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Enzyme-catalyzed polymerization and precipitation of aromatic compounds from wastewater.

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ENZYME CATALYZED POLYMERIZATION AND PRECIPITATION OF AROMATIC COMPOUNDS FROM WASTEWATER

By

James A. Nicell

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ABSTRACT

Horseradish peroxidase (HRP) demonstrates a valuable potential for wastewater treatment by catalyzing the polymerization and precipitation of aromatic compounds from water. It acts on a broad range of compounds including those that are biorefractory or toxic to microbes and retains its catalytic ability over wide ranges of temperature, pH, and contaminant concentration.

Removal efficiency is dependent on the nature of the aromatic substrate and the dose of enzyme used. Optimal catalytic lifetime was achieved in the pH range of 7 to 9 for the eight phenolic compounds used in this study. Enzymatic precipitation should be conducted at temperatures below 35^{0}C to prevent significant thermal inactivation of peroxidase. The stoichiometry of the reaction between aromatic compound and hydrogen peroxide was unity. Enhanced removal of hard–to–remove compounds was accomplished by co–precipitation with other substrates of HRP.

A kinetic model was developed which matches the trends of data collected in a batch reactor under various conditions of enzyme, aromatic substrate and peroxide concentrations. Further development is required to define the mechanisms and kinetics of inactivation to extend application of the model to the design of a full–scale waste treatment system.

The catalytic lifetime of the enzyme may be extended by maintaining a low instantaneous enzyme concentration in the reaction mixture. The enzyme catalyzed polymerization process was implemented in a continuous stirred tank reactor (CSTR) configuration because reactant and enzyme concentrations are lowered immediately upon entering the reactor causing a reduction in free radical inactivation and Compound III formation. Catalytic turnovers achieved in single and multiple CSTR's in series were significantly higher than those observed in batch reactors when sufficient retention time was provided.

Dedicated to my parents,
Patrick and Barbara,
who guided and supported me
through the first two ages which took
a little longer than expected...

"All the world's a stage,
And all the men and women merely players;
They have their exits and their entrances;
And one man in his time plays many parts,
His act being seven ages. At first the infant,
Mewling and puking in the nurse's arms;
Then the whining school–boy, with his satchel
And shining morning face, creeping like a snail
Unwillingly to school. And then..."

William Shakespeare
As You Like It
Act II, Scene VII

And then?

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

1. INTRODUCTION

Enzymes are biological catalysts that regulate the multitude of chemical reactions that occur in a living cell, whether it be plant, animal or microbial. They carry out such cellular processes as energy conversion, food digestion and biosynthesis. Microbial enzyme preparations have been used in the production of food products for many years – long before the nature and function of enzymes were known or understood. Some of the enzymes used in industries and their applications are listed in Table 1–1.

Enzymes that have been isolated from their parent organisms are often preferred over intact organisms containing the enzyme because the isolated enzymes act with greater specificity, their potency can be better standardized, they are easier to handle and store (Vieth and Venkatasubramanian, 1973) and enzyme concentration is not dependent on bacterial growth rates (Bailey and Ollis, 1986). These enzymes also offer a number of advantages over conventional chemical catalysts including: a high degree of specificity; operation under mild conditions reducing energy requirements and corrosion effects of the process; and a high reaction velocity which reduces processing costs.

The advent of new technology for the production, isolation and purification of enzymes has made their use more competitive. These advances are leading to the development of new commercial applications of enzymes (Bailey and Ollis, 1986).

1.1 Application of Enzyme Catalysis to Wastewater Treatment

Aromatic compounds, including phenols and anilines, are present in the wastewaters of numerous industries including petroleum refining, plastics, resins, textiles, iron and steel manufacturers, among others. Many of these are toxic and some are known or suspected carcinogens (Verschueren, 1977).

Table 1–1: Selected Enzymes and Their Commercial Applications

Enzyme Type	Industrial Application
Proteases	Cheese making Digestive aids Meat tenderizing Beer haze removal Cereal Syrups manufacture Leather manufacture Medical uses Detergents
Carbohydrases	Starch hydrolysis Sucrose inversion Fruit juice clarification Vinegar clarification
Nucleases	Flavour control
Other hydrolytic enzymes	Destruction of toxic or undesirable components in foods Hydrolysis of cellulose Candy softening Brewing
Oxidases	Oxidation prevention and color control in foods Clinical diagnostics Sterilization of milk Digestive aids Drying of lacquer Glucose or oxygen removal

References: Vieth and Venkatasubramanian, 1973
Bailey and Ollis, 1986

The levels of aromatic compounds found in industrial waste streams vary from dilute to concentrated as shown by the phenol levels reported in industrial wastewaters listed in Table 1–2. Effective treatment technologies for many of these wastes currently exist; however, any particular industry may encounter difficulties in employing a specific treatment method depending upon the composition of the waste stream. Some examples of current treatment technologies which are available for the treatment of wastewaters containing aromatic compounds are listed in Table 1–3.

This research focuses on the use of horseradish peroxidase (HRP) enzyme for catalyzing the removal of aromatic compounds from aqueous streams. The treatment results in polymerization and precipitation of the organic, which is in contrast to conventional microbiological treatment where metabolic assimilation and possibly oxidation of the organic is catalyzed by multi–enzyme pathways within the organism.

The polymerization of aromatics in the presence of horseradish peroxidase, and other oxidoreductive enzymes, has recently been the focus of much attention due to its potential use for the decontamination of wastewaters. The enzymatic approach is particularly suitable for the treatment of wastewaters because during polymerization the products form insoluble precipitates that can be removed easily from water by sedimentation or filtration. Klibanov *et al.* (1980, 1981, 1982, 1983), Maloney *et al.* (1985, 1986) and Fiessinger *et al.* (1984) have demonstrated the effectiveness of using horseradish peroxidase for the polymerization and precipitation of substituted phenols and aromatic amines from wastewaters and drinking waters. These authors recognized the potential for the enzymatic treatment process and recommended the process for further development. This necessarily includes fundamental chemistry and biochemistry research and engineering development. Fundamental research is on–going as indicated by the

Table 1–2: Levels of Phenols Reported in Industrial Wastewaters (Patterson, 1975)

Industrial Source	Phenol Concentration (mg/L)
Coke ovens	
Without dephenolization	580 – 3900
With dephenolization	4.5 – 332
Oil refineries	
Sour water	80 – 185
Post–stripping	80
General (catalytic cracker)	40 – 50
Mineral oil wastewater	100
API separator effluent	0.35 – 6.8
General wastewater	10 – 100
Petrochemical	
General petrochemical	50 – 60
Benzene refineries	210
Nitrogen works	250
Tar distilling plants	300
Aircraft maintenance	200 – 400
Herbicide manufacturing	210 – 524
Other	
Rubber reclamation	3 – 10
Orlon manufacturing	100 – 150
Plastics	600 – 2000
Fiber board	50
Wood carbonizing	500
Phenolic resin production	1600
Stocking factory	6000
Synthetic phenol	12 – 18
Fiberglass manufacturing	40 – 400

Table 1–3: Common Treatment Technologies for Aromatic Compounds

Recovery Systems
Countercurrent extraction
Pulsed column extraction
Physical/Chemical Treatment Systems
Chlorine oxidation
Chlorine dioxide oxidation (as sodium chlorite)
Ozone oxidation
Hydrogen peroxide oxidation (Fenton's reagent)
Potassium permanganate oxidation
Incineration
Hydrocarbon stripping and combustion
Photocatalytic oxidation
Activated carbon adsorption
Landfilling
Coagulation
Biological Treatment Systems
Biooxidation pond
Aerated lagoon
Stabilization pond
Oxidation ditch
Trickling filter
Activated sludge
Rotating biological contactors

volume of literature produced but, until now, development from an engineering perspective has not been the focus of much attention.

1.2 Advantages of Enzyme–Based Treatment

The potential advantages of an enzyme–based treatment over conventional biological treatment include:

- application to a broad range of compounds, including those which are biorefractory
- action on, or in presence of, many substances which are toxic to microbes
- operation over wide temperature, pH and salinity ranges
- operation both at high and low concentrations of contaminants
- no shock loading effects
- no delays associated with shutdown/startup (acclimatization of biomass)
- reduction in sludge volume (no biomass generation)
- better defined system with simpler process control.

Based on the foregoing, it might, for certain industrial waste sources, be possible to replace conventional biological treatment with the enzyme–based one. Alternatively, the latter might be used in conjunction with the conventional one as a pre–treatment at the source to reduce the burden on the biological treatment system or as a tertiary treatment to deal with biorefractory compounds.

The potential advantages of an enzyme–based treatment over chemical/ physical processes are:

- operation under milder, less corrosive, conditions
- operation in a catalytic manner
- operation on trace level organic compounds and on organics not removed by existing chemical/physical processes
- reduced consumption of oxidants

- reduced amounts of adsorbent materials, such as charcoal, for disposal.

The advantages listed in both categories above touch on a number of the current challenges in the field of industrial wastewater treatment. Government regulations will require that a broad range of organic compounds in concentrations ranging from below μg/L to 1000's of mg/L must be detected and removed from aqueous streams even though they arrive at the detection/removal stage in a solution/suspension which can vary widely in other parameters such as temperature, pH, level of other solutes and particulates (Environment Ontario, 1988). In June 1986, the Ontario Ministry of the Environment announced the Municipal Industrial Strategy for Abatement (MISA) Program whose objective is the virtual elimination of persistent toxic discharges into Ontario waterways (Chapman and Loo–Sy, 1991). Future compliance with the discharge limits imposed by this program will require the development of economically and technologically effective methods for reducing industrial and municipal discharges of targeted pollutants. To the extent that this research attempts to meet these challenges in a general approach, a number of technical benefits are foreseen arising from this research:

- a versatile approach to enhance organics removal;
- the operation of selective treatment units at designated sources within the plant;
- a means of satisfying increasingly stringent regulations with respect to water effluent quality;
- a window on technology in the area of biocatalyst–based reactors which could find other applications such as chemicals production in aqueous media or in water–immiscible solvents and in product purification.

The economic benefits forcseen are in areas where conventional technologies cannot be used or are too costly or inefficient.

The application of the enzyme catalyzed polymerization process may include

certain disadvantages such as high catalyst costs, disposal of solid reaction products and possible formation of hazardous soluble byproducts. Extensive research is required to further define the nature and extent of these disadvantages.

1.3 Research Requirements

Several conclusions may be drawn from the existing literature on enzymatic waste treatment:

(i) The use of some enzymes for degrading or removing toxic organics from wastewaters has been demonstrated under laboratory experimental conditions.

(ii) In order to assess the technical and economic feasibility of enzymatic waste treatment, the following research is needed:

- basic chemistry and biochemistry research to establish the scientific bases and requirements of the process under a variety of waste treatment conditions;
- applied process development research to establish the engineering requirements for the design and operation of enzymatic treatment systems;
- pilot scale studies to confirm engineering design and to provide data for economic analysis of the process.

1.4 Objectives and Scope

In view of the need for research in the area of enzymatic treatment of wastewater, this study attempted to meet the following objectives:

(i) Horseradish peroxidase was evaluated in the laboratory as a catalyst for the polymerization and precipitation or co–precipitation of selected organic compounds as a means of treating industrial wastewaters.

(ii) A preliminary design of a continuous flow reactor was developed and implemented on the bench–scale to apply the process developed above.

The study was conducted in the following phases:

(i) Horseradish peroxidase was evaluated in terms of its ability to catalyze the oxidation of the following compounds and precipitate or co–precipitate the by–products, individually and in combination in the laboratory under controlled conditions such as temperature, pH, substrate and enzyme concentrations, and contact time:

- phenol
- alkyl phenols
- chlorinated phenols

(ii) Process variables were identified which have a significant impact on biocatalyst lifetime and aromatic substrate removal efficiency.

(iii) Methods were developed for keeping the horseradish peroxidase active, protecting it against damaging by–products and preventing its loss from the system.

(iv) Kinetic modelling of the enzyme catalyzed reaction was performed to aid in the selection and design of a continuous flow reactor system.

(v) Enzyme–based bench–scale reactors were developed to treat the above selected compounds. Reactor tests were performed under controlled conditions of temperature, pH, contact time, substrate and enzyme concentrations.

2. LITERATURE REVIEW

Horseradish peroxidase (HRP), also called hydrogen–peroxide oxidoreductase, is a protein with a molecular mass of about 40 000 which contains a single protoporphyrin IX heme group. It is isolated from horseradish roots by extraction, fractionation and chromatography. HRP has been studied extensively because in the presence of hydrogen peroxide it catalyzes many useful reactions such as hydroxylations, N–demethylations, sulfoxidations, or other oxidations of various aromatic substrates (Urrutigoity, 1989), especially phenols and aromatic amines.

2.1 Background on Horseradish Peroxidase

The one–electron oxidation of aromatic substrates (AH_2) catalyzed by peroxidase was proposed originally by Chance (1952) and George (1952, 1953a, 1953b). This process involves changes in the oxidation state of an iron atom located at the center of the catalytic site of the enzyme. The sequence of events involved in this catalysis is quite well understood and is usually depicted by the following mechanism:

$$E + H_2O_2 \longrightarrow E_i + H_2O \qquad (2\text{–}1)$$

$$E_i + AH_2 \longrightarrow E_{ii} + \cdot AH \qquad (2\text{–}2)$$

$$E_{ii} + AH_2 \longrightarrow E + \cdot AH + H_2O \qquad (2\text{–}3)$$

In this catalytic cycle, the native enzyme (E) is oxidized by peroxide to an active intermediate enzymatic form called Compound I (E_i). The Compound I state of HRP accepts an aromatic compound into its active site and carries out its oxidation. The resulting free radical is released back into solution leaving the enzyme in the Compound II (E_{ii}) state. This form of the enzyme oxidizes another aromatic molecule, releasing a second free radical into solution and returning the

enzyme to its base state, thus completing the cycle. The sum of Reactions 2–1 to 2–3 produces the following overall reaction:

$$H_2O_2 + 2\,AH_2 \longrightarrow 2\cdot AH + 2\,H_2O \qquad (2\text{–}4)$$

Independently of the enzyme, the free radicals formed during the cycle diffuse from the enzyme into solution where they react to form polyaromatic products. These polymers are less soluble in water than their monomeric precursors and tend to precipitate from solution. If the resulting polymer does not precipitate, it still has the potential to be accepted back into the active site of the enzyme resulting in the formation of a still larger polymer which has a further reduced solubility.

2.2 Treatment of Wastewater with Horseradish Peroxidase

Horseradish peroxidase (HRP), once activated by hydrogen peroxide, effects the oxidation of a wide variety of phenols, biphenols, anilines, benzidines, and related heteroaromatic compounds (Artiss *et al.*, 1981; Bovaird *et al.*, 1982, Josephy *et al.*, 1982; Ngo *et al.*, 1980; Sawahata and Neal, 1982). Klibanov *et al.* (1983) took advantage of the fact that peroxidase generates phenoxy radicals from phenol plus peroxide and then the phenoxy radicals polymerize. Generation of phenoxy radicals and formation of secondary products was confirmed in an independent report (Sawahata and Neal, 1982). In a series of parallel reports, Klibanov and coworkers (1980, 1981, 1983) demonstrated that the same approach was effective for anilines, hydroxyquinoline and arylamine carcinogens such as benzidines and naphthylamines. In addition, it was shown that compounds such as PCB's, naphthalene and azobenzene, which themselves are not substrates for the enzyme, were very efficiently co–precipitated with the phenols, anilines, etc. which are substrates. The concentration range surveyed was generally up to 100 mg/L (0.3–1 mM) but with phenol up to 5 g/L was checked. In all cases, removal

efficiencies of greater than 98% could be achieved when sufficient peroxide and enzyme were added. "Real" samples in the form of coal–gasification wastewater (2 g/L phenol, 5 g/L ammonia, 19 g/L chloride, 0.1 g/L cyanide, 1.0 g/L thiocyanate; pH 9.0), "flushing liquor" from a coke plant (0.4 g/L phenol, pH 8.6) and effluent from a triarylphosphate plant (0.1 g/L phenols, including cresols and alkylphenols plus triarylphosphates and inorganic phosphate; total of 150 different chemicals; pH 7.8) were treated successfully.

Klibanov argued (1983) that enzyme-catalyzed removal of aromatic compounds from wastewater has many advantages over current methods such as microbial degradation, adsorption on charcoal, chemical oxidation, solvent extraction, membrane processes and irradiation. However, the main drawback of the Klibanov system was that the enzyme was inactivated, presumably by the phenoxy radicals. Therefore, for efficient clearance of 100 mg/L phenol solutions of 2 000 "units" of HRP (one unit is the catalytic activity producing 1 mg purpurogallin in 20 seconds from pyrogallol at pH 6.0, 20°C) or 300 000 "units" of polyphenol oxidase (expressed as tyrosinase activity, one unit is the catalytic activity producing an absorbance increase at 280 nm of 0.001/minute with tyrosine as substrate at pH 6.5, 25°C; reaction mixture 3 mL, pathlength 10 mm) per litre were recommended. For small purchases these amounts of activity presently cost approximately 2 and 100 U.S. dollars, respectively. Even the lower of these numbers is orders of magnitude too high. Although HRP is already robust and inexpensive as enzymes go, any economically feasible enzyme–based wastewater treatment method would require a substantial increase in the catalytic lifetime of the enzyme, estimated at 10 000 reaction turnovers (Klibanov *et al.*, 1983) for soluble peroxidase as used above.

Schmidt and Joyce (1981) used horseradish peroxidase and H_2O_2 to remove low molecular mass colour bodies (lignin fragments and derivatives) from

biologically treated pulp mill effluents. The enzymic precipitation was used to supplement colour body removal by lime precipitation which only accomplished removal of high molecular weight contaminants. Colour removal improved by 50% as compared to removal by lime precipitation alone.

Alberti and Klibanov (1982) demonstrated that crude enzyme preparations in which the horseradish roots were minced, suspended in water, stirred and filtered did not reduce the efficiency of the process. Therefore, the efficiency of the enzyme does not depend on the purity of the enzyme. In the same paper, they reported a preliminary economic analysis of the process based on a rough estimation of the amount of HRP that could be recovered from raw horseradishes and the current cost of horseradish roots and hydrogen peroxide. Their calculations demonstrated that the process costs were comparable with those associated with treatment using hydrogen peroxide and an iron catalyst for oxidation of phenols to CO_2 and water. This cost estimate did not include costs associated with the extraction of the peroxidase from the horseradish roots.

Other workers followed up on the reports from Klibanov's investigations. A French group (Maloney *et al.*, 1985, 1986; Fiessinger *et al.*, 1984) confirmed the MIT work with various chlorophenols and extended it to lower levels of aromatic substrates (as might be found in groundwater; e.g. 2–chloro– and 2,4–dichlorophenol down to 10 and 2.5 μg/L, respectively) using soluble peroxidase. Van der Zee *et al.* (1989) demonstrated that N–substituted aromatic amines were good electron donors and thus are easily oxidized by horseradish peroxidase and H_2O_2.

A list of aromatic substrates for the horseradish peroxidase enzyme which are known to be precipitable from water using HRP catalyzed polymerization is contained in Appendix A. This list is by no means complete but represents those compounds which have been studied in the references cited here.

Horseradish peroxidase is isolated from horseradish cells. A Japanese group

(Taya *et al.*, 1989) has successfully developed a hairy root clone from horseradishes and developed cultures in a bioreactor. These cultures had peroxidase activity comparable to that of the original plant. Development of a peroxidase production system independent of field–grown horseradish is extremely advantageous in terms of the commercial production of HRP.

2.3 Products of Enzyme Catalyzed Polymerization

The enzymatic precipitation of aromatic compounds from solution involves the formation of both soluble and insoluble polyaromatic byproducts. Recent research has concentrated on identifying these products.

Biphenols (o,o'–biphenol and p,p'–biphenol) were formed by the oxidation of phenol (Danner *et al.*, 1973). The p,p'–biphenol was further oxidized to p–diphenoquinone (Sawahata and Neal, 1982). Dec and Bollag (1990) found that the precipitates formed during polymerization of 2,4–dichlorophenol constituted a mixture of polymers with average molecular masses up to 800 for the fraction that was soluble in dioxane. This soluble fraction contained tetramers and pentamers and the insoluble fraction likely contained components of higher molecular mass. Dehalogenation of the aromatic compound was also observed. Potter *et al.* (1985) identified several dimers, trimers and one tetramer in the polymerization of acetaminophen. Dordick *et al.* (1987) carried out the polymerization of phenols in non–aqueous media (dioxane, acetone, etc.) and identified various polymers with average molecular masses ranging from 400 to 26 000. These reports indicate that polyaromatic byproducts continued to act as substrates of the HRP enzyme.

Hewson and Dunford (1976a) reported that Pummerer's ketone, a dimethylated dibenzofuranone, was a product of the oxidation of p–cresol by horseradish peroxidase. Maloney *et al.* (1986) found that when HRP was used to transform a concentrated solution of 2,4–dichlorophenol, dioxins and furan

derivatives were detected among the byproducts. However, these authors note that in realistic wastewater treatment applications chlorinated phenols are present in trace concentrations. In addition, other organics present in solution may enter into the reaction in a manner that significantly alters the byproducts. Oberg *et al.* (1990) summarized what is currently known of the nature of the products of enzyme catalyzed polymerization. They reported in their own findings that enzyme catalyzed polymerization of 2,4,5– and 3,4,5–trichlorophenol formed polychlorinated dibenzo–p–dioxins and dibenzofurans. In previous work, Oberg and Swanson (1987) failed to detect such compounds under similar conditions using other chlorophenolic isomers (pentachlorophenol, 2,3,4,6–tetrachlorophenol and 2,4–dichlorophenol). They attributed this lack of observation to the nature of the substrates used and not to their ability to detect the byproducts (Oberg and Swanson, 1987).

In the treatment of wastewater by enzyme catalyzed polymerization, solid precipitates must be separated from solution and disposed of in an environmentally safe manner. The papers cited above demonstrate the possibility that some of the reaction products (dioxins and furans) are more toxic than their monomeric precursors. Before this process can be implemented on an industrial scale, the nature of the soluble and insoluble products formed in the polymerization process must be characterized. Characterization is necessary to evaluate the true potential of enzymatic polymerization as a wastewater treatment alternative or to identify those situations where it is applicable.

