_2/LPO ratios, and the absorption spectrum of LPO was measured. When Br_2/LPO ratio was not very high (< 26, M/M), no obvious change of the Soret band of LPO (between 388 - 430 nm, maximum absorption at 412 nm) was observed. Instead, an absorption band between 262 - 296 nm, maximum absorption at 282 nm, was decreased and became broader with the increase of the Br_2/LPO ratio (Fig. 23, a, b, and c). If the Br_2/LPO ratio was high enough (> 50, M/M), the Soret band decreased and became broader, with a maximum absorption at 402 nm, and the absorption band between 262 - 296 nm can not be distinguished (Fig. 23, d). The first derivatives of Fig. 23, curve a, c, and d, were also obtained (Fig. 24). These derivative results showed that when Br_2/LPO ratio (M/M) was less than 26, no change was observed at wavelength 388 - 430 nm, but the peak (negative) at 262 - 296 nm was decreased with the increase of Br_2/LPO ratio (Fig. 24, a and b). When Br_2/LPO ratio (M/M) was larger than 50, the peak (negative) at 388 - 430 nm was decreased, and the peak (negative) at 262 - 296 nm was disappeared (Fig. 24, c).

Figure 23. Spectra of the bromine-treated LPO. Stopped-flow accessory was used. Spectra shown were measured at the 200th second after the starting of the reaction. Syringe 1 contained 1.507 μM LPO. Syringe 2 (as blank) contained (a) 0, (b) 1.553×10^{-3}, (c) 0.01553, (d) 0.07764 mM Br_2. Both of the two syringes contained 8 mM acetate buffer, pH 4.0.

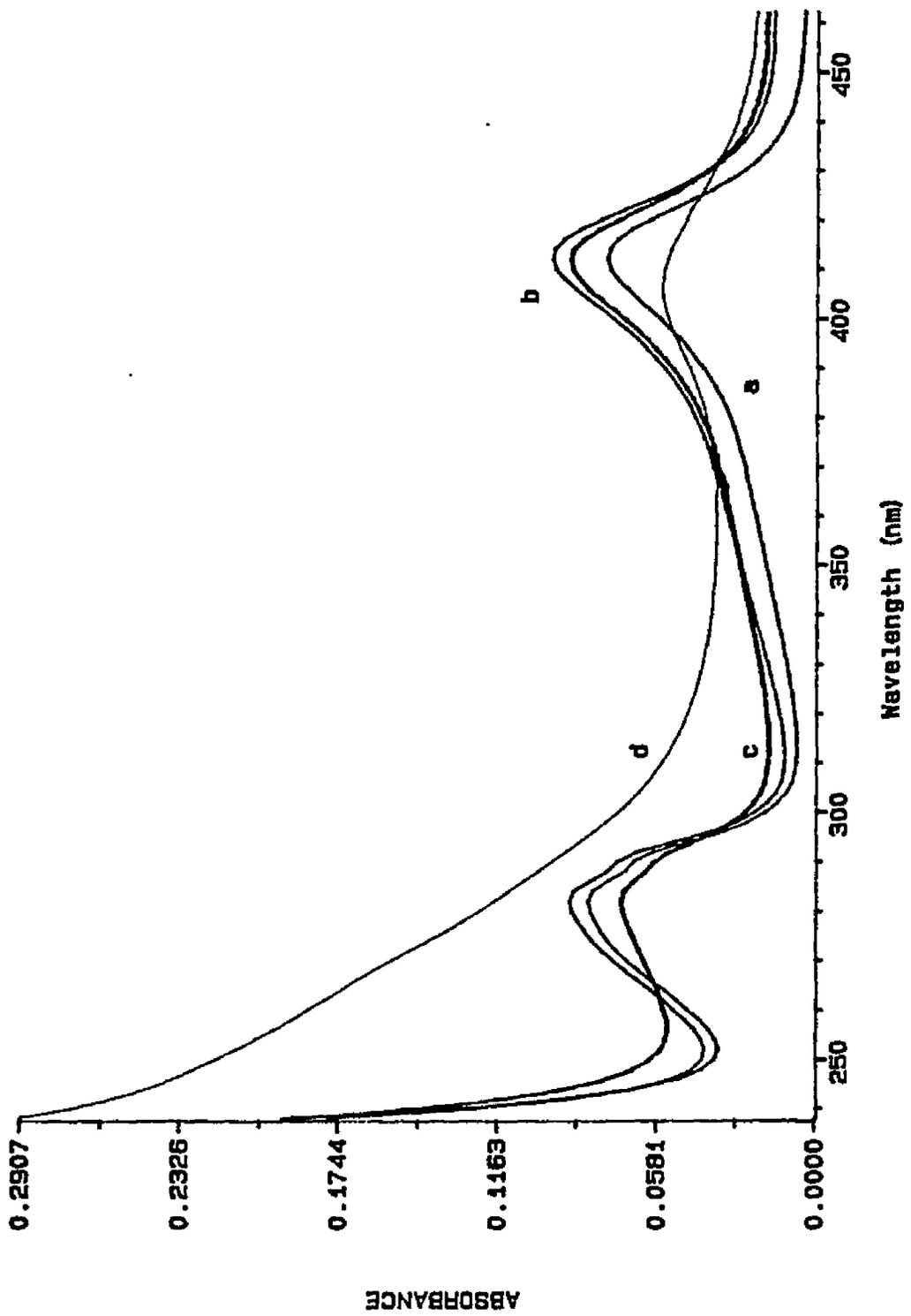
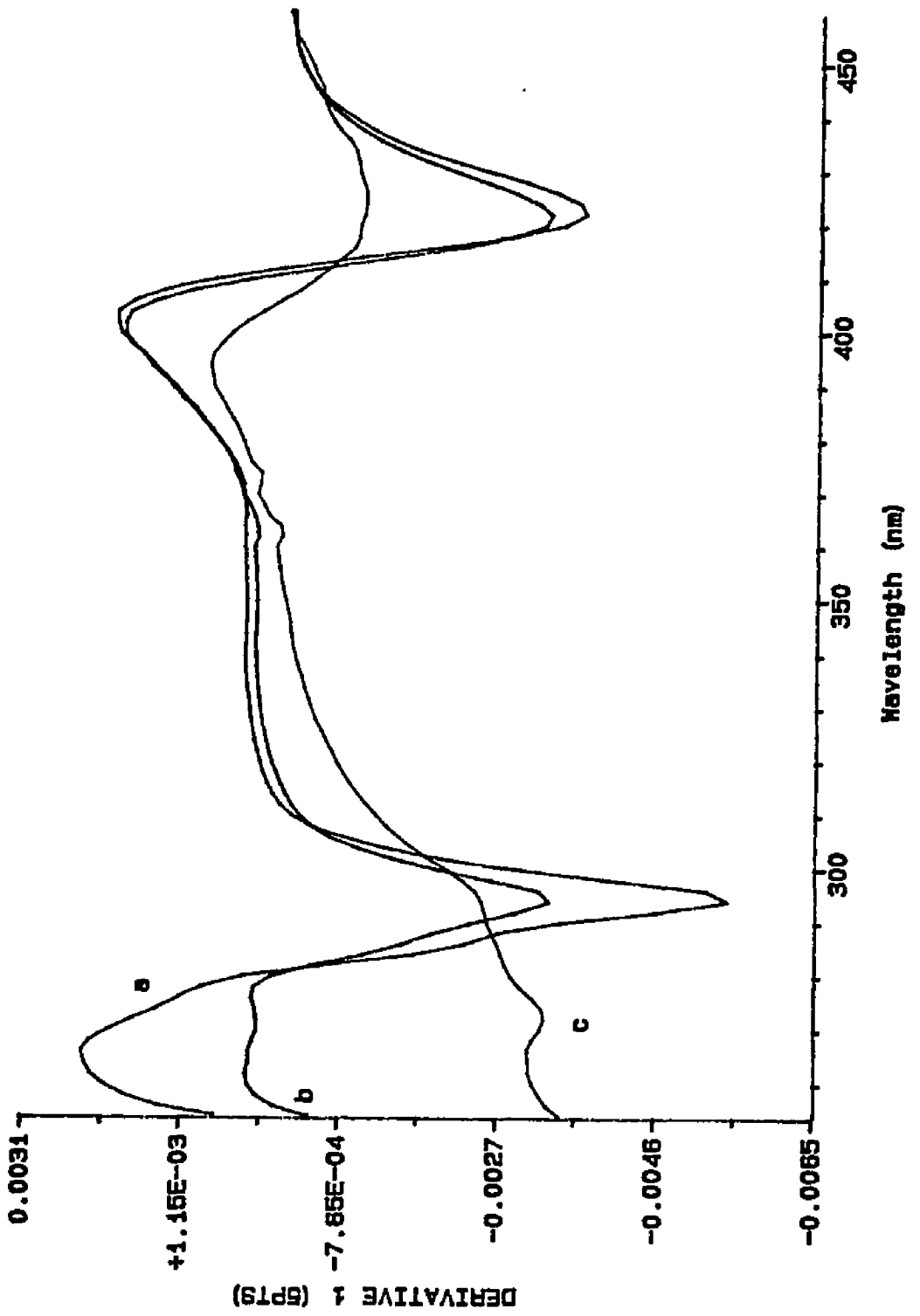


Figure 24. First derivatives of the bromine-treated LPO's spectra. Reaction conditions were the same as Fig. 23. Curve a, b, and c were the first derivatives of curve a, c, and d of Fig. 23, respectively.