2.4 Kinetics of Horseradish Peroxidase

Yamazaki and Nakajima (1986) determined a rate constant for the oxidation of HRP with hydrogen peroxide. Job and Dunford (1976) obtained rate constants for the reaction of many phenols and aromatic amines with Compound I. Dunford's group (Cotton and Dunford, 1973; Critchlow and Dunford, 1972; Hewson and

Dunford, 1976; Perez and Dunford, 1990) examined the effect of pH on rate constants for reactions of Compounds I and II. Dunford and Adediran (1986), Sakurada *et al.* (1990) and Huang and Dunford (1990) reported rate constants for the reaction of a variety of phenols and anilines with Compound II.

These rate constants are insufficient to describe the reacting system in its entirety. While Reactions 2–1 to 2–3 dominate in a mixture of aromatic substrate, peroxide and HRP, a number of side reactions also occur, some of which are responsible for inactivation and inhibition of HRP resulting in a finite lifetime for the catalyst. Baek and Van Wart (1989) modified the widely accepted mechanism proposed by Chance (1952) and George (1952) due to evidence of at least one reversible intermediate step in the formation of Compound I. The possibilities for an intermediate include the formation of an HRP–H_2O_2 complex and an oxidized form of HRP which they designated as Compound 0.

Arnao *et al.* (1990b) reported that in the absence of a reductant substrate and with excess H_2O_2, Compound I can react with hydrogen peroxide to form an intermediate complex:

$$E_i + H_2O_2 \rightleftharpoons (E_i \cdot H_2O_2) \qquad (2\text{–}5)$$

This complex can follow three routes described by:

$$(E_i \cdot H_2O_2) \longrightarrow E + O_2 + H_2O \qquad (2\text{–}6)$$

$$(E_i \cdot H_2O_2) \longrightarrow E_{670} \qquad (2\text{–}7)$$

$$(E_i \cdot H_2O_2) \longrightarrow E_{ii} + O_2^{\cdot} \qquad (2\text{–}8)$$

Reaction 2–6 is a weak catalase reaction in which peroxide acts as a reducing agent resulting in the conversion of peroxide to oxygen and water. In the presence

of stronger reducing agents, the catalase action of peroxidase will be minimal. Reaction 2–7 describes the conversion of peroxidase to inactive verdohaemoprotein, called P–670 (E_{670}), when it reacts with excess H_2O_2 (670 refers to the peak wavelength at which this compound absorbs light). Reaction 2–8 represents an alternate route for the formation of Compound II in the absence of a reductant substrate. Arnao *et al.* (1990b) have provided a summary of kinetic constants at neutral pH and 25^{0}C for these reactions.

Once HRP is in the Compound II state, it can be oxidized by excess peroxide to the Compound III (oxyperoxidase, E_{iii}) state according to a scheme presented by Nakajima and Yamazaki (1987):

$$E_{ii} + H_2O_2 \longrightarrow (E + O_2^{\cdot-} + H^+) \qquad (2\text{–}9)$$

$$(E + O_2^{\cdot-} + H^+) \longrightarrow E_{iii} + H_2O \qquad (2\text{–}10)$$

Compound III is not catalytically active but its formation does not represent a terminal inactivation of HRP since Compound III decomposes spontaneously to native peroxidase (Arnao *et al.*, 1990b) according to:

$$E_{iii} \longrightarrow E + O_2^{\cdot-} \qquad (2\text{–}11)$$

The return to the native enzyme is sufficiently slow that, once in the Compound III form, the enzyme is severely hampered in carrying out the catalytic oxidation of aromatic substrates. This represents a loss in catalytic efficiency. Adediran and Lambeir (1989) and Nakajima and Yamazaki (1987) have described the formation of Compound III in detail and evaluated rate constants for the governing reactions.

Permanent inactivation can result from the return of a free radical to the

active center of the enzyme (Klibanov, 1983) where a bond may form at or near the active site. Such a bond blocks the active site or upsets the critical geometric configuration of the enzyme; thus eliminating the enzyme's catalytic ability. Superoxide anion (O_2^-) produced in Reactions 2–8 and 2–11 can oxidize peroxidase (Arnao *et al.*, 1990b) resulting in the formation of Compound III. However, this radical is an extremely strong oxidizing agent and is likely to react with other aromatic molecules present in solution. This reduces its potential for oxidizing and inactivating HRP.

2.5 Alternatives to Horseradish Peroxidase

The possibility that other enzymes might show similar activity with the same or different classes of organic compounds in wastewater was considered by Klibanov and co–workers. Klibanov's group found similar dephenolization efficiencies for another enzyme, polyphenol oxidase (tyrosinase) from mushrooms (Atlow *et al.*, 1984). This enzyme uses molecular oxygen rather than hydrogen peroxide as oxidant but substantially more of polyphenol oxidase than of peroxidase is required for equivalent removal efficiency. In addition to their studies with polyphenol oxidase, lactoperoxidase, chloroperoxidase and several microbial peroxidases were tested and were found to catalyze precipitation of more limited groups of aromatic compounds (Klibanov *et al.*, 1983).

Haloperoxidase activity (i.e. hypohalous acid addition to carbon–carbon double and triple bonds and to cyclopropane rings) has been demonstrated for a number of enzymes (Geigert *et al.*, 1983a, 1983b). A great deal of recent work on ligninases (or lignin peroxidases – the enzymes catalyzing the initial steps carried out by wood rotting fungi) indicates both peroxidase and haloperoxidase activities on aromatic and ethylenic groups, respectively. Recent reports from Gold's laboratory (Renganathan *et al.*, 1986, 1987) summarize this research. Ligninase

catalyzed oxidation of several phenolic pollutants by hydrogen peroxide has been accomplished, although only partial removal was observed (Aitken *et al.*, 1989). Lewandowski *et al.* (1990) immobilized the white rot fungus *Phanerochaete chrysosporium* which produces ligninase extracellularly and used it to degrade 2–chlorophenol at inlet concentrations up to 520 mg/L.

Similarly, laccases from various soil micro–organisms have been shown by Bollag and coworkers (Hoff *et al.*, 1985) to cause oligomerization of phenols and anilines. Bollag (1990) suggests the use of laccase and other enzymes to catalyze the binding of aromatic pollutants in ground water with the humic materials in soils. In this manner, highly soluble pollutants would partition more efficiently from the aqueous phase allowing decontamination *in situ*. Dec and Bollag (1990) showed that laccase from the fungi *Rhizoctonia praticola* and horseradish peroxidase were effective in polymerizing several phenols producing a mixture of oligomers with average molecular masses of up to 800 for the fraction soluble in dioxane. They also observed that enzymatic polymerization of 2,4–dichlorophenol resulted in 20% dechlorination of the organic. They pointed out that dehalogenated products are usually less toxic and more susceptible to biological degradation. Roy–Arcand and Archibald (1991) also found that laccases from *Trametes* (*Coriolus*) *versicolor* could lower the adsorbable organic halogen content of chlorinated Kraft bleachery effluents by dechlorinating a number of toxic polychlorinated phenols and guaiacols. They concluded that the extent of the dechlorination could be increased through process optimization. Kersten *et al.* (1990) performed some mechanistic comparisons between horseradish peroxidase, lignin peroxidase and laccase in the oxidation of methoxy benzenes.

A French group under Chapsal (Chapsal *et al.*, 1986; Matcham *et al.*, 1987) has explored the possibility of substituting hemoglobin (which is very abundant, inexpensive and also shows peroxidase activity) in place of peroxidase.

Furthermore, these workers immobilized hemoglobin and reported small–scale reactor operation. Their data show that hemoglobin is a catalyst of lower specific activity and requires approximately 15–fold more hydrogen peroxide than peroxidase to catalyze phenol transformation. A recent theoretical paper (Ortiz de Montellano, 1987) suggests in mechanistic terms why hemoglobin is less than ideal for the type of free radical reactions of present interest.

3. MATERIALS AND METHODS

In order to monitor the course of the reacting system and to evaluate the capabilities of horseradish peroxidase under varying conditions, analytical techniques were required to measure hydrogen peroxide, aromatic substrate and enzyme concentrations. Such techniques have been developed and implemented over the course of this study. In addition, experimental protocols were developed for the operation of the batch, semi-batch and continuous flow reactors.

3.1 Materials

Horseradish peroxidase (EC 1.11.1.7, RZ 1.1) and catalase (EC 1.11.1.6) were purchased from Sigma Chemical Co. St. Louis, MO. The specific activity of horseradish peroxidase was approximately 44.3 ± 1.0 U/mg dry solid and 163.3 ± 3.3 U/mg peroxidase (based on the absorbance at 404 nm and a peroxidase extinction coefficient of 102 000 $M^{-1} cm^{-1}$) measured using the assay technique described below. One unit is based on the number of micromoles of 3,5–dichloro–2–hydroxy-benzenesulfonic acid (HDCBS) converted per minute at 25°C and pH 7.4 with 9 mM HDCBS, 2.4 mM 4–aminoantipyrine and 0.1 mM hydrogen peroxide. The nominal activity measured by Sigma Chemical Co. was 80 Units/mg dry solid where one unit will form 1.0 mg of purpurogallin from pyrogallol in 20 seconds at pH 6.0 and 20°C. The nominal activity of the catalase was 9 300 Units/mg dry solid and 12 400 Units/mg protein where one unit will decompose 1.0 micromole of H_2O_2 per minute at pH 7.0 and 25°C while the H_2O_2 concentration falls from 10.3 to 9.2 micromoles per millilitre of reaction mix. Enzymes were stored as dry powder in a freezer at –15°C until needed. Aqueous stock solutions of peroxidase and catalase were prepared weekly and stored at 4°C. Horseradish peroxidase activity was measured daily.

All phenolic compounds used in this study had a purity of 99% or greater and were purchased from Aldrich Chemical Co., Milwaukee, WI. The phenolic compounds used include phenol, 2–chlorophenol, 3–chlorophenol, 4–chlorophenol, 2–methylphenol, 3–methylphenol, 4–methylphenol and 2,4–dichlorophenol. Hydrogen peroxide (30% w/v) was supplied by BDH Chemicals, Toronto. Aqueous solutions of aromatic compounds were prepared as needed and stored at 4°C. Peroxide solutions were prepared daily. The buffers used and the corresponding pH ranges in which they were applied are listed in Table 3–1. Buffers were prepared according to the method of Gomori (1955) and were of equal concentration (0.1 M).

Colour development and absorbance were measured using a Hewlett Packard Diode Array Spectrophotometer Model 8452A (wavelength range 190 to 820 nm with 2 nm resolution) operated from a HP Vectra ES/12 computer. Quartz spectrophotometer cells (macro and semi–micro with 1 cm path length) were supplied by Hellma (Canada) Ltd.

Up to 24 15–mL samples may be centrifuged simultaneously in a Centra–8 centrifuge purchased from the International Equipment Company (IEC) of Needham Hts., MA. Samples were centrifuged at 3 000 gravities for 40 minutes.

Measurements of pH were performed with an Orion Research Microprocessor Ionanalyzer Model 901. Two point calibrations bracketing the pH ranges under investigation were performed with pH standards purchased from BDH Chemicals, Toronto.

The Harvard 22 Syringe Pump, capable of simultaneous infusion of the contents of up to 10 syringes, was purchased from Harvard Apparatus, South Natick, MA. The Becton Dickinson plastic syringes (inert and non–adsorbing) were supplied by CanLab Scientific Ltd., Toronto. A peristaltic pump (Model 89052B) purchased from Hewlett Packard was used to deliver solutions in the continuous flow reactor systems. Food grade silicon and Tygon tubing were used for flexible

Table 3–1: Buffers and the pH Range Over Which They were Applied

Buffering Reagents	pH Range
Hydrochloric acid - potassium chloride	1.0 to 2.2
Citric acid – dibasic sodium phosphate	2.6 to 7.0
Citric acid - sodium citrate	3.0 to 6.0
Acetic acid - sodium acetate	4.0 to 5.6
Monobasic sodium phosphate - dibasic sodium phosphate	5.7 to 8.0
Tris(hydroxymethyl)aminomethane – hydrochloric acid	7.2 to 9.0
Boric acid – borax	7.6 to 9.2
Sodium carbonate - sodium bicarbonate	9.2 to 10.6

joints and peristaltic pump tubing. All elbow joints, valves and connecting tubing (1 mm ID) were made of teflon.

Water bath temperatures were maintained using a Haake D1 Temperature Controller produced by Haake of Germany.

3.2 Analytical Techniques

A complete description of the theory, procedures and calculations associated with the analytical techniques discussed in the following sections may be found in Appendix B.

3.2.1 Horseradish Peroxidase Enzyme

An assay was developed in this laboratory (Artiss *et al.*, 1979) in order to measure the activity/concentration of the HRP enzyme in solution. This assay uses 3,5–dichloro–2–hydroxybenzenesulfonic acid (HDCBS), 4–aminoantipyrine (AAP) and hydrogen peroxide (H_2O_2) as colour generating substrates. Excess amounts of these substrates force the initial rate of reaction to become directly proportional to the amount of enzyme present. The rate of reaction is measured by monitoring the rate of formation of the non-precipitating product which absorbs light at a peak wavelength of 510 nm.

This assay must be conducted under very specific conditions of substrate concentrations, pH and temperature due to the dependence of activity measurements on these variables. It has been found that the presence of even slight amounts of other substrates of HRP such as the phenolic compounds used in this study interfere with this assay and do not provide reliable measurements of activity. This is demonstrated in Figure 3–1 in which the assay solution has been spiked with various concentrations of 4–chlorophenol between 0 and 0.2 mM (25.7 mg/L) and the resulting activity appears to change even though the same amount of enzyme is

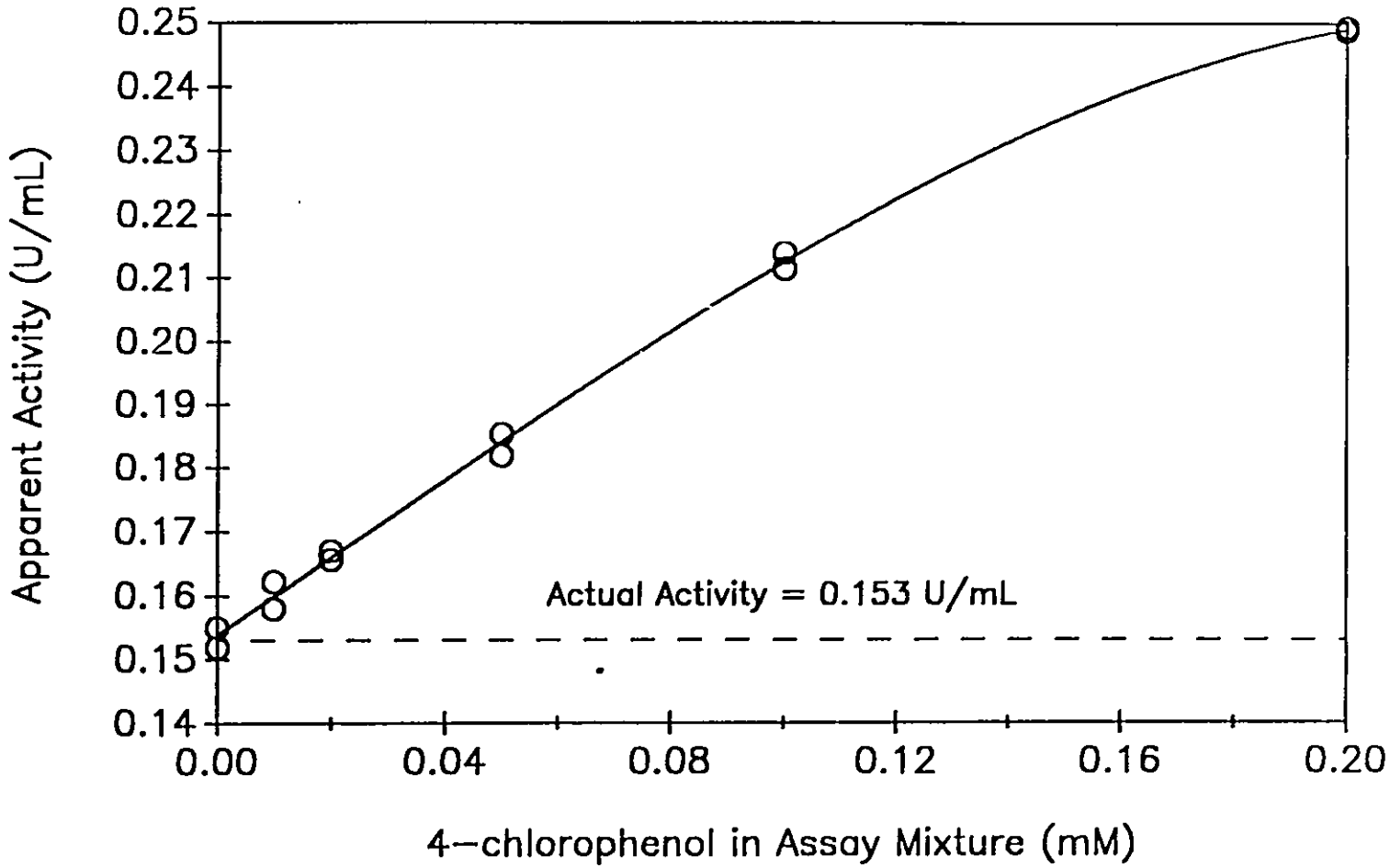


Figure 3-1: The Effect of 4-Chlorophenol Concentration on Activity Measurements of HRP

present in all cases. In this case, a 2% increase in the total aromatic concentration (0.2 mM 4–chlorophenol added to 9.0 mM HDCBS) caused a 70% increase in the rate of colour formation at 510 nm. This drastic effect complicates the measurement of enzyme activity in a reaction mixture since aromatic compounds are present. Therefore, only initial concentrations of HRP, measured before the addition of an aromatic substrate, can be obtained using this assay.

An attempt was made to develop an alternative method for measuring enzyme activity. This method replaces the HDCBS normally used in the activity assay with the aromatic substrate under study – the intention being that the aromatic compound could not interfere with an assay based on itself. However, this assay requires that saturation be achieved with respect to all substrates. Saturation was never satisfactorily achieved with any of the aromatic compounds studied. Therefore, the assay development was abandoned.

3.2.2 Aromatic Compound

In order to determine reactor efficiency and kinetics of the enzyme–catalyzed polymerization of aromatic substrates, it is necessary to be able to make quick and reproducible measurements of the aromatic concentration in solution as the reaction proceeds. It was hoped that this could be accomplished spectrophotometrically since aromatic compounds absorb ultraviolet light in quantities that are directly related to their concentration. Calibration curves at the appropriate peak wavelengths were developed for all aromatic substrates examined in this study. The calibrations were conducted at various pH's since pH influences the peak wavelength and amount of light absorbed by the compounds.

The concentrations of phenolic compounds used in this study were expressed in terms of molar quantities for convenience of calculation. A one millimolar solution (a typical concentration used in this study) corresponds to: 94.1 mg/L of

phenol, 128.6 mg/L of 2–, 3– and 4–chlorophenol, 108.2 mg/L of 2–, 3– and 4–methylphenol, and 163.0 mg/L of 2,4–dichlorophenol.

While no interferences were observed due to the presence of HRP, H_2O_2 or the buffers used to maintain pH, there was a possibility that the reaction products, which themselves are aromatic, might remain in solution to an extent that they would also absorb light at the peak wavelength, thus giving a false measurement of the amount of aromatic compound remaining in solution. In order to confirm or dispel this possibility, a colorimetric assay was developed in which the phenol was made to react with 4–aminoantipyrine and hydrogen peroxide using HRP as catalyst. The colour generated is directly proportional to the concentration of the aromatic substrate. This assay has been applied to reaction mixtures in which 4–chlorophenol was removed to various extents using hydrogen peroxide and HRP. The amount of 4–chlorophenol remaining in solution was measured using both the colorimetric assay and the method based on the absorbance of UV light. It was found that there was no significant difference between the results obtained by the two methods except at low 4–chlorophenol concentrations where the colorimetric assay was subject to wide scatter. In addition, it was found that the UV absorbance method has the advantage of being simpler, quicker and more reproducible than the colorimetric assay. Therefore, the UV absorbance method was used to monitor substrate concentrations.

Unfortunately, the UV absorbance method cannot be used to measure the aromatic substrate concentration on a continuous basis. This complication arises from the formation of precipitate which is opaque to the UV light. Continuous filtration of the sample was attempted in order to remove the precipitates in advance of spectrophotometric measurements. However, it was found that the colloidal particles could not be adequately removed by filtration alone. Rather, it was necessary to first coagulate the particles by adding alum and adjusting the pH

to approximately 6.3 to optimize floc formation. No interference in the colorimetric method was observed when alum was present in solution. Following centrifugation, an aliquot of the supernatant is taken for spectrophotometric measurement. The spectrophotometer calibration corresponding to each compound at a pH of 6.3 was used to determine the residual aromatic substrate concentration.

3.2.3 Hydrogen Peroxide

A colorimetric assay was developed for the measurement of hydrogen peroxide (H_2O_2) concentration using HRP as catalyst and HDCBS and AAP as colour generating substrates. The assay conditions were such that the only substrate present in limiting quantities was hydrogen peroxide. Therefore, the degree of colour formation at 510 nm is directly proportional to the amount of peroxide present in the assay mixture. The concentration of peroxide in the original sample was calculated based on the volume of sample added to the assay mixture. The degree of colour formation at 510 nm as a function of peroxide concentration in the assay mixture is shown in Figure 3–2. Measurements were found to be extremely reproducible with a standard deviation that is typically less than two percent over the concentration range of 5.0 to 50 μM peroxide in the assay mixture.

The H_2O_2 assay was tested to determine if the presence of varying amounts of horseradish peroxidase and phenolic substrates had any effect on the assay calibration. Since no interferences were observed, it was concluded that this assay could be used to produce accurate measurements of peroxide concentration in a reaction mixture.