HYDROGEN PEROXIDE/BROMIDE SYSTEM WITH BROMINE-TREATED LPO AS ENZYME

When Br^- and H_2O_2 concentrations were kept constant, a mixture of LPO and Br_2 with various Br_2/LPO ratios was added to the reaction solution to start the reaction, and the formation of tribromide (Br_2/Br_3^- species) was measured. The results were compared with that for the reaction catalyzed by LPO which was not treated with bromine. It was found that when the Br_2/LPO ratio was increased, the amount of Br_3^- formed and the initial Br_3^- formation rate were decreased compared with that for the untreated LPO catalyzed reaction (see Fig. 25 -- at which reaction condition the Br_3^- can not be accumulated. Curve a: formation of Br_3^- using untreated LPO as the enzyme; curve b, c, and d: formation of Br_3^- using Br_2-treated LPO as the enzyme, and the Br_2/LPO ratio was increased from b to d).

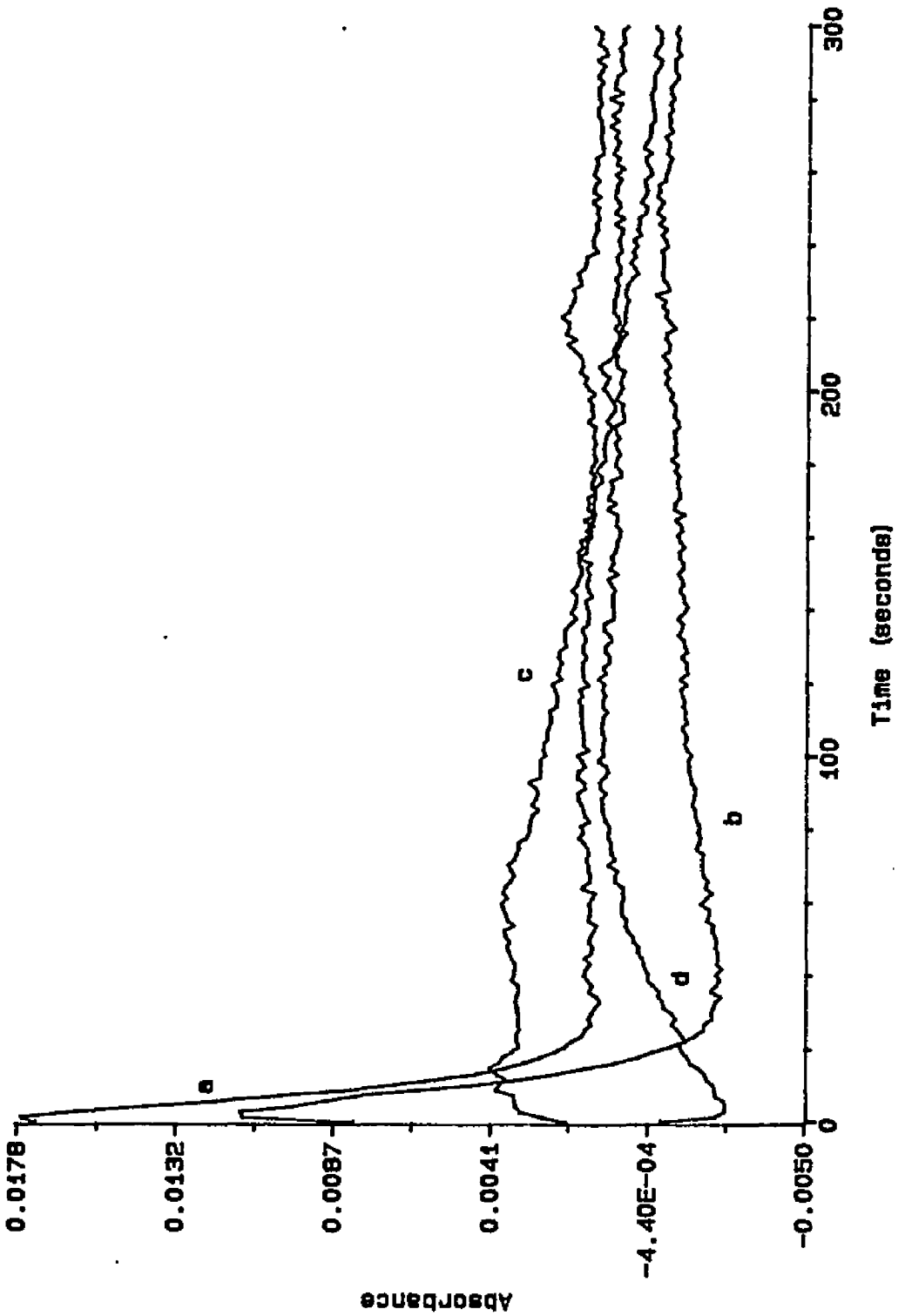
L-TYROSINE/HYDROGEN PEROXIDE/BROMIDE SYSTEM WITH BROMINE-TREATED LPO AS ENZYME

Bromine-treated LPO was used in the L-tyrosine/hydrogen peroxide/bromide system. The decrease of the initial MBT formation rate and the decrease of the concentration of MBT formed were observed with the increase of the Br_2/LPO ratio (data not shown).

LPO/HYDROGEN PEROXIDE/BROMIDE SYSTEM

If LPO was added twice (the first portion was added at

Figure 25. Br_3^- formation monitored at 267 nm in the hydrogen peroxide/bromide system with bromine-treated LPO as the enzyme. 1.0 ml solution containing 0.065 M KBr, 1 X 10^{-5} M H_2O_2, and 8 mM acetate buffer, pH 4.0, was added into a microcuvette as blank. Reactions were started by adding 0.05 ml (a) 1.507 µM (untreated) LPO, (b) solution containing 1.507 µM LPO and 1.553 X 10^{-3} mM Br_2, (c) solution containing 1.507 µM LPO and 0.01553 mM Br_2, or (d) solution containing 1.507 µM LPO and 0.07764 mM Br_2



the beginning to start the reaction, and the second portion was added when the reaction was close to equilibrium), a rapid increase of the Br_3^- at 267 nm (reformation of Br_3^-) was observed with the addition of the second portion of LPO (Fig. 26).

LPO/L-TYROSINE/HYDROGEN PEROXIDE/BROMIDE SYSTEM

A process similar to the LPO/hydrogen peroxide/bromide system was obtained: a rapid increase at the MBT's absorption of 290 nm was observed when the second portion of LPO was added (Fig. 27).

DISCUSSION

In the CPO/hydrogen peroxide/bromide system or CPO/L-tyrosine/hydogen peroxide/bromide system, there are Br^- and Br_3^-/Br_2/HOBr species in equilibrium. Br_2 and HOBr cannot be distinguished in our studies, because of that equilibrium and the instability of HOBr.

Kanofsky (21) reported that in the CPO/hydrogen peroxide/bromide (or chloride) system, singlet oxygen is produced (see Chapter 1, introduction). That report is consistent with our results (see results of CPO/hydrogen peroxide/bromide system, in which reaction 1, 2, 3, 4, and 9 were probably involved); and, we further found that the $[Br^-]/[H_2O_2]$ ratio (M/M) must be $(6.2\pm0.2) \times 10^3$ or higher in order to maintain the accumulation of the Br_2/Br_3^- species. In addition, since there is a linear relationship

Figure 26. Formation of Br_3^- monitored at 267 nm in the LPO-catalyzed hydrogen peroxide/bromide system. 1 ml solution containing 0.065 M KBr, 8 mM acetate buffer, pH 4.0, and 1 X 10^{-4} M H_2O_2 was added into a microcuvette as blank. 0.02 ml 0.1 μM LPO was added two times into the microcuvette: at 0 second, and at the 270th second, respectively.