3.3 Reactor Operation

Three types of reactor systems were created including batch, semi–batch and continuous stirred tank reactors. A detailed explanation of the operation of each

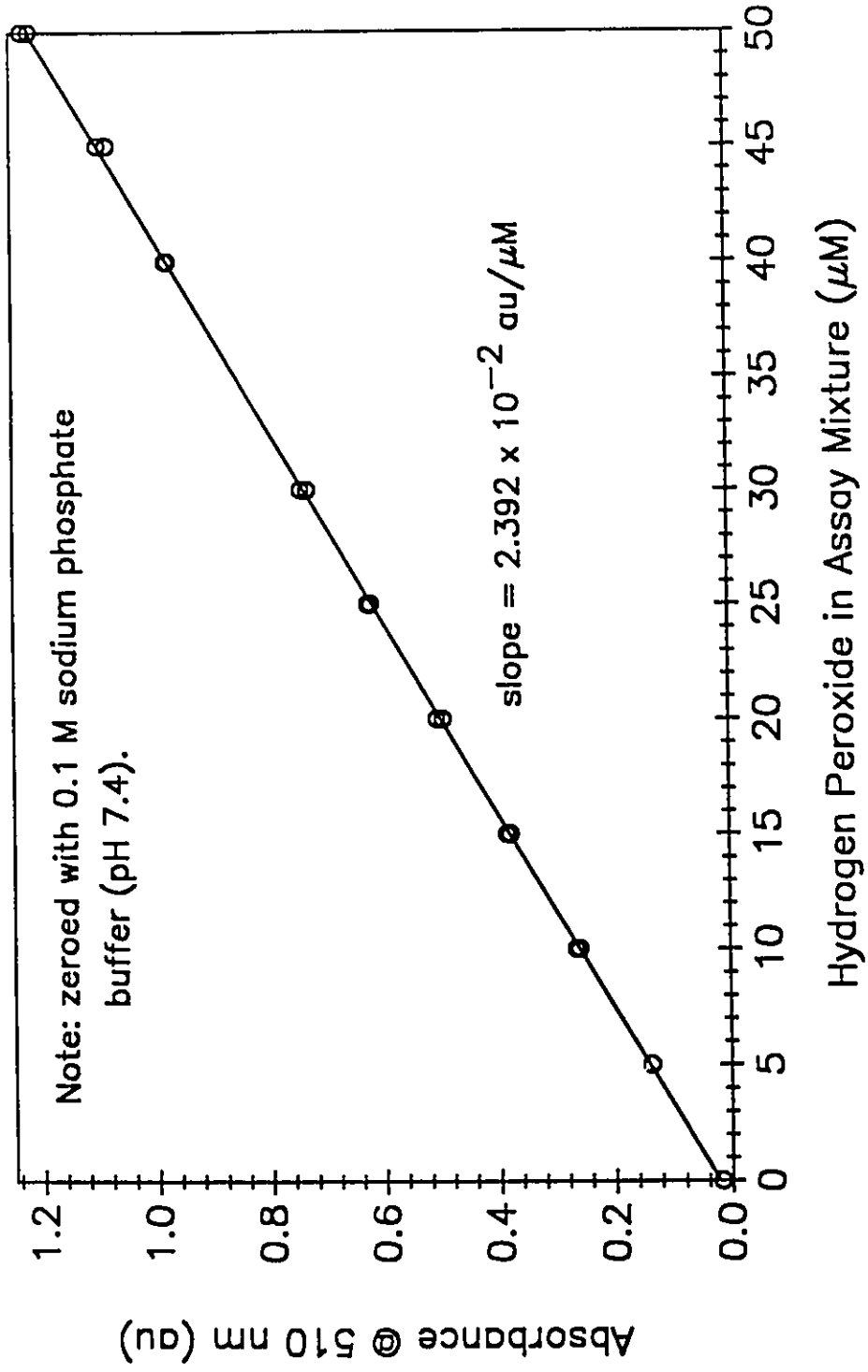


Figure 3–2: Hydrogen Peroxide Assay Calibration Curve

reactor system is included in Appendix C.

3.3.1 Batch Reactor Operation

The batch system shown in Figure 3–3 was used to determine: removal efficiencies of phenolic compounds from aqueous solution; catalytic lifetime of the enzyme at various conditions of temperature, pH and substrate dose; reaction stoichiometry; and, conversion as a function of time for kinetic modelling.

The batch reactor consisted of a vial containing 30 milliliters (mL) of a mixture of aromatic substrate, hydrogen peroxide, peroxidase enzyme and buffer where applicable. The procedure begins by combining the aromatic substrate and enzyme in the desired amounts with distilled water or a buffer solution at the appropriate pH. The reaction was initiated by the addition of hydrogen peroxide. The reacting solution was mixed vigorously using a magnetic stirrer and teflon coated stir bar. The reaction was stopped when desired by the addition of a dose of catalase enzyme. Catalase catalyzes the rapid conversion of hydrogen peroxide to oxygen and water (Ortiz de Montellano, 1983) according to:

$$2\,H_2O_2 \longrightarrow O_2 + 2\,H_2O \qquad (3\text{–}1)$$

Therefore, a large dose of catalase consumes the hydrogen peroxide remaining in the reactor and halts the peroxidase reaction.

The additional absorbance of UV light in the reaction mixture as a result of catalase addition was found to be insignificant. Where extremely large doses of catalase were required to halt a reaction (mixtures with high H_2O_2 concentrations greater than 3 mM), the UV absorbance was corrected by monitoring the absorbance at 404 nm. The absorbance at 404 nm is proportional to the absorbance of the catalase in the UV range at which the aromatics studied absorb light. The

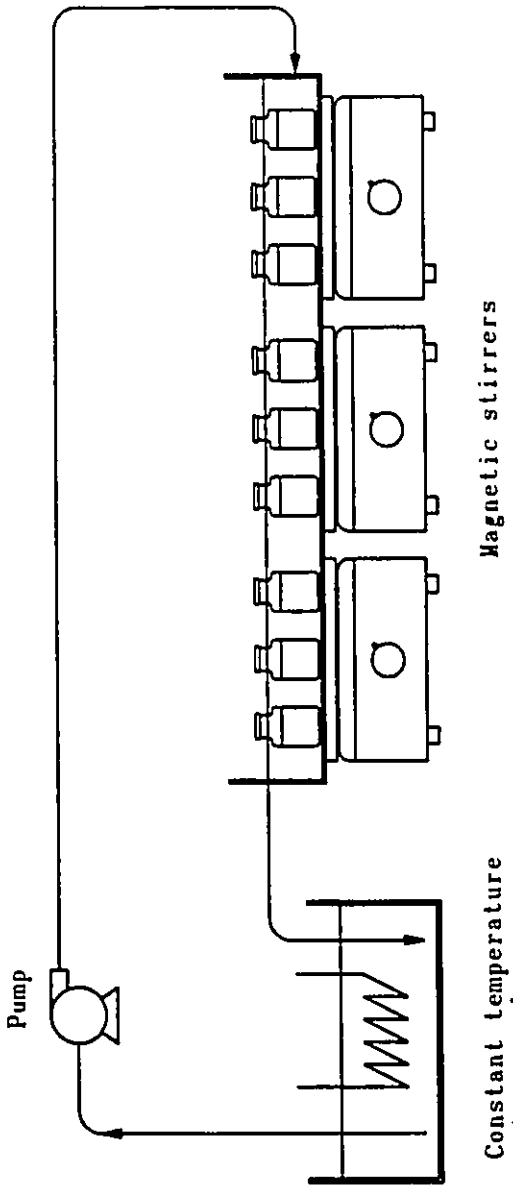


Figure 3-3: Batch Reactor System

corrections made never exceeded 3% of the observed absorbance.

The temperature of the system was maintained at the desired level using a constant temperature water bath. This water bath coul l accommodate the simultaneous operation of up to 35 30–mL batch reactors with a fixed temperature between 10°C and 40°C. Insulation was placed between the magnetic stirrers and the water bath to prevent the formation of local temperature gradients.

For temperatures up to 80°C a second apparatus was constructed which provided better heat transfer between the water bath and batch reactors and had less heat loss through the liquid surface and sides of the water bath. This bath was only used for high temperature batch reactor studies since it could accommodate only 8 samples at a time.

3.3.2 Semi–Batch Reactor Operation

The reactor system shown in Figure 3–3 was modified for semi–batch reactor operation by adding the horseradish peroxidase or hydrogen peroxide to the vials over time as shown in Figure 3–4. This was accomplished using a variable–rate syringe pump for the simultaneous injection of either peroxide or enzyme to a maximum of ten vials. As with the batch system, the reaction was halted by the addition of catalase.

3.3.3 Continuous Stirred Tank Reactor (CSTR) Operation

The continuous stirred tank reactor (CSTR) used in this study is shown in Figure 3–5. This reactor consisted of a graduated syringe with a volume that can be varied between approximately five and one–hundred and forty milliliters. The synthetic wastewater was pumped into the reactor using a peristaltic pump operating at high speed to minimize pulsating flow in the reactor. This influent consisted of aqueous aromatic substrate, hydrogen peroxide, and buffer when

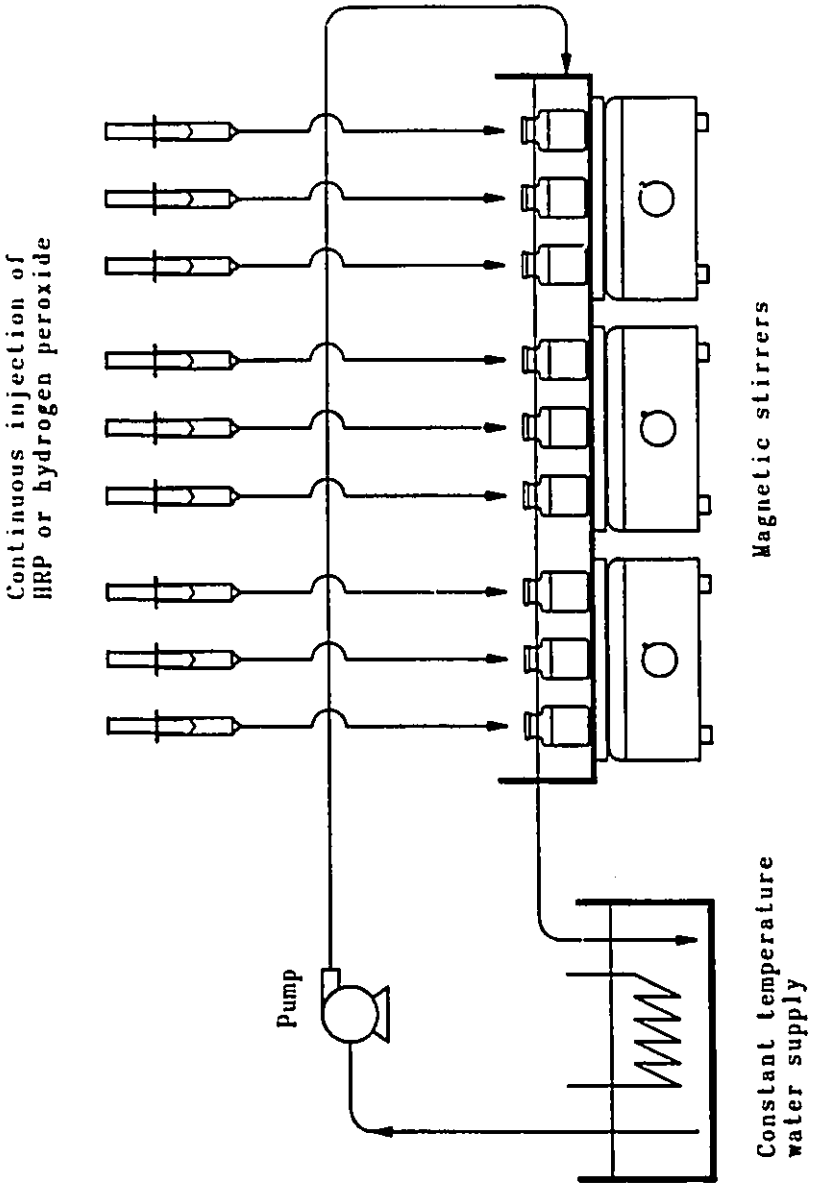


Figure 3-4: Semi-Batch Reactor System

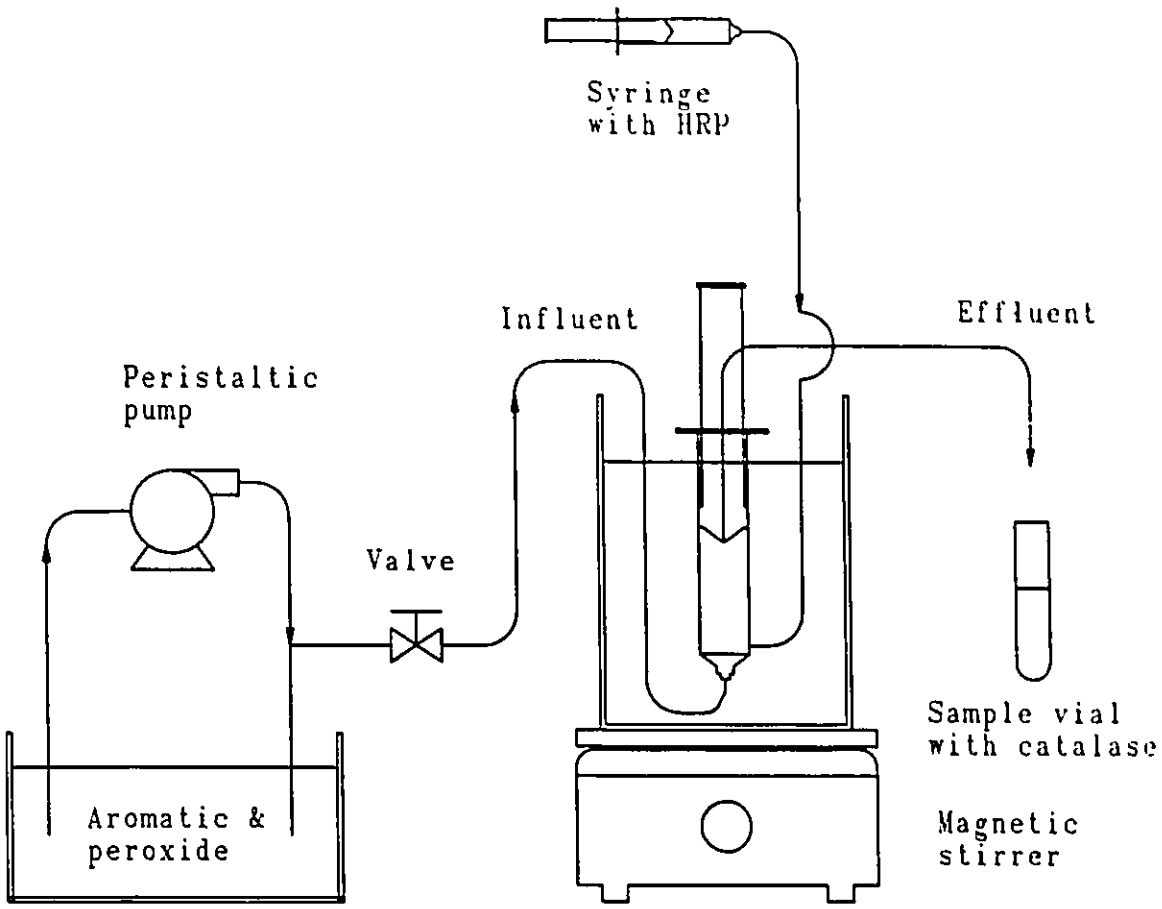


Figure 3–5: Single Continuous Stirred Tank Reactor System

required. The HRP enzyme was introduced into the side of the reactor using a syringe pump. The synthetic wastewater stream entered at the base of the reactor at the appropriate flowrate. The reactor contents were mixed vigorously with a teflon coated stir bar propelled by a magnetic stirrer. The contents were stirred with sufficient energy to prevent any settling or accumulation of precipitate in the reactor. This magnetic stirrer also provided mixing action to the constant temperature water bath. The reactor had a fixed volume, therefore effluent exited from the top of the reactor at the same rate at which the influent was introduced. Effluent flowrates were checked periodically.

Once the system reached steady state, samples were collected from the effluent stream by diverting a measured volume into a vial containing a dose of catalase enzyme. While the sample was being collected the catalase rapidly consumed the peroxide remaining in the exit stream and halted the polymerization reaction. The time to stop this reaction was extremely small with respect to the retention time in the CSTR.

The continuous flow system could also be operated as a series of CSTR's in which the effluent from one reactor passed directly into a subsequent reactor or was available for sampling. The options existed to continuously inject either peroxidase enzyme or peroxide or both to each reactor in the series.

3.4 Sources of Error

Experimental observations are subject to errors which result from (i) personal errors in reading and interpreting measurements, (ii) accuracy and reproducibility of measurements and (iii) calibration error. Experiments and calibrations were reproduced to verify experimental values and trends in the data. Not all experiments were repeated due to time constraints; however, repetition was performed when data indicated trends which required detailed interpretation. The

reproducibility of typical measurements are discussed. All standard deviations listed below result from five repetitions of the same measurement under fixed conditions of pH, temperature, peroxide and aromatic compound concentration, and enzyme dose and concentration, where applicable.

Measurements of activity were reproducible with standard deviations in the range of 0.5 to 1.8% for a 60–fold dilution of stock horseradish peroxidase (0.4 mg/mL) when the activity measurement was repeated five times. Measurement of the activity of samples prepared one day apart resulted in standard deviations between 1.0 and 2.3%.

Measurements of peroxide concentration using the peroxide assay had a typical standard deviation of 2.0% for samples prepared one day apart with or without the presence of an aromatic substrate. Measurements of aromatic concentration by spectrophotometric analysis had standard deviations between 0.1 and 0.5% for the same sample and between 0.2 and 1.0% for samples prepared one day apart.

Measurements of residual aromatic compound concentration following treatment in a batch reactor for five hours had a typical standard deviation of 2.5% (based on the influent concentration) between experiments. Similarly, transient aromatic concentrations measured for kinetic analyses had standard deviations between 2 and 4%. Measurement of residual aromatic compound concentrations in both single reactors and multiple continuous stirred tank reactors in series were extremely reproducible with a standard deviation of 2.5% between experiments at least one week apart.

4. INACTIVATION OF HORSERADISH PEROXIDASE

Chemical species other than substrate can combine with an enzyme to alter, inhibit or destroy its catalytic ability. In many industrial processes, the rate at which the enzyme activity declines is a critical characteristic since the economic feasibility of the process may hinge on the useful lifetime of the enzyme biocatalyst (Bailey and Ollis, 1986). Such is the case with the enzymatic treatment of wastewater where the catalyst is susceptible to inactivation by temperature extremes, mechanical deformation and chemical denaturation.

Protein structures are stabilized by weak forces which often are important to its catalytic ability. However, this weak stability also implies that the enzyme is susceptible to perturbations in geometry and chemical structure resulting from changes in its chemical and physical environment with a resulting loss in native enzyme activity. For example, the pH and temperature of the surrounding environment can reduce the probability of certain types of interactions or alter the structure of the enzyme. In addition, mechanical forces can disturb the elaborate shape of an enzyme molecule to such a degree that inactivation occurs. The mechanical fragility of enzymes can impose limits on the fluid forces (shearing) which can be tolerated in enzyme reactors being stirred to increase substrate mass–transfer rates.

In an enzyme reactor, combinations of mechanical, chemical and thermal processes interact to influence enzyme activity. Investigation of the extent of these influences is required to determine the operating capabilities of HRP and to provide means for the extension and/or optimization of catalytic lifetime.

4.1 Stability of Horseradish Peroxidase

Storage of the enzyme in distilled water at neutral pH and 4°C for one month produced no significant loss in activity. In addition, storage of the aqueous enzyme buffered at pH 7.4 at room temperature for 2 days with or without vigorous mixing (30 mL solution stirred with a $^1/_8$" by $^1/_2$" magnetic stir bar at 2 400 rpm) produced only a 5% loss in activity. The dry enzyme retains its activity when stored for several years at –15°C (Biozyme, 1989) and for many weeks when stored at 37°C.

The activity of horseradish peroxidase measured using the assay discussed in Appendix B is a function of pH as demonstrated in Figure 4–1. Concentrations of AAP, HDCBS and peroxide were maintained at the levels prescribed by the assay procedure but the buffer was varied to provide the appropriate pH's. The buffers used and their corresponding pH's are as follows: hydrochloric acid-potassium chloride (pH < 2.2), citric acid-sodium citrate (pH 3 to 4), acetic acid–sodium acetate (pH 4 to 5.6), monobasic sodium phosphate-dibasic sodium phosphate (pH 5.7 to 8.1), boric–acid borax (pH 7.6 to 9.2) and sodium carbonate-sodium bicarbonate (9.2 to 10.6). Buffers were of equal concentration (0.1 M) and were overlapped to ensure that the changes in activity were associated with the pH and not the buffer used.

The enzyme activity had a broad optimum over a pH range of 5.7 to 8.5 with optimal activity occurring at neutral pH. HRP retained more than 90% of its activity after incubation for 48 hours at pH's between 6 to 9 and room temperature but was severely reduced outside this range. Return of the enzyme to neutral pH produced no recovery in activity.

Figure 4–2 demonstrates the inactivation of the enzyme over time as it is incubated at temperatures between 5°C and 72°C at neutral pH for periods up to one hour in duration. All activities were measured at 25°C. The curves demonstrate that the enzyme is susceptible to inactivation at elevated

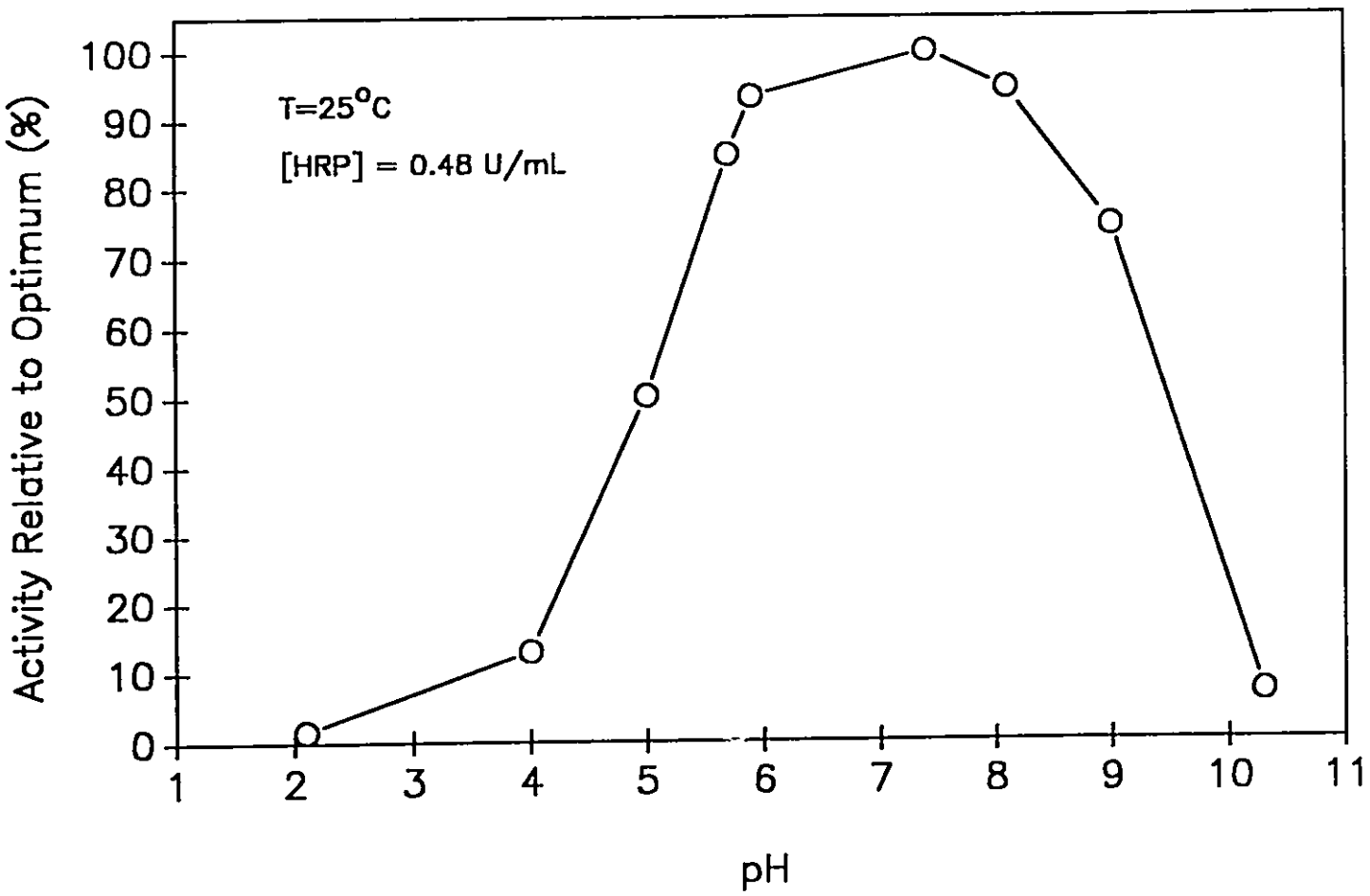


Figure 4–1: Horseradish Peroxidase Activity as a Function of pH

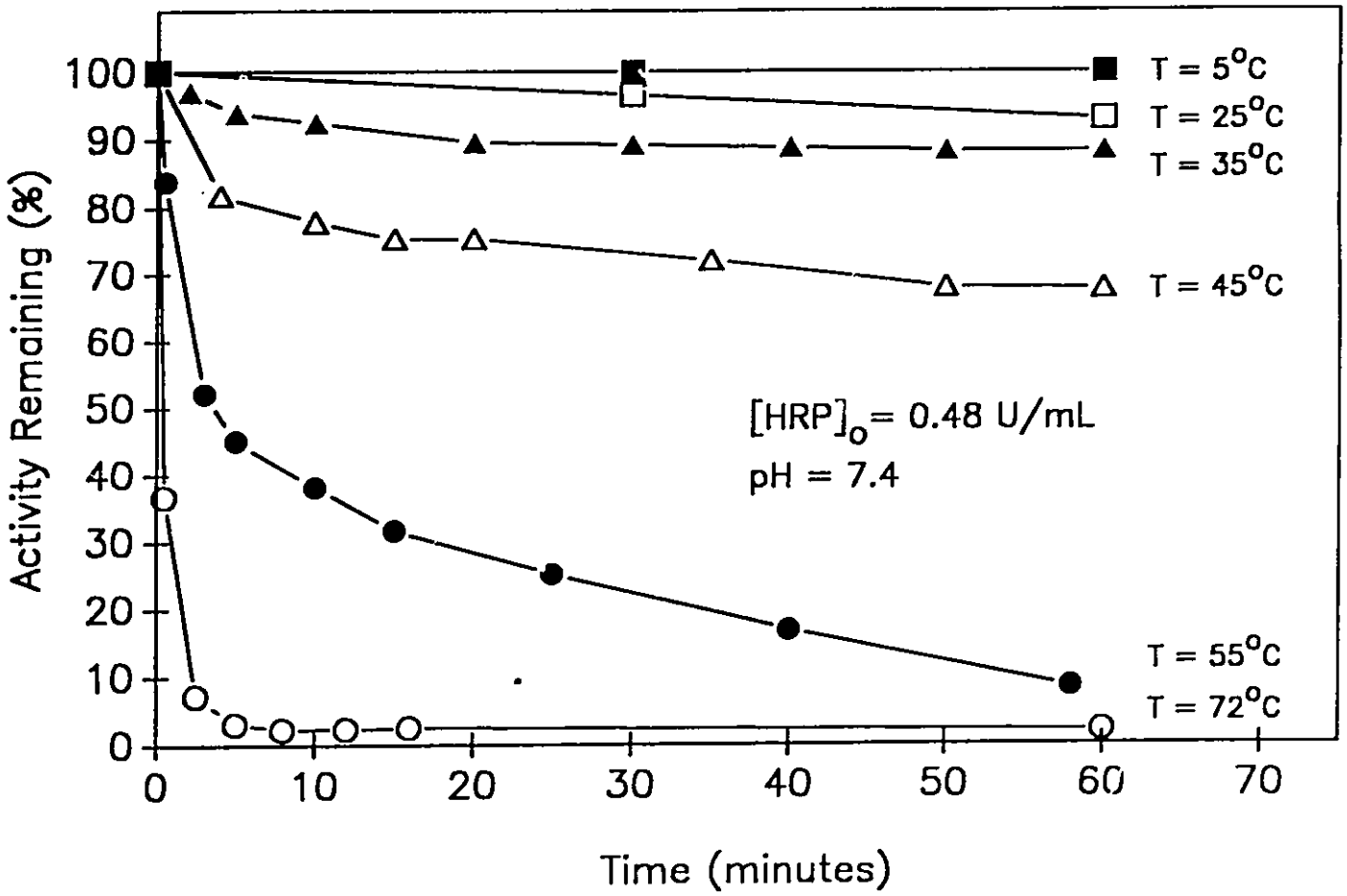


Figure 4–2: HRP Activity as a Function of Time and Temperature

temperatures. HRP retained all of its activity when stored at 5°C and 70% or more of its activity after incubation at temperatures ranging from 25°C to 45°C for one hour. Above 45°C the residual activity declined rapidly with increasing temperature. Virtually all enzyme activity was lost immediately at 72°C. No recovery of activity was noted upon returning the incubation mixtures to room temperature. The rate of reaction (relative activity) for HRP increases with temperature up to a maximum at 50°C and then rapidly falls off past 50°C as shown in Figure 4–3.

The thermal inactivation of HRP was not first order with respect to enzyme concentration as has been reported by Lee *et al.* (1984). A first–order process would have produced straight lines on a plot of the logarithm of the percentage of activity remaining versus time. Instead, at each of the temperatures examined, a rapid initial inactivation followed by a more gradual loss in activity was observed. This biphasic, non–first–order inactivation may be the result of a mixture of isoenzymes of which some are heat-resistant and others are heat-sensitive. The heat–sensitive enzyme rapidly loses activity leaving the stronger, more resistant enzyme to be inactivated at a slower rate. Further research is required to determine if certain isoenzymes of peroxidase have greater stabilities under extremes of heat.

Results indicate that the horseradish peroxidase enzyme has the capability to operate over wide ranges of pH and temperature and can retain its activity over long storage periods before use.

4.2 Exposure of HRP to Single Substrates

Studies have been performed to determine how the activity of the enzyme responds to varying concentrations of the materials involved in the polymerization reaction catalyzed by HRP. It has been found that the horseradish peroxidase enzyme is relatively unaffected by storage in distilled water or phosphate buffer at

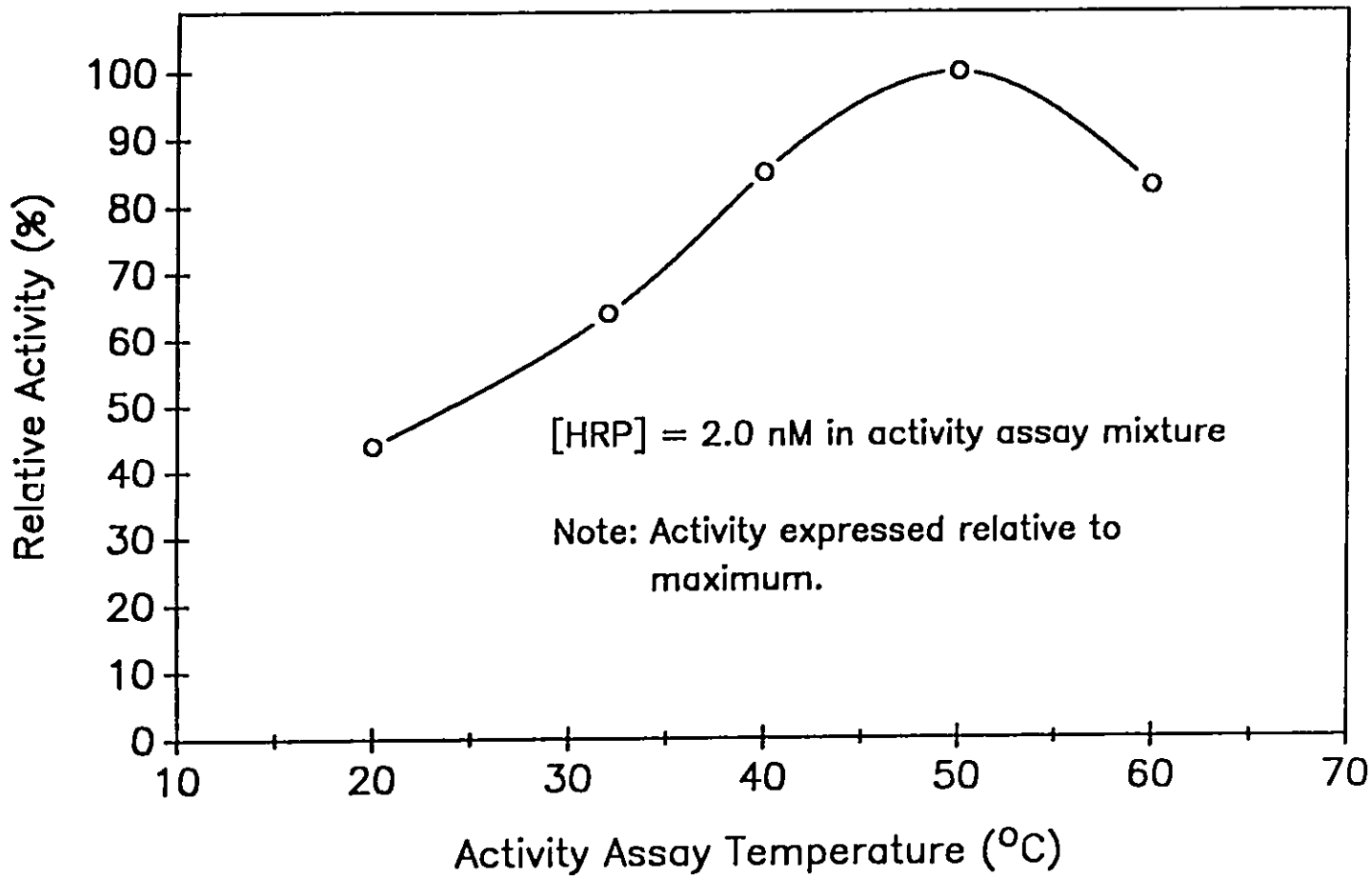


Figure 4–3: Relative Activity of Horseradish Peroxidase as a Function of Temperature

neutral pH over extended periods of time. In addition, no additional change in activity is observed over a 3 hour period when HRP is exposed to various concentrations of phenolic substrates up to concentrations of 5 mM (471 mg/L phenol, 643 mg/L chlorophenol, 541 mg/L methylphenol).

In contrast, when HRP is placed in solution with hydrogen peroxide alone, a sudden drop in activity followed by a more gradual decline is observed as shown in Figure 4–4. The magnitude of the sudden drop is a function of peroxide concentration. It is likely that more than one process is responsible for the enzyme inactivation as seen by the sudden drop and gradual decline sections of the curves in Figure 4–4. A quick but reversible deactivation process which drives to an equilibrium level could be responsible for the large drop in activity while the gradual decline may be due to a second, slower process. Inactivation of HRP in the presence of peroxide alone may be due to one or more of the following reasons:

(i) horseradish peroxidase may be oxidized to the catalytically inactive Compound III state;

(ii) Compound I may be oxidized to another inactive state as a result of altering or stripping away the heme group at the active site;

(iii) since HRP is made up of amino acid residues including the phenolic tyrosyl residue, one enzyme may be using another as substrate resulting in inactivation.

Compound II formation is required before Compound III can be produced according to the mechanism described by Reactions 2–9 and 2–10. This normally occurs when an aromatic compound is available to be oxidized by Compound I (Reaction 2–2); however, aromatic substrates were not present in these experiments. The first explanation listed above requires a mechanism in which Compound II can be formed from Compound I in the absence of aromatic substrate. Such a mechanism, proposed by Arnao *et al.* (1990b), is described by Reactions 2–5

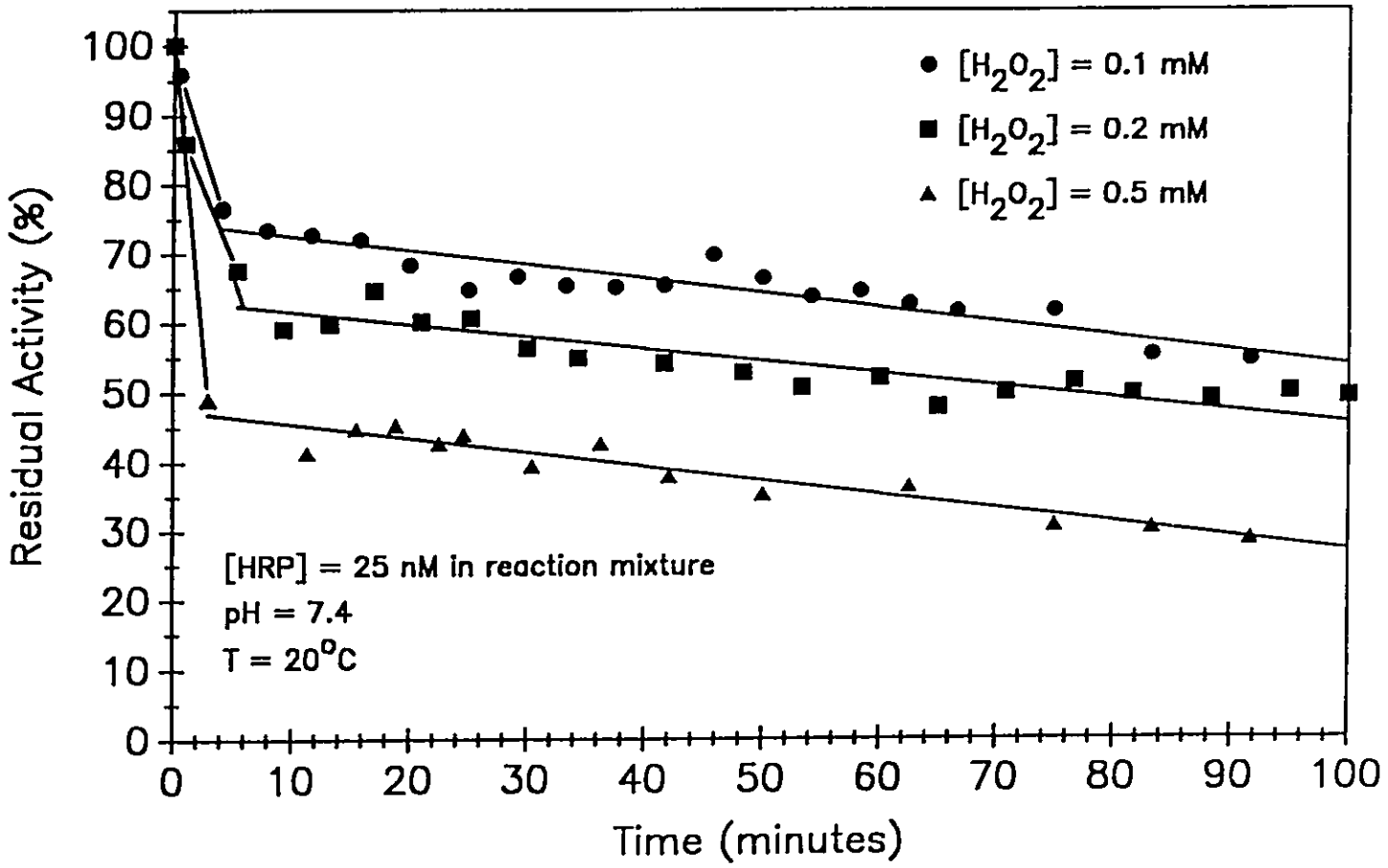


Figure 4–4: Inactivation of HRP by Hydrogen Peroxide in the Absence of an Aromatic Substrate

and 2–8.

The second explanation in which Compound I is oxidized to an inactive state is supported by evidence for the formation of the inactive form of HRP called P–670. Measurements of kinetic rate constants for the formation of P–670 through Reactions 2–5 and 2–7 has been performed by Arnao *et al.* (1990b).

The third explanation is unsatisfactory since it is unlikely that the first quick phase of the inactivation process can be explained by the uptake of one enzyme as a substrate for another since the enzyme is present in extremely small concentration (25 nM). No evidence has been demonstrated in literature to support this theory.

A combination of Compound III and P–670 formation would explain the trends of the curves in Figure 4–4. The dependence of the magnitude of the rapid drop on peroxide concentration can be explained by reversible formation of Compound III. For higher H_2O_2 concentrations, the equilibrium level of Compound III formed increases. An irreversible slow deactivation of enzyme by oxidation to P–670, or some other inactive form of HRP, would account for the gradual decline in activity. Kinetic modelling will be performed to determine if inactivation through Compound III and P–670 formation can account for the inactivation observed.

4.3 Effect of Peroxide Concentration on Activity

The treatment of wastewater with horseradish peroxidase and hydrogen peroxide would be extremely expensive if, upon the introduction of enzyme and oxidant to the treatment process, the majority of the enzyme was inactivated as was observed in Figure 4–4. Experiments were performed to determine if inactivation occurs when both substrates, aromatic compound and peroxide, are present in solution. For this experiment, a non–precipitating colour–generating substrate system consisting of AAP and HDCBS was used. This is the substrate system used

to measure the activity of the enzyme as described in Appendix B. Successive activity measurements were performed on a stock solution of HRP but in each measurement the assay recipe was varied by increasing the peroxide concentration incrementally. The rate of colour formation was monitored over a 3 minute period (before significant substrate depletion) to determine if the rate of colour formation and, hence, the activity changed with time. The results are shown in Figure 4–5.

Activity increased with peroxide concentration until it reached a level at which the enzyme became saturated with peroxide. At this concentration sufficient peroxide was available such that the enzyme's catalytic activity was no longer limited by the rate of diffusion of peroxide to the active site. Enzyme activity decreased with peroxide concentration beyond the saturation level. The loss in enzyme activity can be attributed to the rapid formation of significant quantities of catalytically inactive Compound III. For each activity measurement beyond saturation, the rate of colour formation remained constant over a 3 minute period indicating that the enzyme was not inactivated with time as was observed in the absence of aromatic substrate. This demonstrates that there is a competition between aromatic compound and peroxide for the enzyme resulting in protection of the enzyme from inactivation. This phenomenon can be explained by the reaction scheme shown in Figure 4–6 where there are two possible pathways that the enzyme can follow once it is in the Compound I (E_i) state. One route leads to the formation of Compound II and the production of a free radical from an aromatic compound such as AAP. The second route leads to the formation of E_{670}, E, or E_{ii} along paths which do not require the presence of an aromatic substrate. The mechanism describing the protection of HRP by an aromatic substrate is proposed in recent publications by Arnao *et al.* (1990a, 1990b).