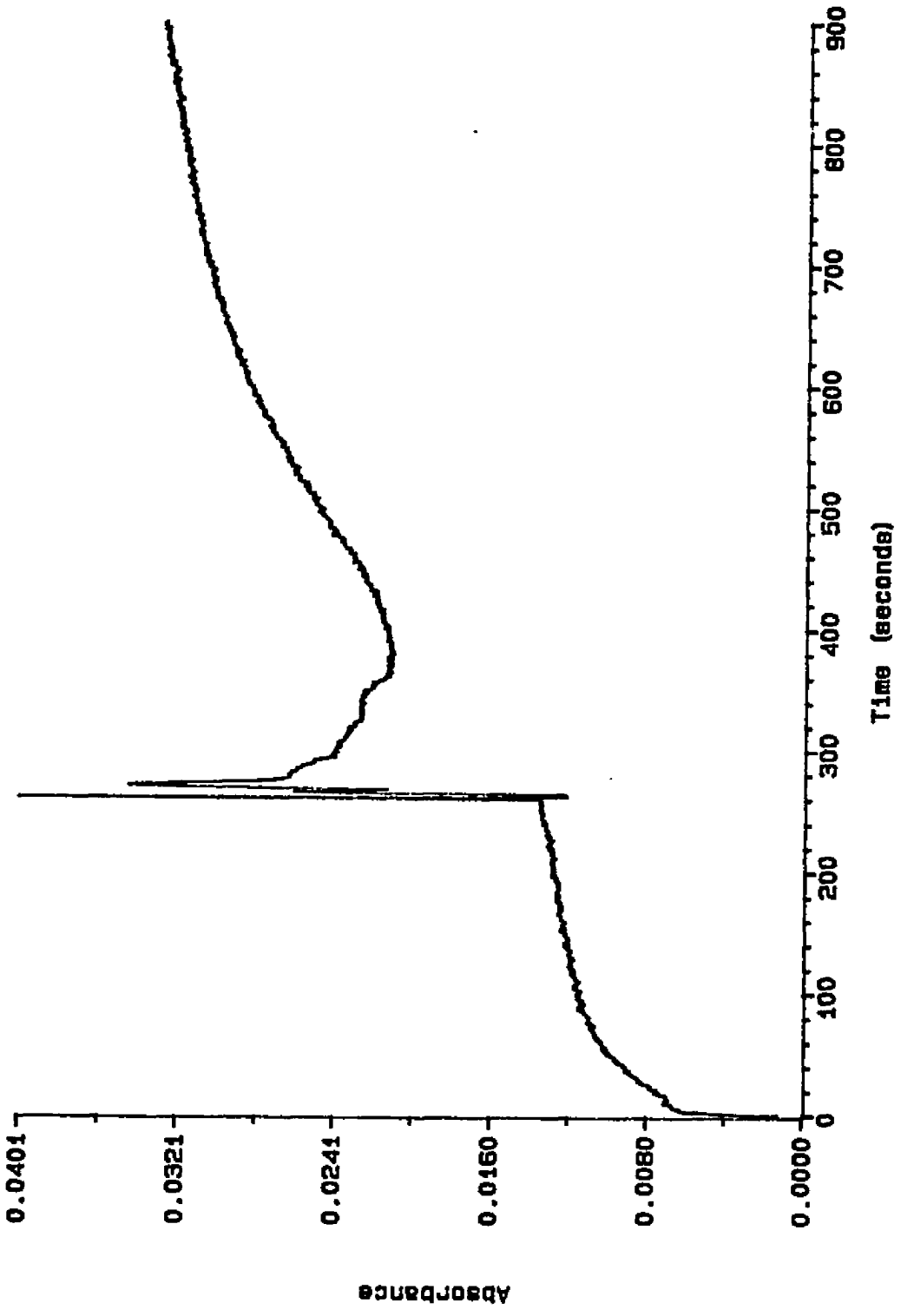
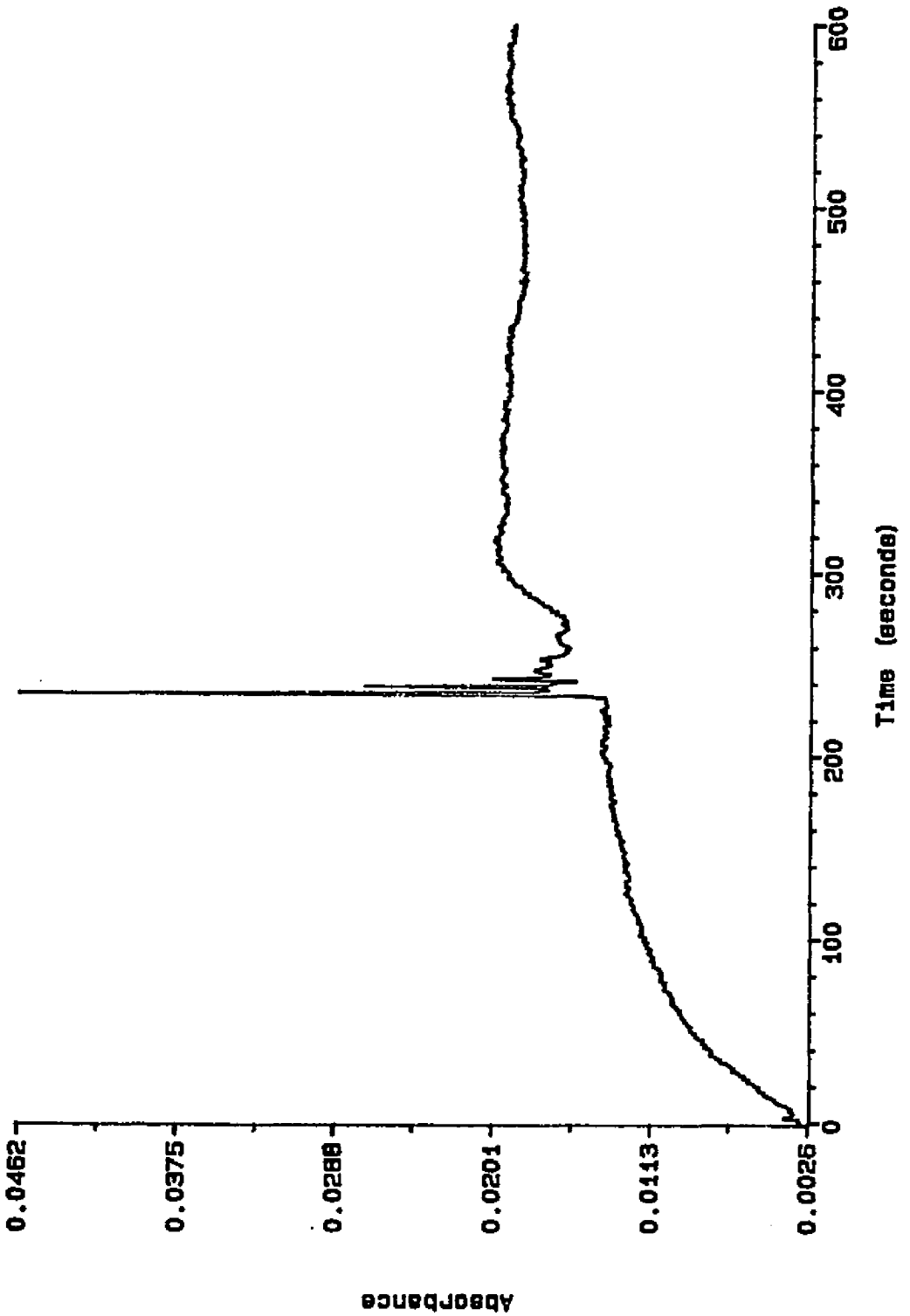


Figure 27. Formation of MBT monitored at 290 nm in the LPO-catalyzed L-tyrosine/hydrogen peroxide/bromide system. 1 ml solution containing 0.385 mM L-tyrosine, 0.065 M KBr, 8 mM acetate buffer, pH 4.0, and 1 X 10^{-3} M H_2O_2 was added into a microcuvette as blank. 0.02 ml 0.1 μM LPO was added two times into the microcuvette: at 0 second, and at the 230th second, respectively.



between 1/[CPO] and $1/[Br_3^-]_{max}$ (Fig. 7), and since there is a rapid increase of the Br_3^- 267 nm absorption observed with the second addition of CPO (Fig. 8 & 9), the deactivation of CPO in the CPO/hydrogen peroxide/bromide system would be reasonable. This is further proven by treating CPO with Br_2 or Br_3^-, and using the treated CPO in the reaction system instead of using the untreated CPO (Fig. 12, 13, 14, & 15). As a result, the destruction and deactivation of CPO by treating CPO with Br_2 are observed, and this destruction is accompanied with the decrease (or disappearance) of the Soret (heme) band and the increase (or formation) of the Br_3^- (267 nm) peak. This indicates that the CPO deactivation is due to the oxidation of its heme structure by Br_2. On the other hand, the CPO destruction and deactivation are not observed by treating CPO with Br_3^-.

Similar results in the L-tyrosine/hydogen peroxide/ bromide system using treated or untreated CPO as the catalyst (Fig. 10, 16, 17, & Table 1, d -- f) also indicates the destruction and deactivation of CPO by free bromine, a strong oxidizing species.

From our results, since no MBT is formed when tyrosine is mixed with Br^- or Br_3^-, it is clear that the free molecular Br_2 is the bromination agent in the nonenzymic chemical bromination reaction (reaction 5). Comparing Table 2 (g -- i) and Table 2 (j -- l), the addition of H_2O_2 will slightly decrease the amount of MBT formed as well as the

initial MBT formation rate, and this could be understood because reaction 2 may occur and so the bromine concentration is decreased. As shown in Table 1 (a -- c), we found that the MBT formation was favored by the condition of $[Br^-] >> [H_2O_2] >> [TYR]$, compared with the condition of $[Br^-] = [H_2O_2] >> [TYR]$. This can be easily explained as reaction 2 is not favored by the condition of $[Br^-] >> [H_2O_2]$, so more Br_2 exists in this system and MBT formation is favored.