According to this mechanism, in the absence of strong reducing agents (aromatic substrates), peroxidase will act like catalase, slowly breaking down H_2O_2

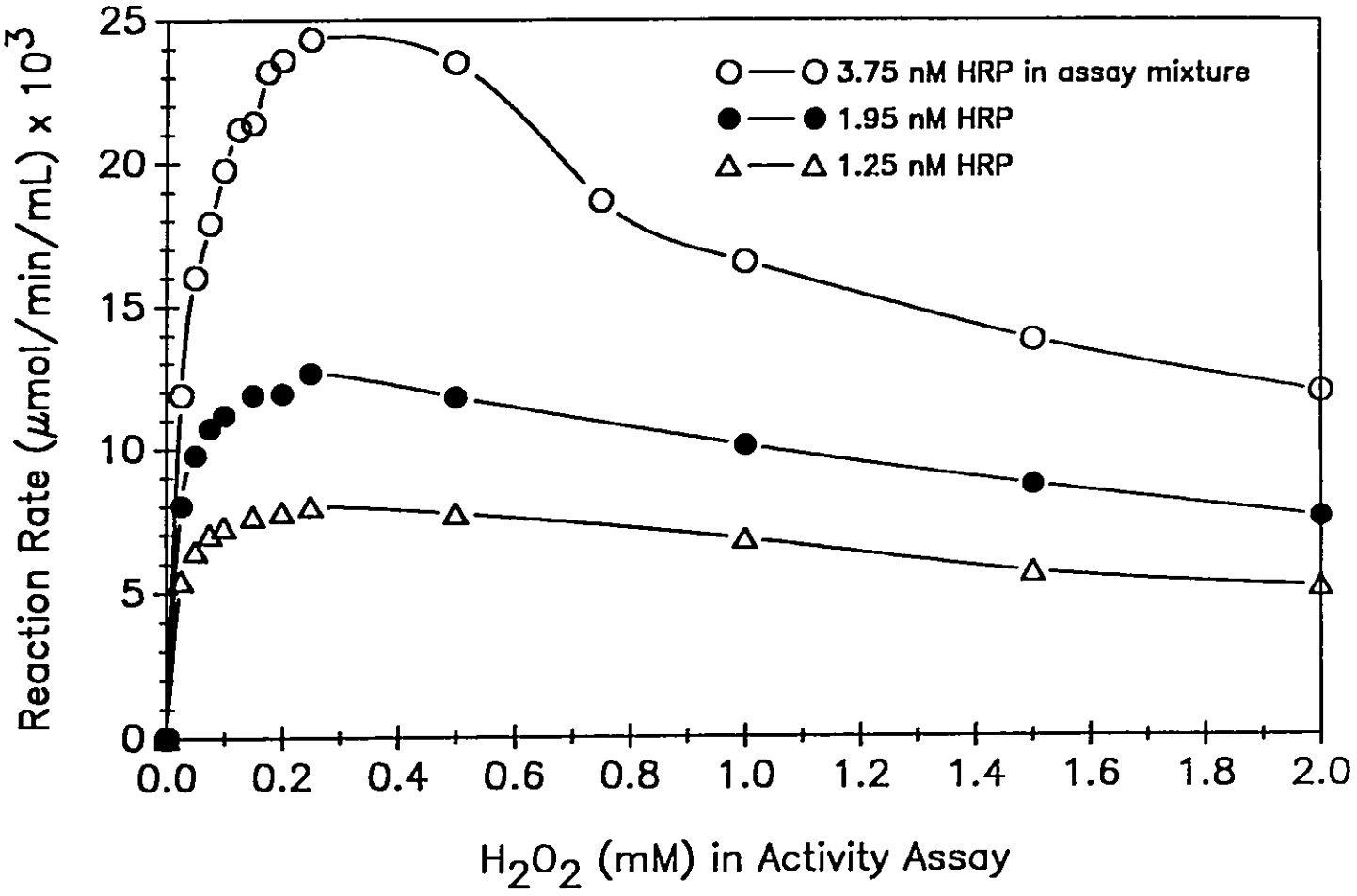


Figure 4–5: Effect of Peroxide Concentration on HRP Activity

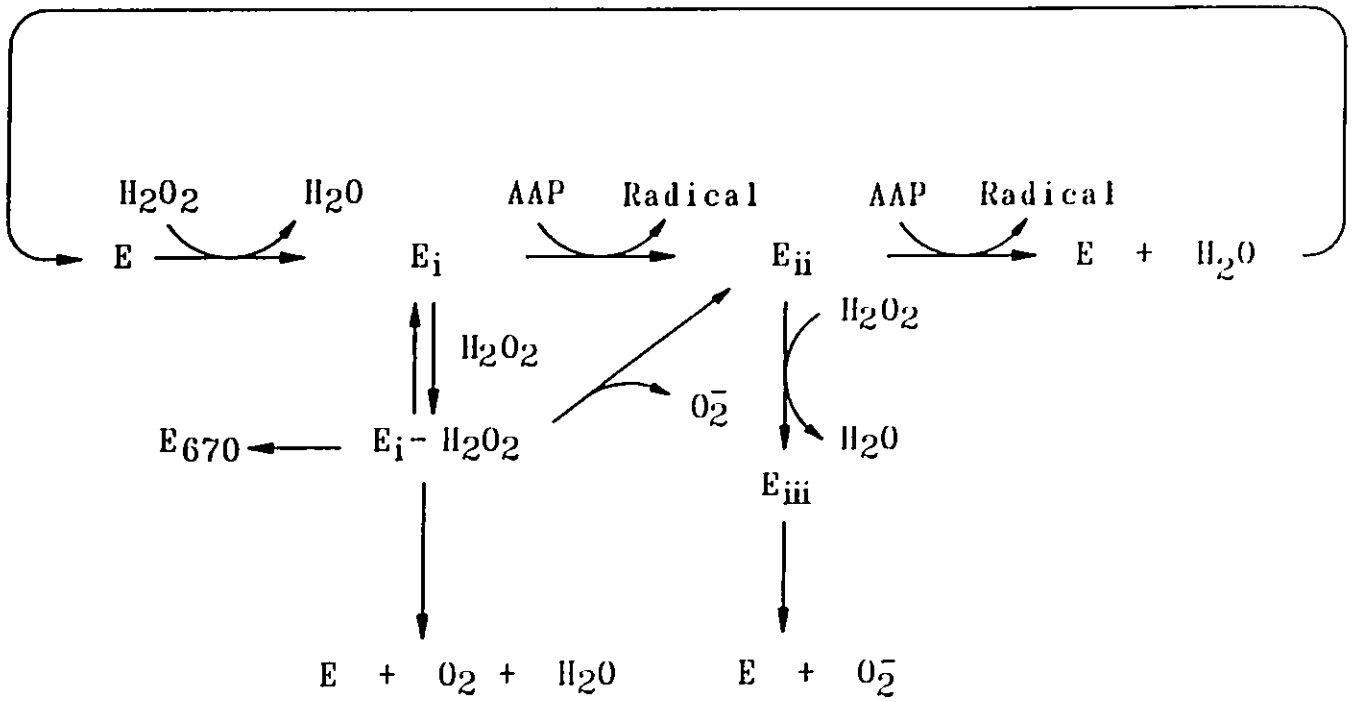


Figure 4–6: Protection of Horseradish Peroxidase in the Presence of an Aromatic Substrate (AAP)

(a weak reducing agent) to oxygen and water and gradually becoming inactive through the formation of Compound III and inactive P–670. However, in the presence of strong reducing agents, the catalase reaction and the formation of inactive enzyme are avoided because HRP favours the turnover of aromatic substrate. Arnao *et al.* (1990b) concluded that for every 450 catalytic turnovers of aromatic substrate only one inactivation results (pH 7.0, 25°C). Compound III formation may also decline as a result of aromatic compounds being present in solution; however an alternate route for the formation of Compound III, described by Reactions 2–9 and 2–10 and included in Figure 4–6, still exists.

5. BATCH REACTOR STUDIES

The batch system shown in Figure 3–4 was used to evaluate the effects of process conditions on the catalytic efficiency of horseradish peroxidase. A batch system was chosen as opposed to a continuous flow system due to its simplicity: there are no flowrates to monitor; temperature control is simple; small quantities of solutions are used; there is no wait for the system to come to equilibrium; and a large number of reactors can be operated simultaneously. The process conditions examined include enzyme and substrate concentrations, pH, and temperature. In addition, batch studies were used to generate transient data and demonstrate the co–precipitation phenomenon. Semi–batch experiments were performed to evaluate the effect of instantaneous concentration of peroxide and HRP on the catalyst efficiency.

5.1 Effect of Enzyme Dose and Aromatic Substrate Concentration

The degree to which an aromatic substrate can be removed from solution depends on the amount of catalyst added since the catalyst has a finite lifetime. The catalyst lifetime, to be termed "catalytic turnovers", is defined as the number of aromatic molecules precipitated from solution per molecule of enzyme provided. It represents a measure of the number of times the enzyme carries out or "turns over" its catalytic cycle before it becomes inactive.

Due to a finite catalyst lifetime, it is expected that as the dose of enzyme provided to the reaction mixture increases the degree of removal would also increase. This was found to be the case as shown in Figures 5–1 and 5–2 for 4–chlorophenol and phenol, respectively, treated in a batch reactor. Retention times in excess of 12 hours were used to ensure that the reaction had gone to completion. Excess peroxide was provided so that only the aromatic substrate and

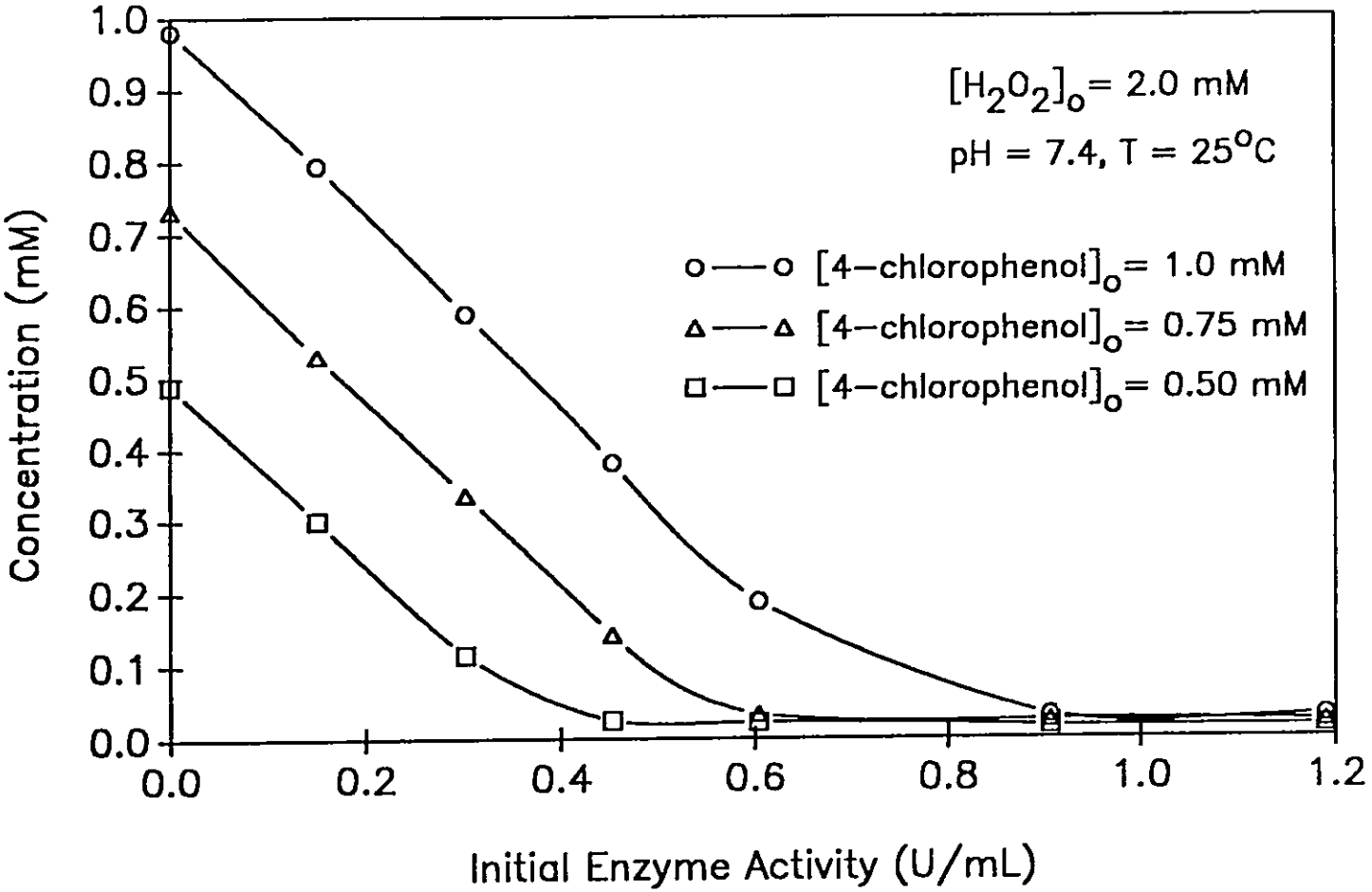


Figure 5–1: Removal of 4–Chlorophenol in a Batch Reactor as a Function of HRP Dose After Complete Reaction

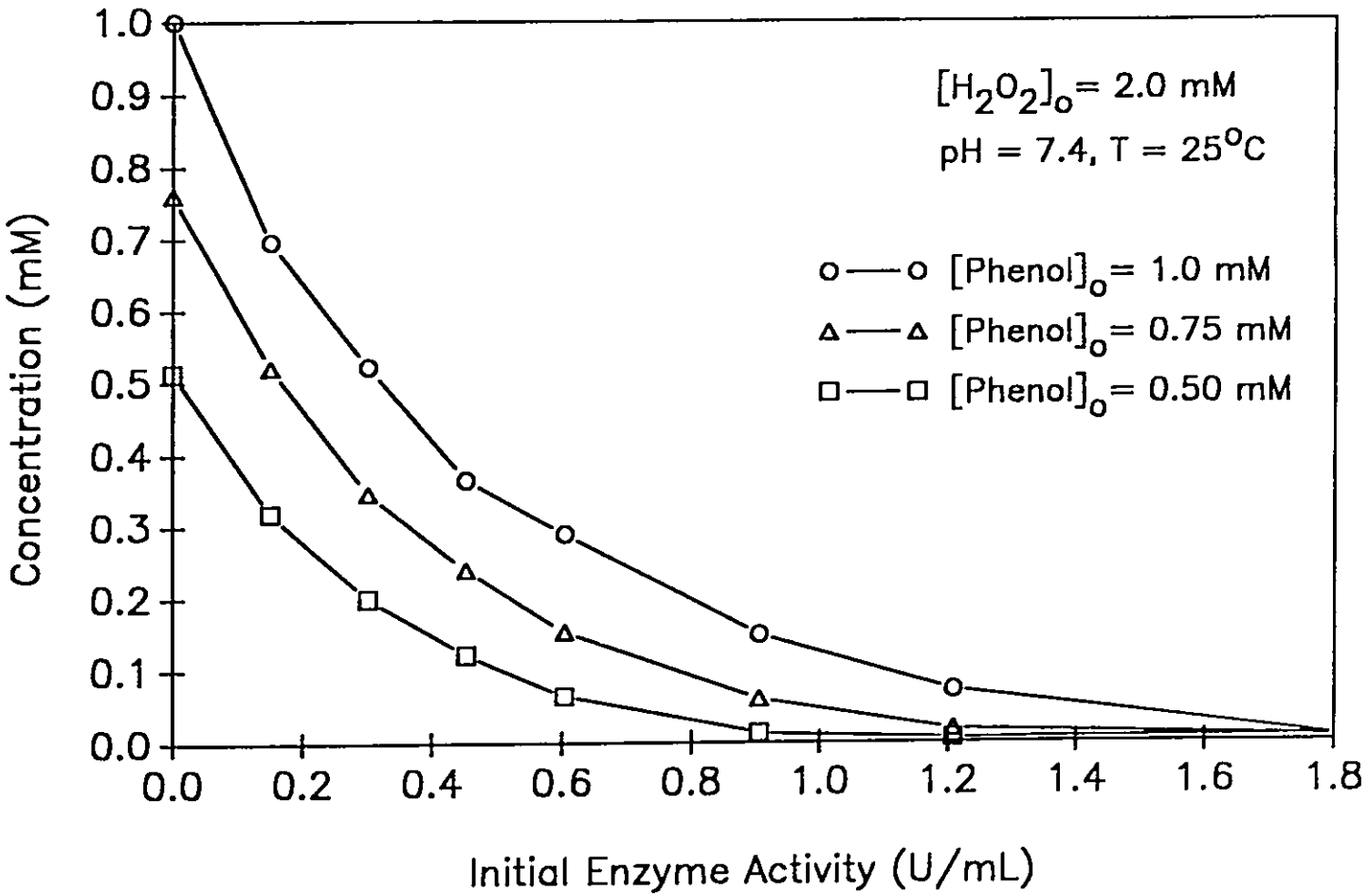


Figure 5–2: Removal of Phenol in a Batch Reactor as a Function of HRP Dose After Complete Reaction

enzyme were limiting.

Figure 5–1 demonstrates that the removal of 4–chlorophenol increases linearly with enzyme dose until the amount of substrate becomes limiting. This is true for all three starting concentrations of 4–chlorophenol. The linear profiles indicate that the number of reactions catalyzed by the enzyme is independent of enzyme dose and 4–chlorophenol concentration. This is demonstrated more clearly in Figure 5–3 where the catalytic turnovers are constant with respect to enzyme dose and 4–chlorophenol concentration while the 4–chlorophenol is not limiting. In contrast, the curves of Figure 5–2 show that as the enzyme dose increased there were diminishing returns on the amount of phenol removed from solution per amount of enzyme added. This means that the number of reactions turned over by the enzyme is greater when the enzyme concentration is low and when the aromatic compound concentration is high as shown in Figure 5–4.

The differences in the behavior of HRP with respect to phenol and 4–chlorophenol catalysis can be explained by inactivation due to free radicals which can reenter and block the active site of the enzyme. The rate of reaction for 4–chlorophenol is approximately one order of magnitude higher than that for phenol at neutral pH (Dunford *et al.*, 1976, 1986; Sakurada *et al.*, 1990). Therefore, it is likely that there is a greater time delay during which the active site of HRP remains unoccupied by phenol in comparison to 4–chlorophenol. A longer delay may provide the free radicals, formed during the phenol polymerization, a greater opportunity to return to the active site resulting in free radical inactivation of the enzyme. In such a case, the probability of a phenoxy radical finding an enzyme is greater at high enzyme concentrations resulting in a shorter average catalyst lifetime. In addition, as the reaction progresses the amount of phenol in solution decreases making it more difficult for the free radicals to find each other and easier for them to find an unoccupied enzyme. Since the 4–chlorophenol polymerization is very rapid, the free

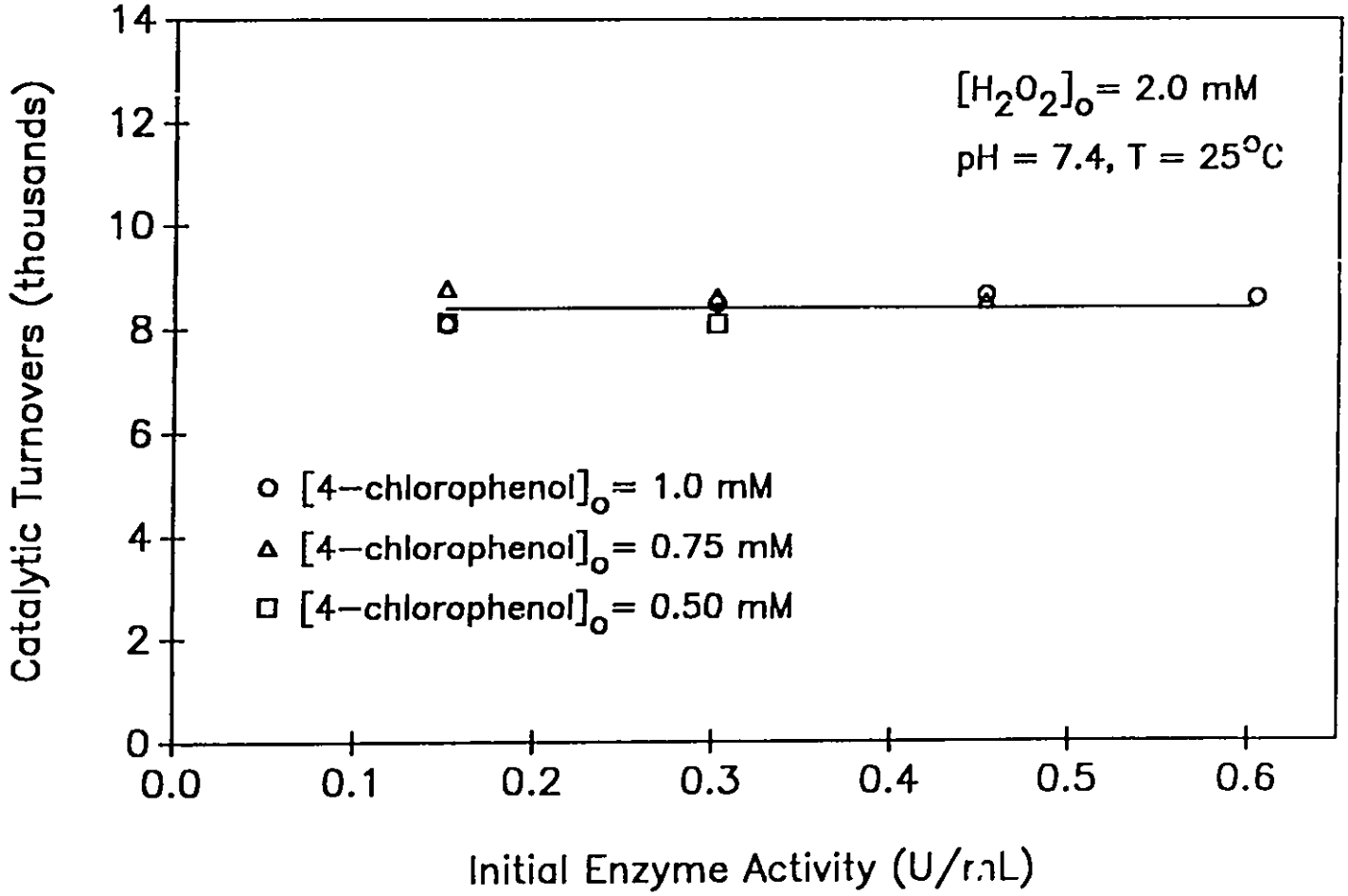


Figure 5–3: Catalytic Turnovers as a Function of 4–Chlorophenol and HRP Dose in a Batch Reactor

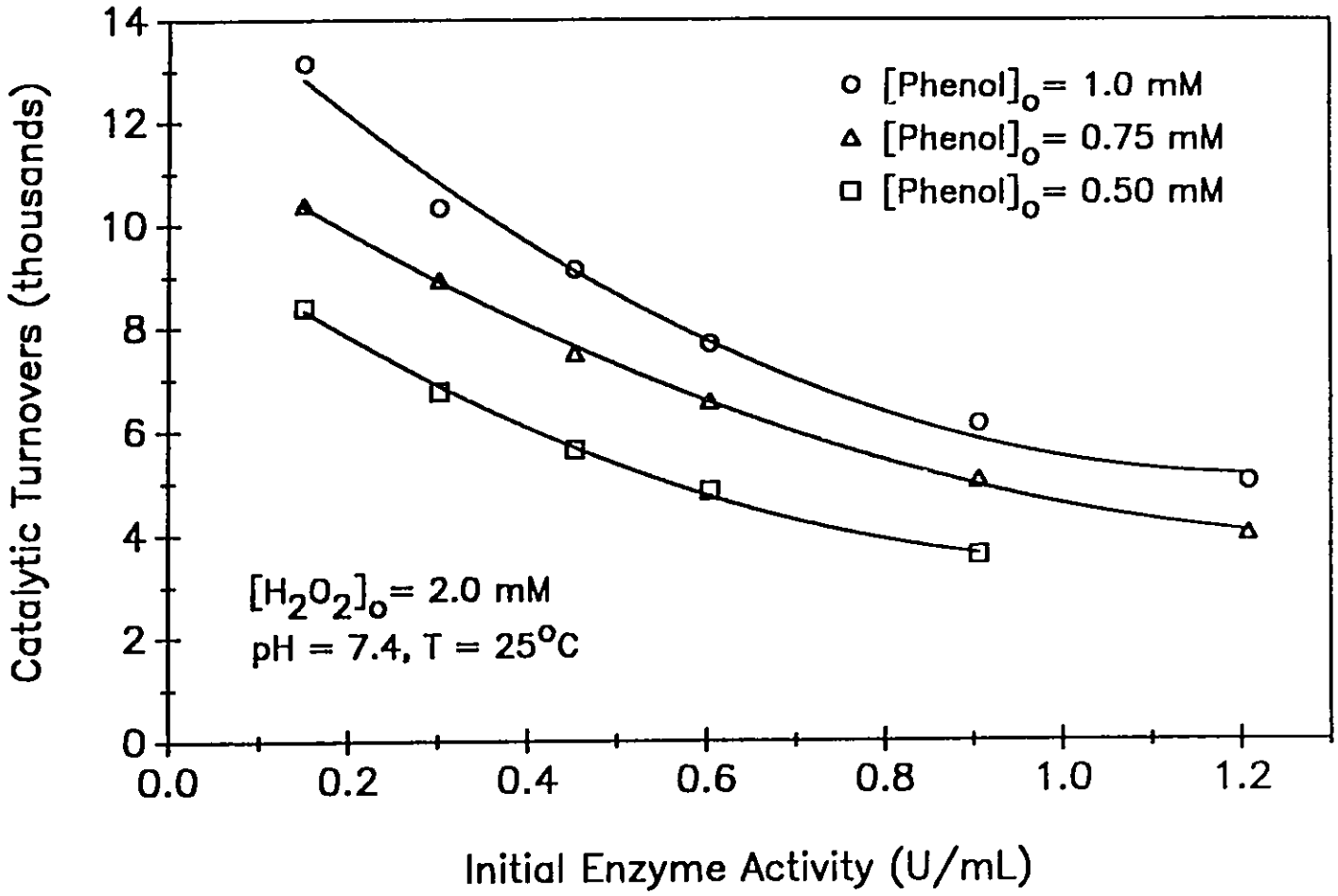


Figure 5–4: Catalytic Turnovers as a Function of Phenol and HRP Dose in a Batch Reactor

radicals are formed quickly and will likely polymerize rapidly due to their high concentration.