In both the CPO/hydrogen peroxide/bromide system and the CPO/L-tyrosine/hydrogen peroxide/bromide system, we found that the same minimum Br_2/CPO ratio (53±3, M/M) will totally inhibit product (Br_3^- or MBT) formation (i.e., deactivation of CPO), and the same maximum Br_2/CPO ratio (4.3±0.8, M/M) has no effect on product formation (compared with the reaction catalyzed by the untreated CPO). Furthermore, these minimum and maximum Br_2/CPO ratios correspond to the total destruction of the heme structure or unchanged heme structure, respectively. Because of these, combined with the fact that the addition of CPO to the L-tyrosine/bromine/hydrogen peroxide system does not affect the chemical (nonenzymic) MBT formation process, we concluded that in the CPO/L-tyrosine/hydrogen peroxide/bromide system, the actual bromination of tyrosine is a nonenzymic chemical reaction between free molecular Br_2 and tyrosine (reaction 5). The reaction catalyzed by CPO is the

conversion of Br^- to Br_2/Br_3^- species in the presence of H_2O_2 (reaction 8), and, then, the product of this reaction, Br_2, oxidizes and deactivates CPO. In this system, our results excluded the possibility of the existence of reaction 6 and 7. We did not find any evidence supporting the presence of Compound EOX (reaction 3), which means we still can not conclude whether the CPO-catalyzed reaction (reaction 8) is reaction 2 or reaction 3 and 4.

The above conclusion could be further confirmed by the lack of stereospecific difference in the bromination rates of D- and L-tyrosine (Table 1, a - c, and Table 3).

In the bromide-dependent reactions, as soon as Br_2 is formed by reaction 1, several reactions involving Br_2 can occur, depending on the reaction conditions:

--- combining with Br^- to form Br_3^-;

--- reacting with tyrosine to form MBT;

--- reacting with CPO and forming Br_3^-;

--- reacting with H_2O_2 to form Br^-.

Because of the above reactions and their reaction velocities, no accumulation of free molecular Br_2 can be observed. For the same reason, it is difficult or impossible to directly relate the tyrosine bromination rate with the Br_3^- formation rate. Attempts to compare the formation rate of oxidized halogen species (Br_2/Br_3^-) with the reaction rate (formation rate) of halogenated substrate (MBT) did not yield data that would allow a conclusion as to

whether HOBr/Br_2/ Br_3^- or EOX (EOBr) is the halogenating species.

When the enzyme is saturated by Br^-, our results show that the K_m for H_2O_2 is 4.4 X 10^{-4} M. If the enzyme was saturated by H_2O_2, the K_m for Br^- is 3.2 X 10^{-2} M. So, the binding of Br^- and CPO, if it exists, is very weak compared with that of H_2O_2 and CPO.

As our results showed, unlike the deactivation and destruction of CPO in the presence of free molecular Br_2, no irreversible change was observed on the structure of HPO when HPO was mixed with free molecular Br_2. The decrease, shift, increase, and "reshift" (shift back) of the Soret band (Fig. 18) indicated that there were some structural changes on HPO when it was mixed with Br_2, and HPO possessed a recoverable function or structure so the above changes was not irreversible.

When bromine-treated HPO was used as the enzyme for both the hydrogen peroxide/bromide system and the L-tyrosine/hydrogen peroxide/bromide system, no difference was observed in the formation of Br_3^- or MBT, compared with the same system using untreated HPO as enzyme, respectively. In the HPO/hydrogen peroxide/bromide system or the HPO/ L-tyrosine/hydrogen peroxide/bromide system, the second and third addition of HPO to the reaction system did not affect the formation of Br_3^- or MBT, and no rapid absorption increase was observed (Fig. 20, and 21). Moreover, in the

HPO/L-tyrosine/hydrogen peroxide/bromide system, we observed that in the reaction (bromination) process, at the beginning of the reaction, the Soret band of HPO was in the "shifted" absorption range, and it would gradually shift back to the original absorption range when the reaction was close to equilibrium. In this system, further addition of HPO would only increase the Soret band at the original absorption maximum, but it would not shift the Soret band (Fig. 22). This directly indicated that HPO can at least recover itself during the bromination process.

Compared with the corresponding reaction systems catalyzed by CPO, the above results were quite different. From the above experiment results, although so far there are not enough evidences to allow us to say that HPO in the "shifted" state still can catalyze the bromination reaction, it is very possible that the HPO in the "shifted" state still has some catalyzing ability (probably HPO was partly inhibited by Br_2, but that inhibition was not irreversible), and there is a very high probability that HPO is involved in the bromination reaction, in another words, the bromination of tyrosine is an enzymatic reaction.

In the HPO/hydrogen peroxide/bromide system, as soon as the second or third portion of HPO was added to the reaction system, it was observed that the absorption of Br_3^- at 267 nm was decreased first, and, then, it would increase to the original absorption level again (see Fig. 20). A possible

explanation for this phenomena is that Br_3^-, or Br_3^-/Br_2 species, would bind with HPO first, which made the HPO's Soret band shifted, and, then, since that binding was not irreversible, Br_3^-/Br_2 species was released from HPO and the HPO's Soret band shifted back to its original wavelength range. If that suggestion is true, there must be an enzyme intermediate (a complex formed when Br_3^-/Br_2 species bind with HPO) existed in the reaction system. As our result showed, the changing process of HPO's Soret band from its original position to the "shifted" position was much faster than the whole reaction process (from the beginning of the reaction to the equilibrium). So, if the enzyme intermediate existed, that intermediate must still possess some catalyzing ability.

Based on these data, a possible reaction route for the HPO-catalyzed bromination reaction was proposed as follow:

$HPO + H_2O_2 \text{ ---> Compound I}$ (1)

$\text{Compound I} + H^+ + Br^- \text{ (--->}$

$\text{HOBr + Compound I) ---> EOX}$ (3)

(EOX: the probable enzyme intermediate formed when Br_3^-/Br_2 species bind with HPO)

$EOX + H^+ \text{ ---> HOBr + HPO}$ (4)

(when the organic substrate, AH, is absent)

$EOX + AH \text{ ---> AX + HPO + } H_2O$ (10)

(when the organic substrate, AH, is present)

In that proposed reaction route, reaction (1) and (3)

are very fast, and the HPO's Soret band will be observed in the "shifted" position at the beginning of the reaction. When reaction (4) or (10) occur, HPO will be released gradually and a "shift back" process of the Soret band will be observed. When the reaction is closed to equilibrium, the Soret band will be found in the original wavelength range.

Further experiments are needed to prove the above suggestion.

The activity of LPO was lost by mixing LPO with Br_2, which was similar to that of CPO. Unlike CPO, when LPO was mixed with Br_2, if the Br_2/LPO ratio was not very high (< 50, M/M), its Soret band was not affected, but the decrease of the absorption arround 262 - 296 nm was observed, which indicated that the mechanism of the deactivation of LPO by Br_2 was different from that of CPO.

Further studies on the mechanism of the HPO-catalyzed bromination and the relative structural change of HPO, as well as the deactivation mechanism of LPO will be the object of future studies.

Since the ratio of Br_2/CPO to completely deactivate CPO is low (considering the amount of CPO used in the bromination reaction), the formation of Br_2 is limited. Moreover, there is a good chance for the occurrence of reaction 2, which lowers the Br_2 concentration further. As a result, in the bromide-dependent system, the bromination

of tyrosine is restricted. Increasing the CPO or Br^- concentration, or, merely using HPO instead of CPO, may increase the MBT formation.

Further experiments are needed to identify the two unknown products in the reaction system. But this would not affect our conclusion about the reaction mechanism.

CHAPTER 5

SUMMARY AND CONCLUSIONS

The haloperoxidase-catalyzed bromination reactions appear to be more complicated than the haloperoxidase-catalyzed iodination reactions. The main reason is that the free molecular bromine is a much stronger oxidizing agent and is more active.

Free molecular bromine plays a key function in the haloperoxidase-catalyzed bromination reactions, but the properties of enzymes are also important and will also affect the reaction mechanism. In the CPO-catalyzed bromination reaction system, we found that CPO is deactivated by Br_2 irreversibly, and this deactivation is associated with the destruction of CPO's heme structure. As the result, CPO only catalyzes the conversion of Br^- to Br_3^- /Br_2 species, and the actual bromination reaction is a nonenzymatic chemical reaction between Br_2 and the organic substrate (tyrosine). It was also found that the Br^- or Br_3^- ions will not deactivate CPO, and the nonenzymatic bromination reaction can not occur between Br^- and tyrosine or Br_3^- and tyrosine --- Br_2 is the only brominating agent.

In the haloperoxidase-catalyzed bromination system, the function of HPO is quite different from that of CPO, even though they both are haloperoxidases. Br_2 also introduces some structural changes on HPO, which is also related with

HPO's heme structure; but, unlike CPO, HPO can not be deactivated irreversibly by Br_2, and HPO can recover during the reaction process. Although more experiments and studies are still needed, we proposed that in the HPO-catalyzed bromination system, an enzyme intermediate is probably formed when HPO bind with Br_3^-/Br_2 species, and the bromination reaction is catalyzed by this enzyme intermediate, which means the actual bromination is probably an enzyme-catalyzed reaction. Further studies are necessary to approve that proposal.

Compared with HPO, LPO is similar to CPO: an irreversible deactivation of LPO was observed when LPO was mixed with Br_2. Since the changes of LPO's Soret band was different from that of CPO's, its deactivation process is probably different. More work is needed in order to find the actual deactivation process, and also to determine whether the bromination reaction is an enzyme-catalyzed reaction or not, when LPO is used as the enzyme.

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