The concentration to which aromatic substrates could be removed from solution was found to be independent of the starting concentration when the reaction was provided sufficient time to go to completion. The peak absorbance of a solution decreased to a residual level which could not be reduced any further by the addition of more enzyme or hydrogen peroxide. This may be attributed to the formation of reaction products which are soluble in water to a small extent and which are not substrates of the enzyme. These products still absorb light at the same wavelength as the parent aromatic substrate and interfere with the spectrophotometric measurement of the residual amount of substrate. Therefore, the removal efficiencies determined using the spectrophotometric method are in reality measurements of the reduction in absorbance at a peak wavelength and are thus conservative estimates of substrate conversion. The degree of removal achieved for other phenols as a function of enzyme dose is shown in Appendix D.

5.2 Reaction Stoichiometry

The overall reaction for the peroxidase catalyzed oxidation of aromatic compounds by hydrogen peroxide is:

$$H_2O_2 + 2\,AH_2 \longrightarrow 2\cdot AH + 2\,H_2O \qquad (5\text{–}1)$$

Reaction 5–1 states that two free radicals are generated for every molecule of peroxide consumed. The free radicals polymerize spontaneously. Therefore, the stoichiometric ratio of peroxide consumed per aromatic molecule precipitated would be $^1/_2$ provided the resulting dimer is completely insoluble in water. In reality, dimers which remain substantially soluble will react again to form trimers,

tetramers or larger polymers which eventually precipitate. As the polymer grows in size, the ratio of consumption of peroxide to aromatic molecules should approach unity as a limit provided each of the aromatic molecules in the polymers are linked through a single bond. This is illustrated in Figure 5–5 for a solution containing a single ring substrate which polymerizes to form longer and longer chain polymers resulting in increased consumption of peroxide.

Experiments were performed to determine the stoichiometric consumption of peroxide to accomplish the precipitation of phenol, 4–chlorophenol, 4–methylphenol, and 2,4–dichlorophenol. The removal of these compounds as a function of peroxide dose at pH 7.4 are shown in Figures 5–6 through 5–9. Reactant mixtures were created with a range of peroxide and peroxidase concentrations varying from limiting to excess quantities of each. Reactions were allowed to go to completion.

Figure 5–7 demonstrates that the removal of 4–chlorophenol was linear with peroxide dose and produced a stoichiometry of one while the aromatic substrate concentration was not limiting. In contrast, phenol consumption was not linear with peroxide dose as shown in Figure 5–6; rather, the stoichiometry of the reaction appeared to vary with the amount of peroxide supplied to the reaction mixture. The extrapolation of the curves of Figure 5–6 indicates that as more peroxide is supplied to the reactor the ratio of peroxide consumption to phenol removal approaches unity. The lack of removal over the low range of peroxide concentration indicates that this peroxide is initially used to form a soluble product, probably a dimer. As the reaction is allowed to progress further with the addition of more peroxide an insoluble product is formed. The trend of the curve shows that as a greater degree of removal of phenol is accomplished the stoichiometry of removal approaches unity. Similar results were obtained for 4–methylphenol and 2,4–dichlorophenol as shown in Figures 5–8 and 5–9, respectively. No dependence of reaction stoichiometry on pH was observed.

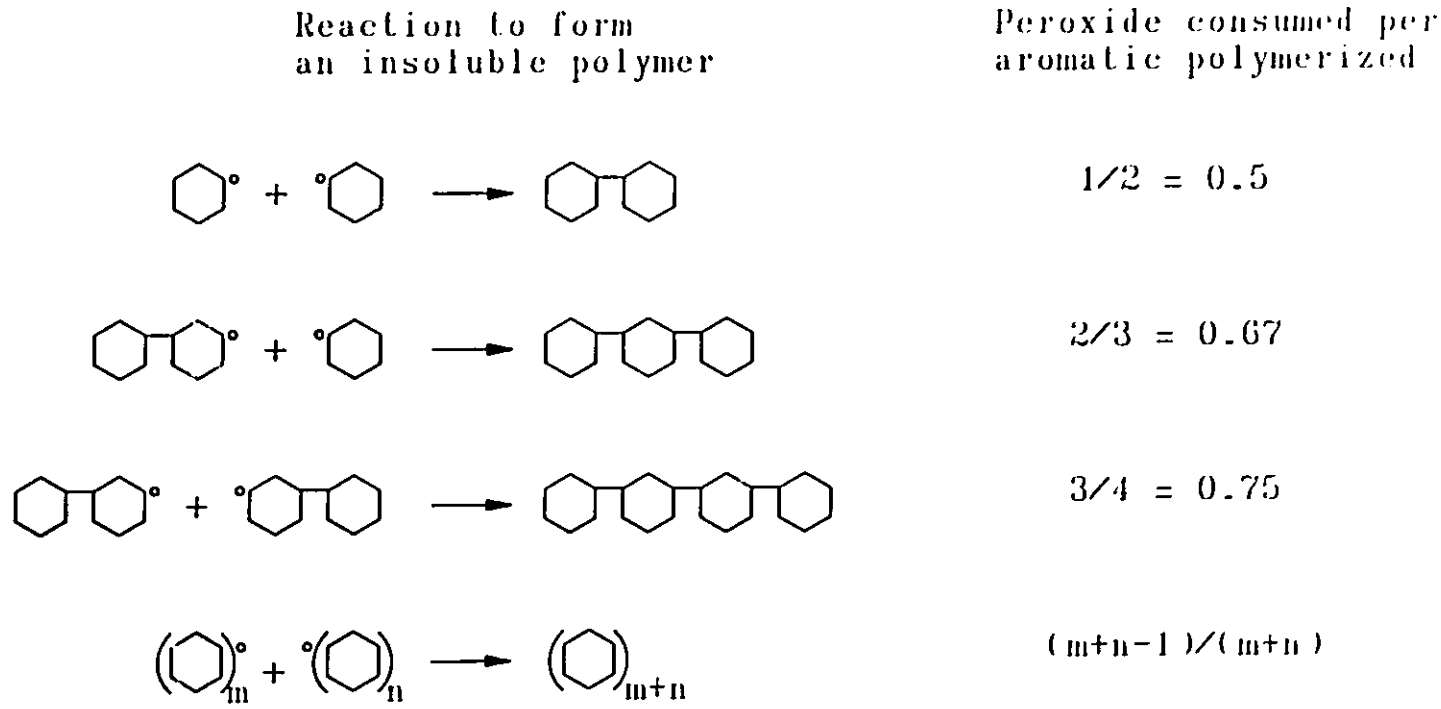


Figure 5–5: Dependence of Reaction Stoichiometry on Polymer Size

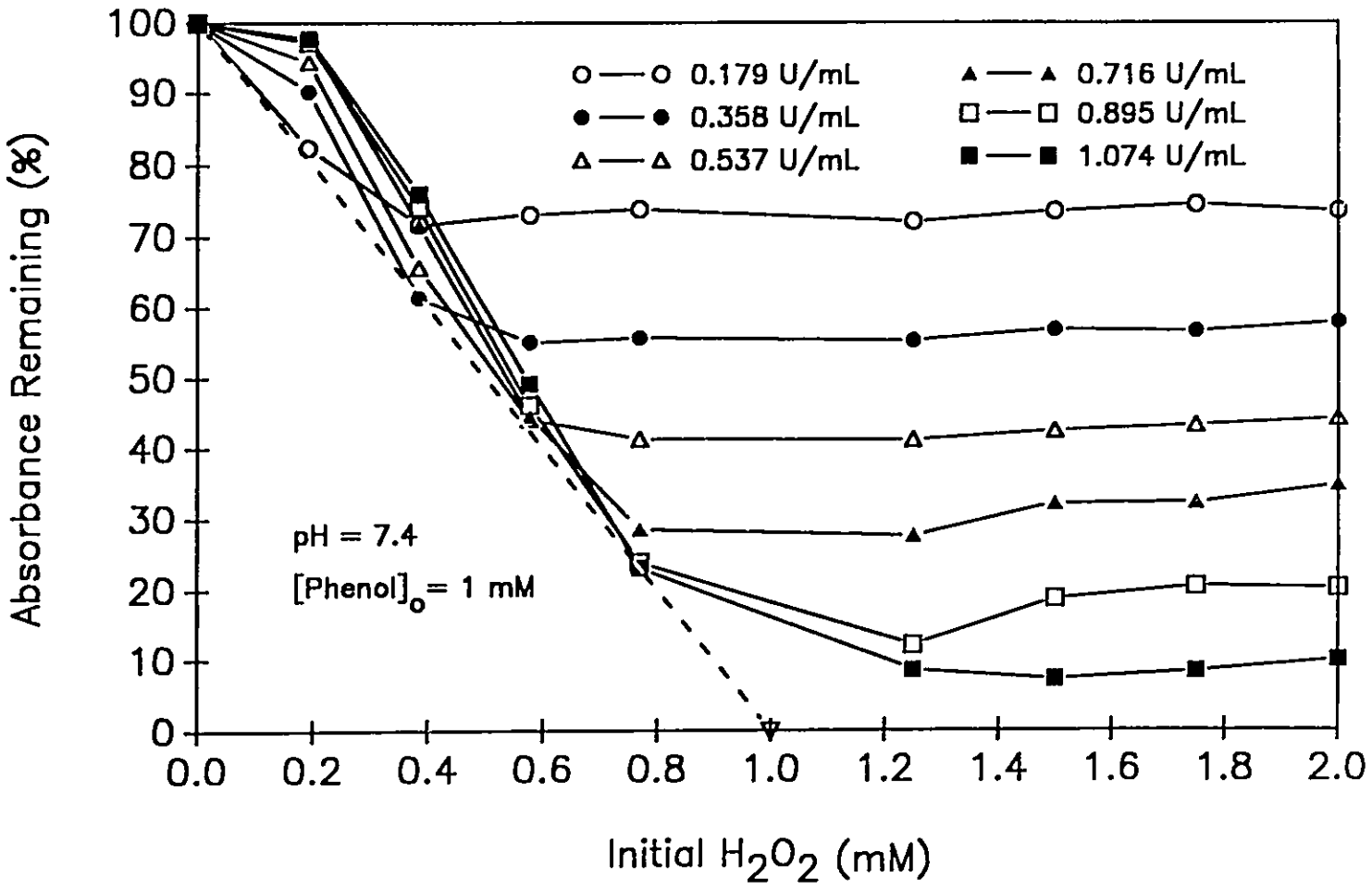


Figure 5-6: Removal of Phenol as a Function of Peroxide and HRP Dose at 25°C

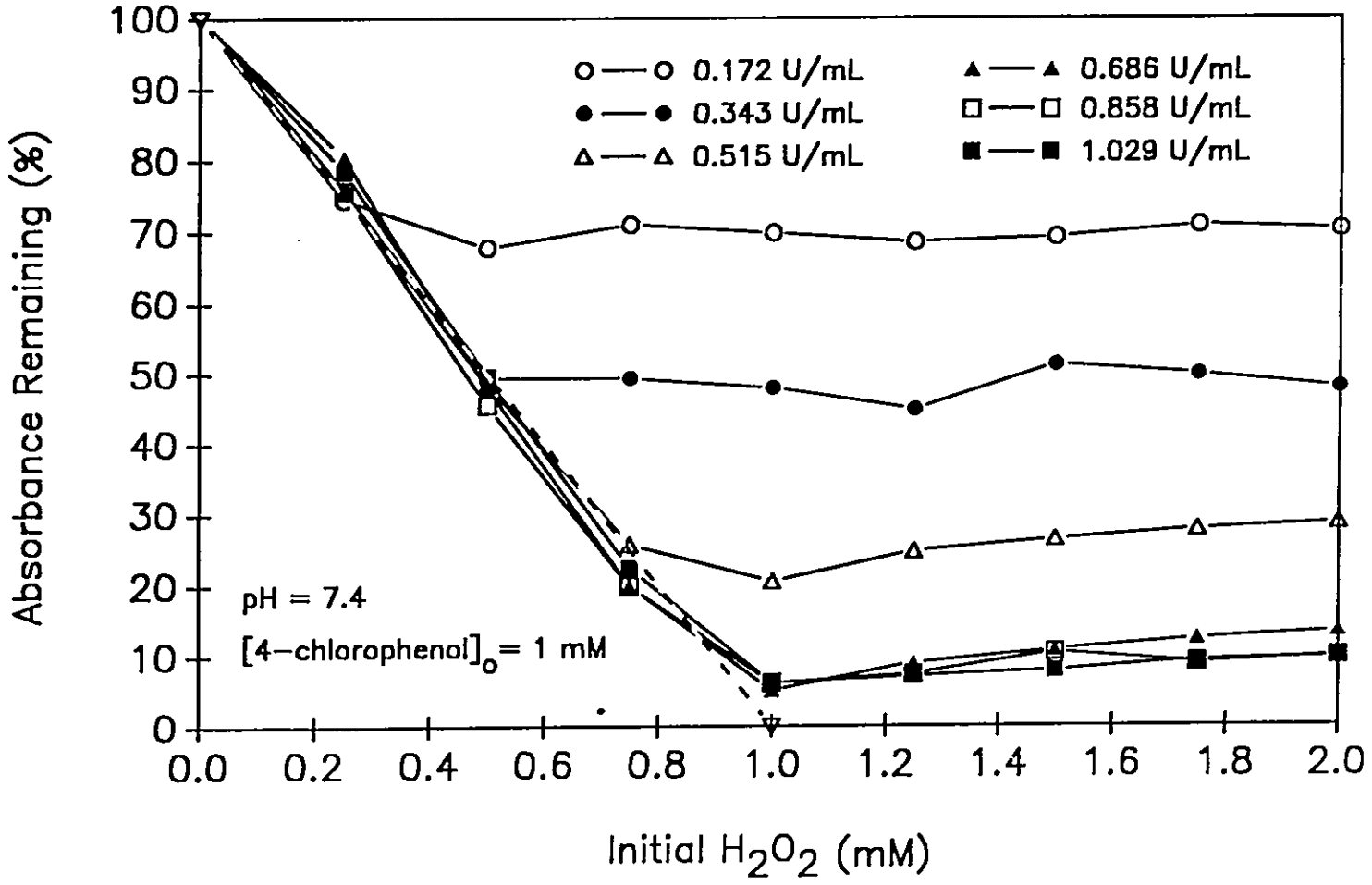


Figure 5–7: Removal of 4–Chlorophenol as a Function of Peroxide and HRP Dose at 25°C

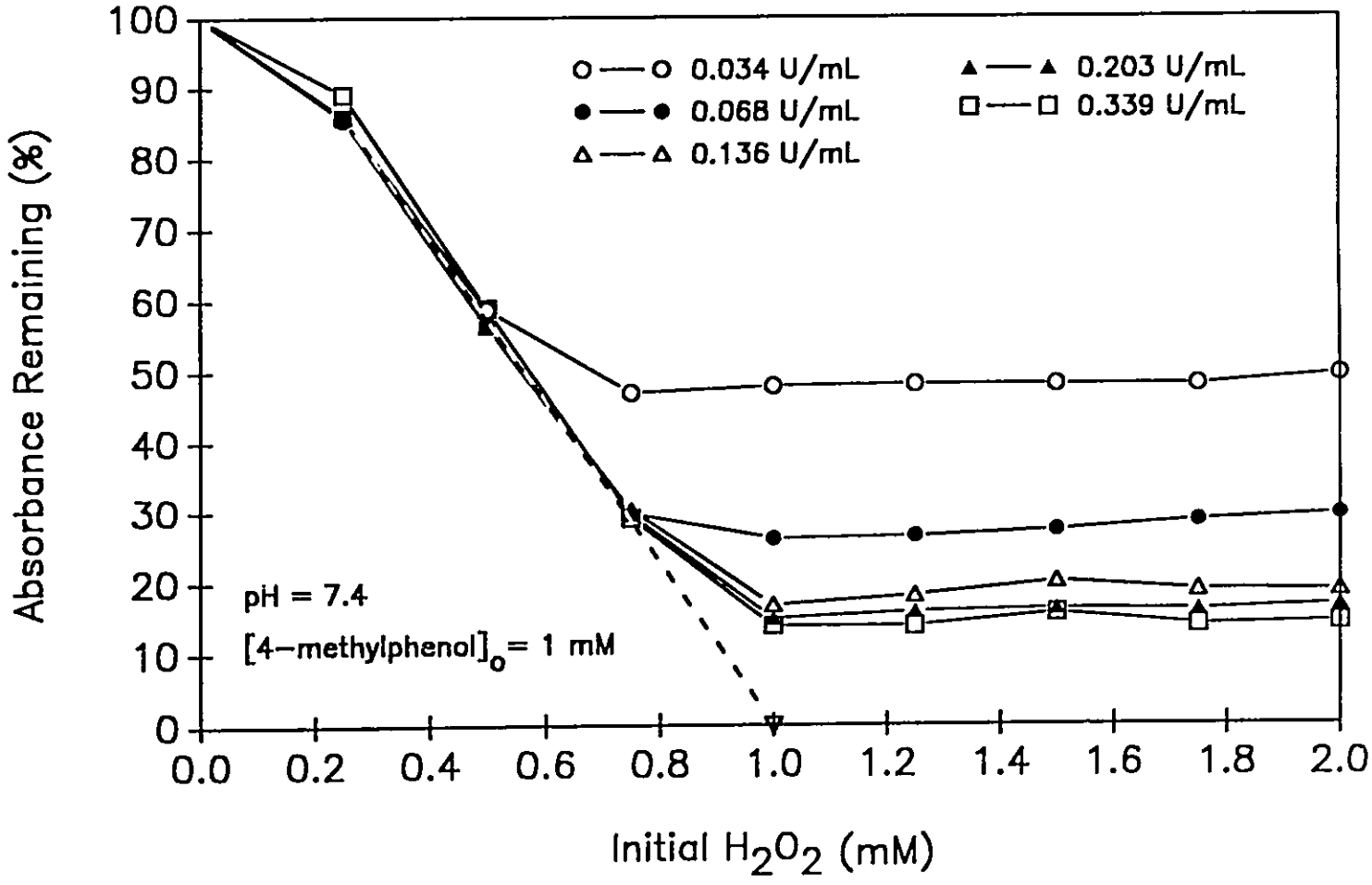


Figure 5-8: Removal of 4-Methylphenol as a Function of Peroxide and HRP Dose at 25°C

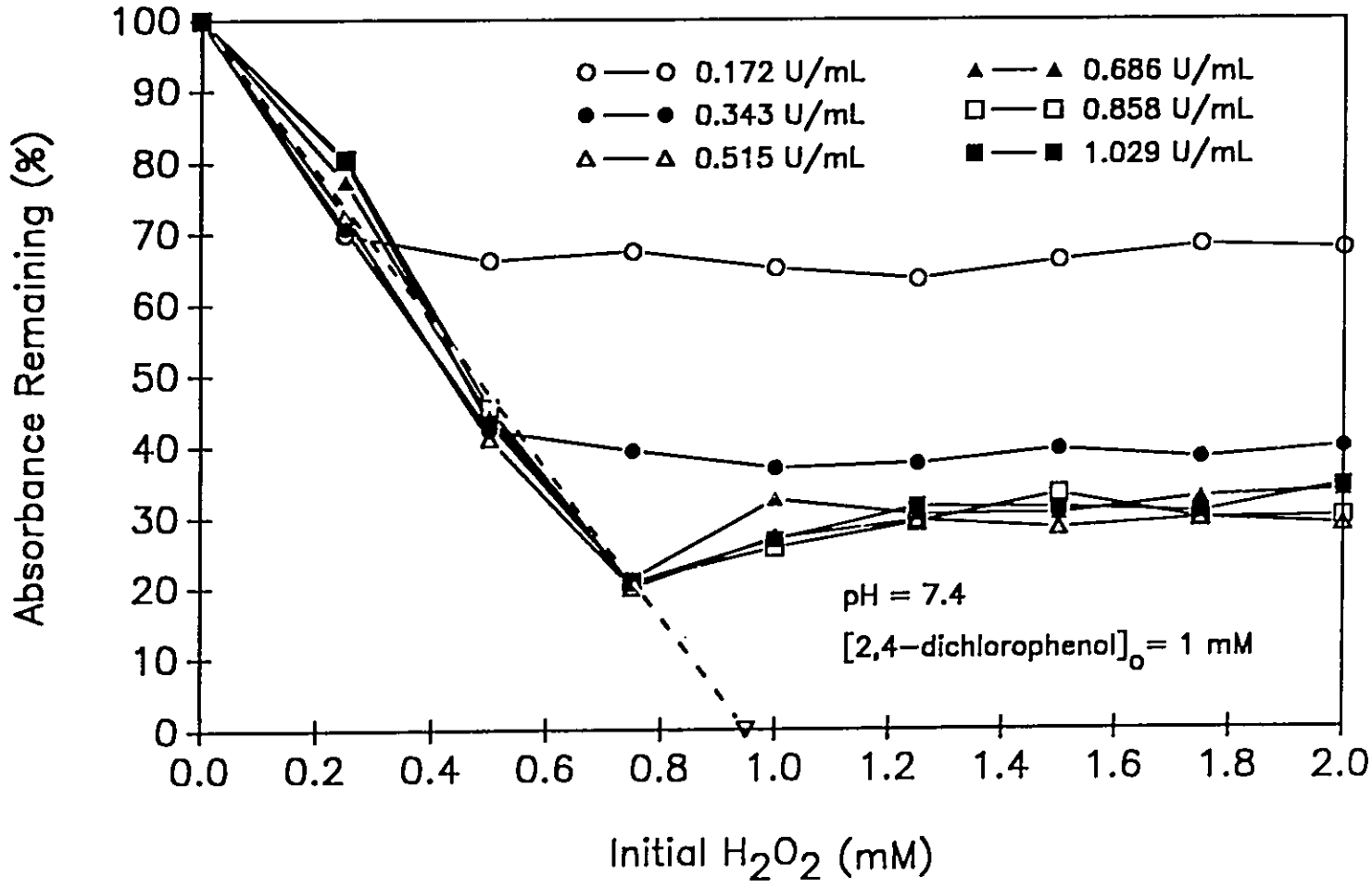


Figure 5–9: Removal of 2,4–Dichlorophenol as a Function of Peroxide and HRP Dose at 25°C

The measured stoichiometry is inconsistent with the stoichiometry predicted by Reaction 5–1 indicating that the polymerization and precipitation process is not as simple as a single bond formation between two aromatic molecules. The catalase reaction of peroxidase (Equations 2–5 and 2–6) cannot account for the additional consumption of peroxide as indicated by the relative magnitude of the reaction rate constants for the reaction of Compound I with an aromatic substrate or with peroxide, both acting as reducing agents. At neutral pH and 25°C, HRP operates in the catalytic cycle (Reactions 2–1 to 2–3) with rate constants on the order of 10^6 to 10^7 M^{-1} s^{-1} (Job and Dunford, 1976; Sakurada *et al.*, 1990). Compound I converts peroxide to oxygen and water under the same conditions with a rate constant on the order of 10^2 to 10^3 M^{-1} s^{-1} (Nakajima and Yamazaki, 1987). This difference of four orders of magnitude in rate constants cannot account for a two–fold difference between the measured and predicted stoichiometry. Other explanations must be forthcoming.

A one–to–one reaction stoichiometry is supported by the formation of long chain polymers which force the stoichiometry to approach unity. Dec and Bollag (1990) observed the formation of polymers from 2,4–dichlorophenol with average molecular masses up to 800 for the fraction soluble in dioxane. It is likely that the insoluble fraction contains polymers of even higher molecular mass. Therefore, a stoichiometry much greater than $^1/_2$ and approaching unity is expected. Other explanations which involve modifications of the catalytic mechanism and which account for the observed stoichiometry are offered by Santimone (1975) and Hewson and Dunford (1976b).

Explanations for the one–to–one stoichiometry observed for the reaction of guaiacol with peroxide have been proposed in the literature. Santimone (1975), citing other authors, has stated that the free radicals which are expected to undergo a polymerization reaction such as:

$$2 \cdot AH \longrightarrow A_2H_2 \qquad (5\text{–}2)$$

can also undergo a dismutation reaction described by:

$$2 \cdot AH \longrightarrow AH_2 + A \qquad (5\text{–}3)$$

which results in the formation of the parent compound (AH_2) and an aromatic byproduct (A). Summation of Reactions 5–1 and 5–3 produces the overall reaction:

$$H_2O_2 + AH_2 \longrightarrow A + 2\,H_2O \qquad (5\text{–}4)$$

The stoichiometry of Reaction 5–4 corresponds the observed stoichiometry but Santimone (1975) was not satisfied with this radical dismutation reaction because it did not account for the fact that Compound I was fully reduced to Compound II by half a mole of guaiacol. An alternative reaction scheme was proposed which was more consistent with their observations:

$$E + H_2O_2 \longrightarrow E_i + H_2O \qquad (2\text{–}1)$$

$$E_i + AH_2 \longrightarrow E_{ii} + \cdot AH \qquad (2\text{–}2)$$

$$E_i + \cdot AH \longrightarrow E_{ii} + A \qquad (5\text{–}5)$$

$$E_{ii} + AH_2 \longrightarrow E + \cdot AH + H_2O \qquad (2\text{–}3)$$

$$E_{ii} + \cdot AH \longrightarrow E + A + H_2O \qquad (5\text{–}6)$$

Summation of these reactions produces Reaction 5–4.

Santimone (1975) has argued that while the above mechanism is different from the Chance–George mechanism (Equations 2–1, 2–2 and 2–3), the mechanisms are still consistent since in both cases the reduction of the electron donor involves the

participation of both Compounds I and II. However, this reaction scheme does not account for polymer formation since the product is a monomer (A). The reactions listed above do not discount the possibility of polymer formation. It must be noted that as the proportion of free radicals ($\cdot AH$) that form polymers increases so does the deviation from a one–to–one stoichiometry. Therefore, the scheme would not fit the measured stoichiometry when significant quantities of polymers are formed.

Hewson and Dunford (1976b) provided an alternate explanation for the stoichiometry observed by Santimone (1975) and for the stoichiometry they observed for 4–methylphenol. They proposed the following scheme:

$$E + H_2O_2 \longrightarrow E_i + H_2O \qquad (2\text{–}1)$$

$$E_i + AH_2 \longrightarrow E_{ii} + \cdot AH \qquad (2\text{–}2)$$

$$\cdot AH \longrightarrow {}^1/_2\,(AH)_2 \qquad (5\text{–}7)$$

$$E_{ii} + {}^1/_2\,(AH)_2 \longrightarrow E + \text{Products} \qquad (5\text{–}8)$$

Which may be summed to produce the following overall reaction:

$$H_2O_2 + AH_2 \longrightarrow H_2O + \text{Products} \qquad (5\text{–}9)$$

Reaction 5–9 supports the one–to–one stoichiometry observed for guaiacol, 4–methylphenol and the phenolics examined in this study and is consistent with the Chance–George mechanism. Also, Hewson and Dunford (1976b) support the proposed mechanism with the identification of intermediate products of guaiacol and 4–methylphenol oxidation which are consistent with this mechanism. This mechanism is also consistent with evidence of large polymer formation since the ${}^1/_2\,(AH)_2$ in Equations 5–7 and 5–8 can be replaced by polymers of greater molecular mass (i.e. ${}^1/_3\,(AH)_3$, ${}^1/_4\,(AH)_4$, or larger).

The reason for the one–to–one stoichiometry is not fully understood. It is possible that one or more of the above explanations are valid. More research is required in which the intermediate and final products of the polymerization of a variety of aromatic substrates are identified.

Additional experiments were performed to determine the effect of adding peroxide in concentrations far in excess of those required by the reaction stoichiometry. The results for phenol are shown in Figure 5–10. The mixtures were provided with a 5–hour reaction time. Results indicate that at high peroxide concentrations (in excess of equimolar) the removal of aromatic substrate decreased. Removal efficiency did not improve significantly with an 18–hour retention time. Decreased catalyst turnovers at high peroxide concentration may be a consequence of high rates of formation of Compound III. This would result in a reaction rate that is dependent on the slow return of the enzyme from the catalytically inactive Compound III form to the active native enzyme.

5.3 Effect of pH

Enzymes often function only when certain ionizable side chains are in a specific form. Since the characteristics of ionizable side chains of amino–acids depend on pH, enzyme activity is usually pH–dependent. In addition, at extremes of pH the structure of the protein may be disrupted and the protein denatured (Palmer, 1985). Therefore, investigation of the effect of pH on the activity of horseradish peroxidase is required to define the pH range over which HRP will function and to determine the relative importance of pH in optimizing the enzyme's catalytic lifetime.

The removal of phenol as a function of pH and enzyme dose when treated in a batch reactor is shown in Figure 5–11. The initial concentrations of phenol and hydrogen peroxide were 1 mM and 2 mM, respectively. These species were present

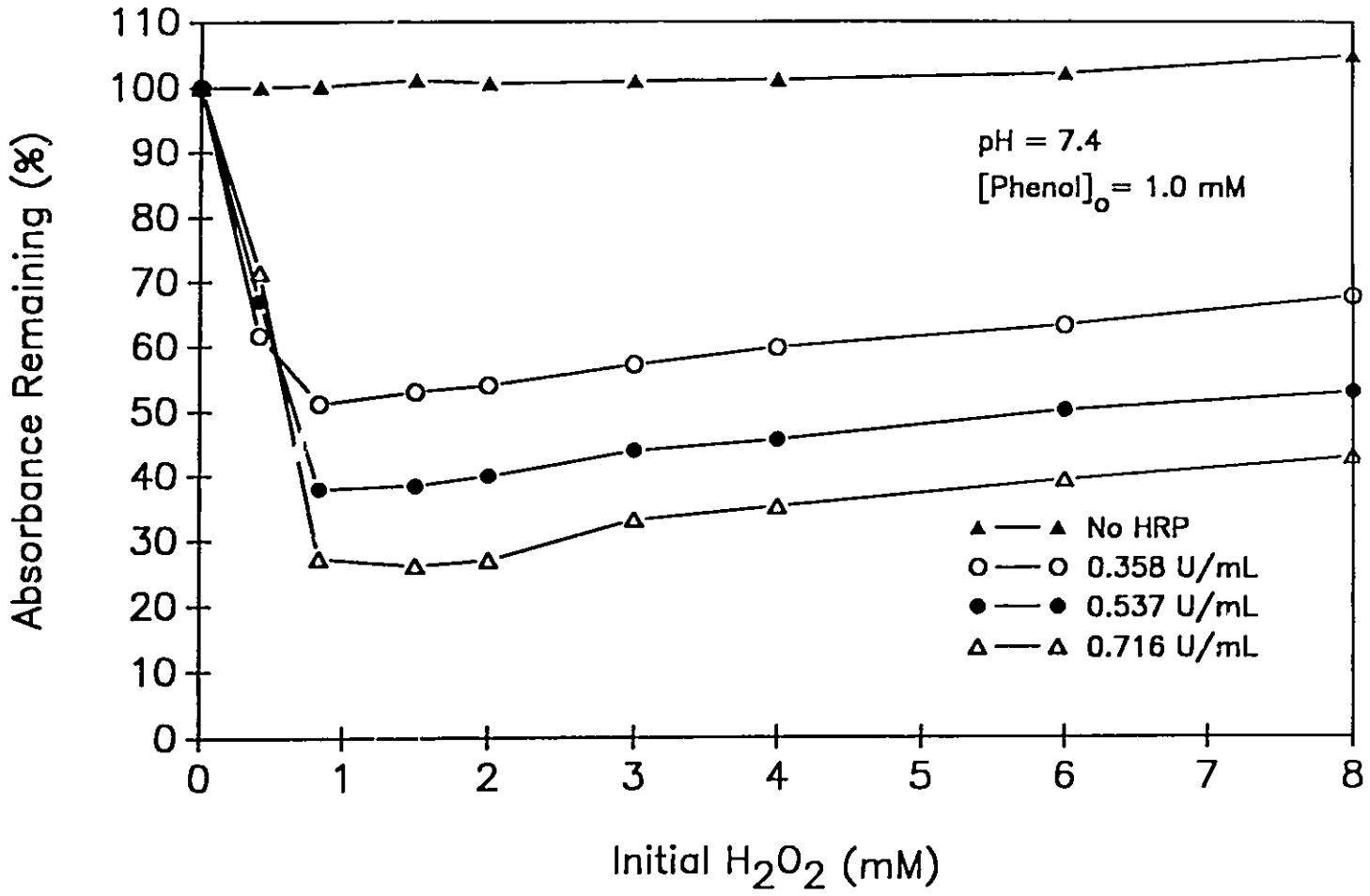


Figure 5–10: Effect of Excess Peroxide on Phenol Removal in a Batch Reactor at 25°C

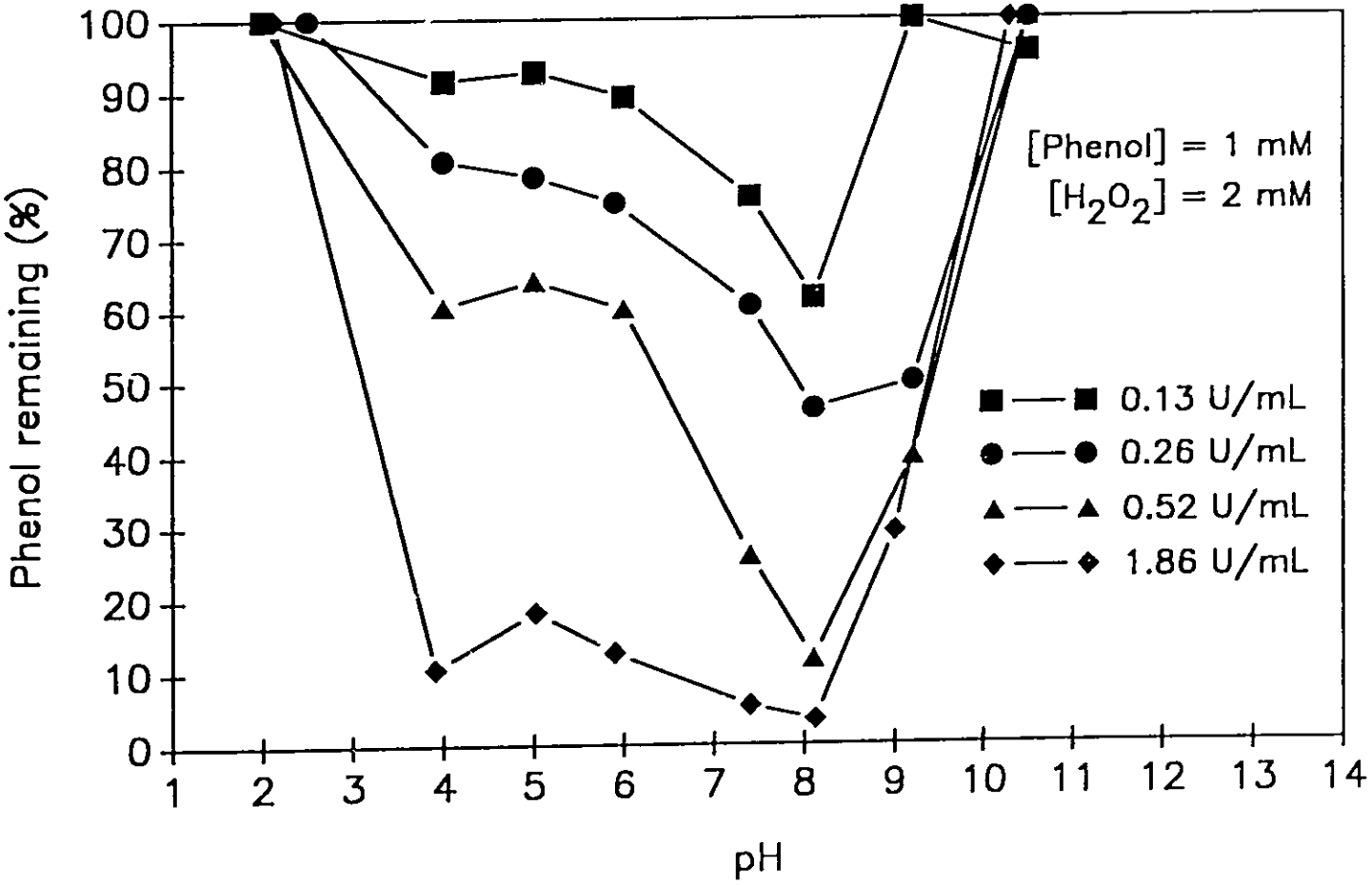


Figure 5–11: Phenol Removal as a Function of pH and HRP Dose in a Batch Reactor at 25°C

in sufficient amounts so that the only limiting participant in the reaction was HRP. Reaction time was sufficient to allow the reaction to go to completion. The buffers used to maintain the pH's were hydrochloric acid–potassium chloride (pH 2), citrate (pH 3 to 4), acetate (pH 4 to 6), phosphate (pH 6 to 8) and carbonate (pH 9 to 10). Buffers were overlapped to ensure that catalytic efficiency was dependant only on the pH and not on the buffer used.

As shown in Figure 5–11, the clearance of phenol from solution based on the residual absorbance at the peak wavelength is clearly a function of pH. Corrections were made for the variation of extinction coefficient with pH. The enzyme demonstrated some catalytic ability over a pH range of 4 to 10 but only accomplished reasonably good removal over the pH range of 6 to 9 with an optimum occurring at a pH of 8. Below a pH of 2 and above 11 no catalytic ability was apparent. Several samples had final absorbances which were greater than the initial. This is due to the formation of a colored product which did not precipitate from solution (even following alum treatment) and which absorbed light to a greater extent than the original aromatic substrate at its peak wavelength. Therefore, it was assumed that no removal of the organic was accomplished.

Figure 5–12 illustrates the effect of pH on the number of catalytic turnovers achieved by peroxidase for phenol, 3–chlorophenol and 4–methylphenol. This figure demonstrates that significant improvement in process efficiency, in terms of catalyst requirements, can be achieved by carrying out the polymerization at the optimal pH corresponding to each compound.

The results of the pH optimization studies for all aromatic substrates investigated are contained in Appendix D. The optimal pH's for all compounds occurred within a range of 7 to 9 as listed in Table 5–1.

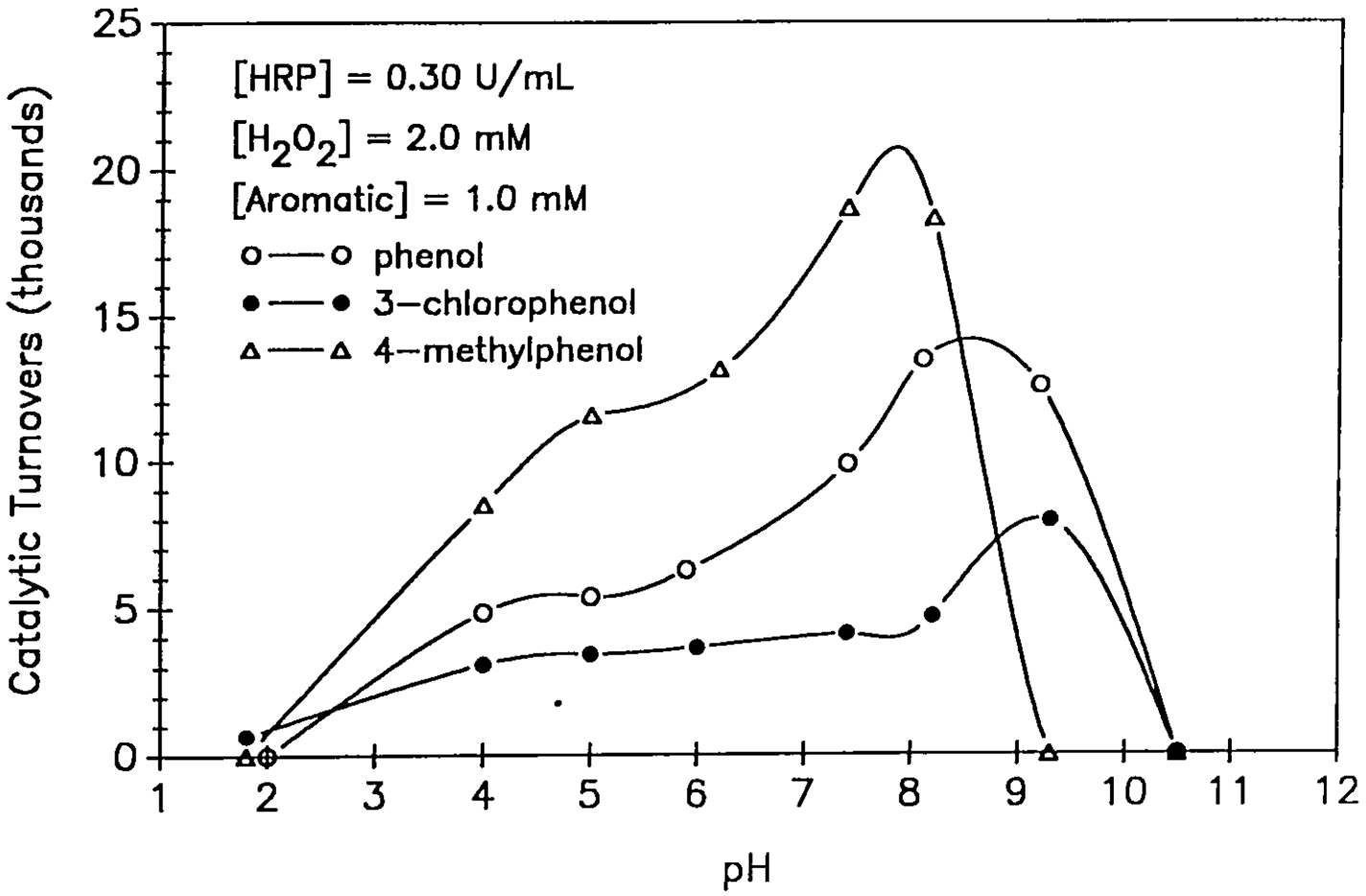


Figure 5–12: Catalytic Turnovers as a Function of pH for the Treatment of Several Phenols in a Batch Reactor at 25°C

Table 5–1: Treatment of Phenols in a Batch Reactor

Compound	Optimal pH	Best Absorbance Reduction (%)	Catalytic Turnovers *
phenol	8	99.7	15 600
2–chlorophenol	8	96.6	10 700
3–chlorophenol	9	98.0	4 900
4–chlorophenol	9	98.6	14 500
2,4–dichlorophenol	8	83.0	18 100
2–methylphenol	9	90.0	8 400
3–methylphenol	9	94.4	27 200
4–methylphenol	7	83.0	40 800

[Aromatic] = 1.0 mM $[H_2O_2]$ = 2.0 mM T = 25°C

* Note: Catalytic turnovers measured when sufficient enzyme is provided to achieve 50% removal of aromatic substrate

5.4 Effect of Temperature

As discussed previously, HRP was found to be susceptible to rapid inactivation at high temperatures. Since the time frame of the inactivation of HRP at temperatures below 70°C was on the order of minutes to hours, and since the catalytic reaction is very rapid, it was hoped that the enzyme would still be able to achieve good removal of substrates at high temperatures before it becomes thermally denatured. Additional experiments have been performed to determine the efficiency of enzyme catalyzed polymerization at temperatures between 5°C and 65°C. The results are shown in Figure 5–13 for the treatment of 4–chlorophenol at neutral pH in a batch reactor.

The same degree of removal was achieved at all temperatures when sufficient enzyme was provided but the cost associated with the removal increased significantly at elevated temperatures due to increased catalyst requirements. For example, the average number of turnovers decreased from approximately 9500 in the range of 5°C to 35°C to 4100 at 65°C. Therefore, while still capable of catalyzing the polymerization reaction, more than twice the HRP is required to precipitate the same amount of 4–chlorophenol at 65°C as at 35°C. This represents a loss in catalytic efficiency and a corresponding increase in cost. These results indicate that the clearance of substrate should be conducted at temperatures below 35°C to prevent significant thermal inactivation of peroxidase.

5.5 Treatment Achieved in a Batch Reactor

Experiments were performed to determine removal as a function of enzyme dose at the optimal pH and the maximum removal under non–limiting conditions of HRP and peroxide. The reactions were given sufficient time to go to completion (in excess of 12 hours). The profiles of removal versus enzyme concentration at optimal pH's are located in Appendix D.

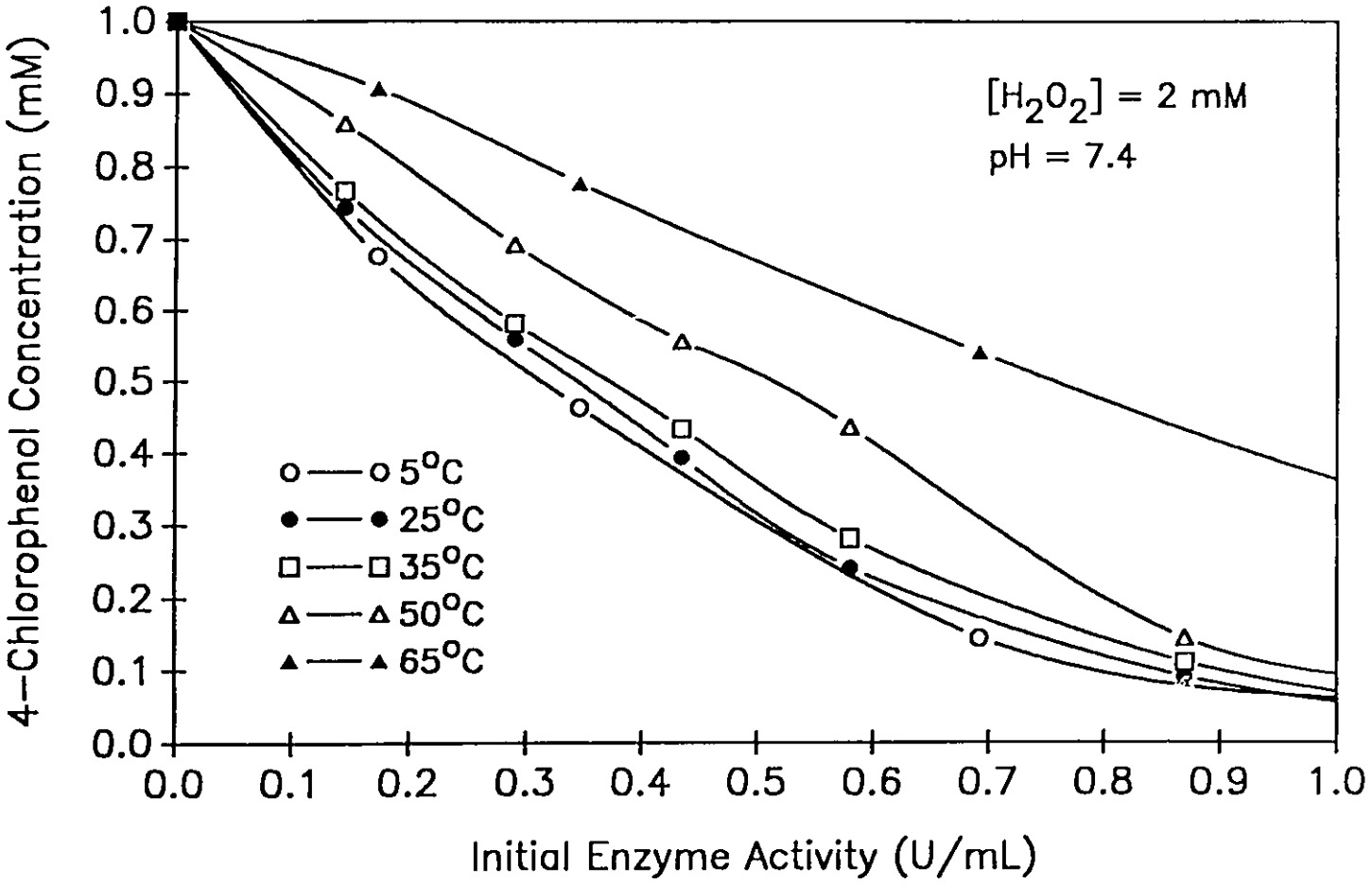


Figure 5–13: Removal of 4–Chlorophenol as a Function of Temperature in a Batch Reactor

Examples of aromatic substrate removal as a function of enzyme dose are shown in Figure 5–14 for 3–chlorophenol, 4–methylphenol and phenol. This graph demonstrates that phenol can be effectively precipitated from solution with a moderate dose of enzyme. 3–Chlorophenol can also be removed from solution to a great extent, however the catalytic turnovers are substantially lower than for phenol. In contrast, the maximum clearance of 4–methylphenol can be achieved with a much smaller dose of enzyme as compared to the other two phenols. However, this maximum removal is not as good as shown by the residual level of 4–methylphenol. This residual, while represented as 4–methylphenol, is probably a byproduct of the reaction which remains soluble and which is not a substrate of the enzyme. Thus, no further removal can be accomplished through the addition of more enzyme or oxidant. Therefore, even though a compound is extremely reactive toward horseradish peroxidase, there is no relationship between this reactivity and the degree of removal that can be achieved.

Table 5–1 lists the residual levels of each aromatic substrate when treatment is carried out at their respective optimal pH's with excess peroxide and peroxidase. This table also lists the catalytic turnovers achieved by peroxidase in accomplishing 50% removal of aromatic substrate at the optimal pH and under fixed initial peroxide and aromatic compound concentrations.

5.6 Contact Time

In any chemical process, the conversion achieved in a reactor is dependant on the reaction time. Since kinetic information was not available for the oxidation of phenolic compounds by horseradish peroxidase, it was necessary to perform experiments to estimate the time scale of the polymerization reaction. This was accomplished by varying the reaction time for mixtures with fixed starting concentrations of HRP, H_2O_2 and phenolic and measuring the residual concentration

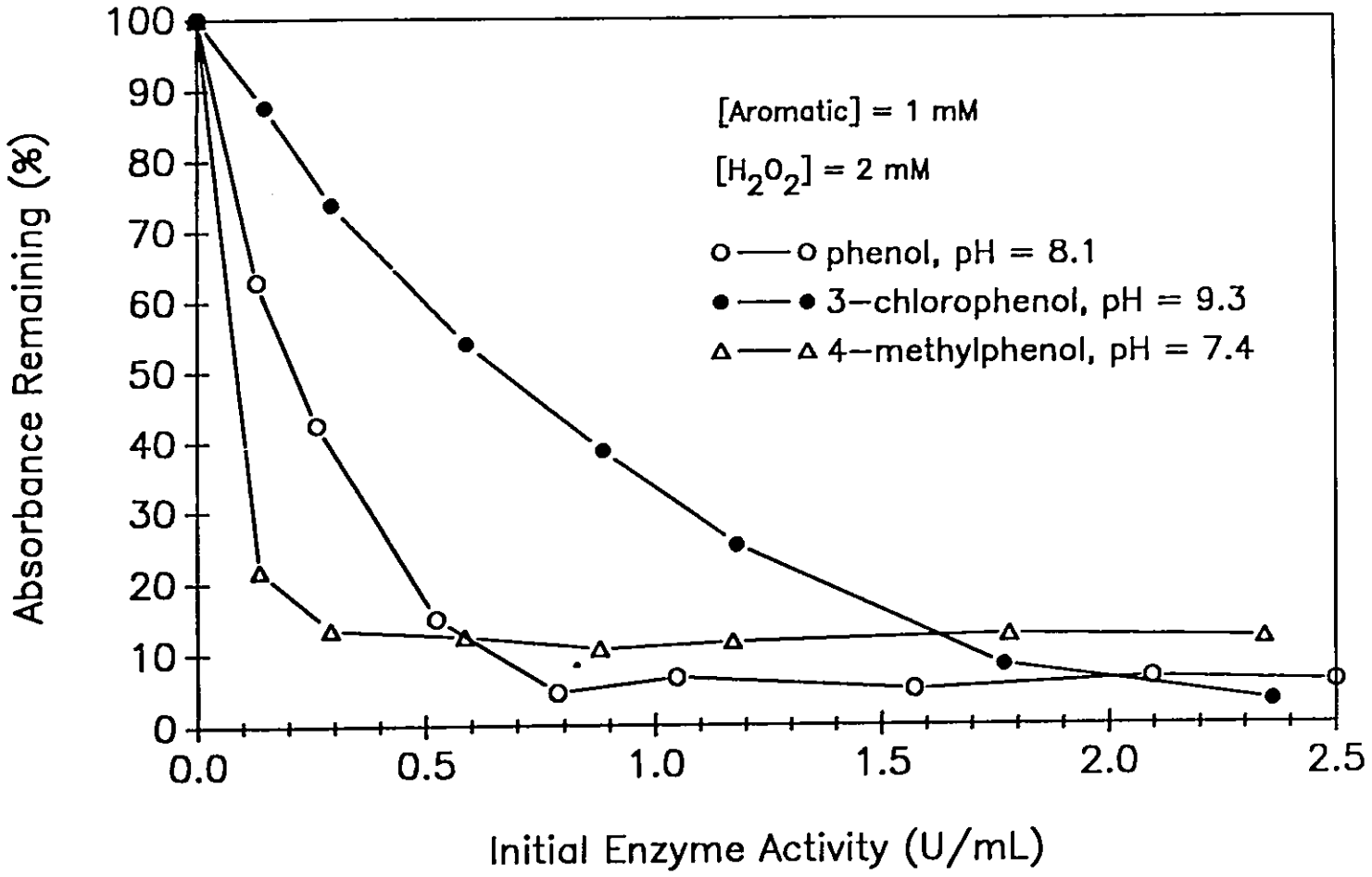


Figure 5–14: Removal of Phenol, 3–Chlorophenol and 4–Methylphenol at their Respective Optimal pH's and 25°C

of the aromatic compound. The reaction was halted by the addition of a large dose of catalase. Results for the conversion of 4–chlorophenol as a function of time and enzyme dose are shown in Figure 5–15.

The majority of the removal of 4–chlorophenol was accomplished in the first minute, indicating an extremely fast reaction rate. This initial rapid phase is followed by a very slow removal process. The dramatic slowdown in the rate of reaction can be attributed to the simultaneous decrease in the concentration of all participants in the reaction (aromatic substrate, peroxide and active enzyme). In addition, Figure 4–5 has shown that the formation of Compound III could be an important cause of reduced reaction rates. Figure 5–16 demonstrates that the removal of 4–chlorophenol is linear with respect to the logarithm of time. Therefore, the reaction rate is decreasing geometrically. This behavior is inconsistent with simple reaction rate equations such as first–order processes which produce linear curves on plots of the logarithm of concentration versus time.

Kinetic analysis and modelling of the enzyme catalyzed reaction are required to explain the effect of substrate concentrations and enzyme inactivation on reaction rates. This modelling, discussed in a later section, attempts to explain the behavior observed in Figure 5–16.

5.7 Effect of Precipitating Versus Non–precipitating Environment

Removal of aromatic substrates as a function of pH was demonstrated previously. In all pH studies, buffers were overlapped to ensure that observed effects were associated with pH and not the buffer chosen. However, three of the buffers used, monobasic sodium phosphate–dibasic sodium phosphate buffer (phosphate buffer), boric acid–borax buffer (borate buffer) and tris(hydroxymethyl)-aminomethane-hydrochloric acid buffer (tris buffer) produced results that were not simply pH dependant.

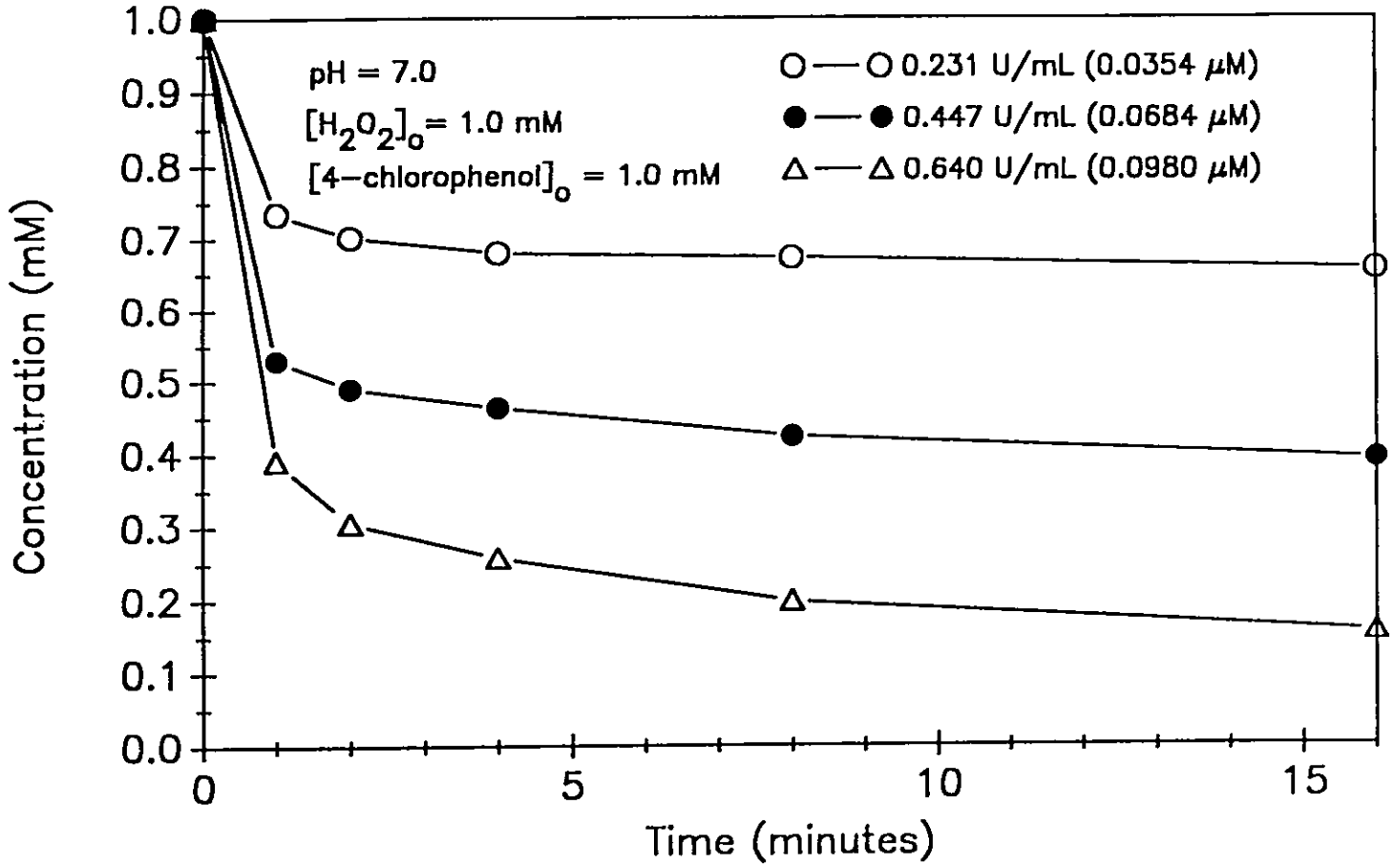


Figure 5–15: 4–Chlorophenol Removal as a Function of Reaction Time and HRP Dose in a Batch Reactor at pH 7.0 and 25°C

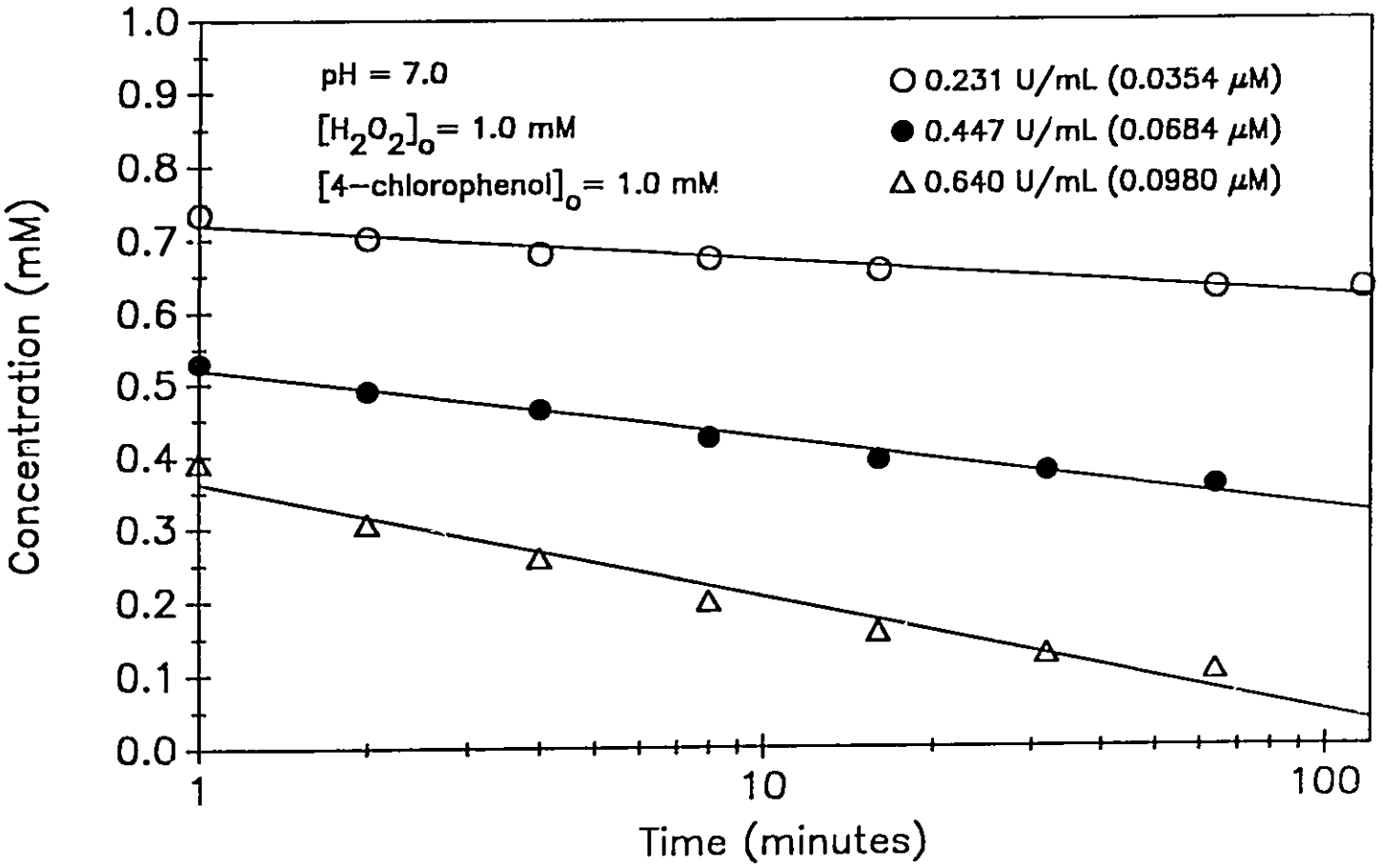


Figure 5–16: 4–Chlorophenol Removal as a Function of the Logarithm of Reaction Time and HRP Dose in a Batch Reactor at pH 7.0 and 25°C

For example, Figures 5–17, 5–18 and 5–19 illustrate the removal of phenol, 2–chlorophenol and 4–chlorophenol at pH 8.1 using three different buffers; phosphate, borate and tris. It should be noted that this pH is not the optimal pH for 2–chlorophenol and 4–chlorophenol. In all three cases, the best catalytic turnovers were achieved with borate, followed by tris and then phosphate buffer. Precipitate formed spontaneously and very rapidly in the presence of phosphate buffer leaving a clear uncolored solution. No solid was formed in the presence of borate buffer without subsequent coagulation and flocculation with alum. Prior to coagulation the solution was dark in color and contained no solids that could be caught on a filter with a 0.45 μm diameter pore size. A small amount of precipitate and much colour developed spontaneously in the presence of tris buffer. Colour was successfully removed by alum coagulation. Colour removal by coagulation and the lack of filter solids on the 0.45 μm filter indicate that the polymers created in the process formed colloidal particles. A colloid is defined as a particle with a diameter in the range of 10^{-8} to 10^{-5} m (Amirtharajah and O'Melia, 1990). Colloidal particles remain suspended in solution until particle aggregation is enhanced by the addition of a coagulating agent.

The differences in removal achieved with these buffers appeared to result from differences in precipitating versus non–precipitating environments; better removal was achieved when the product did not precipitate spontaneously. It is possible that the borate and tris buffers are capable of chelating with the polymers formed in the catalytic process; borate being the stronger chelating agent. This would allow the products to remain soluble resulting in the formation of polymers with higher molecular masses while maintaining a higher concentration of aromatic substrate in solution to protect the enzyme from free radical inactivation. It is also suspected that as polymers precipitate from solution the peroxidase may be trapped by solid particles as they form. This could be avoided by delaying the formation of

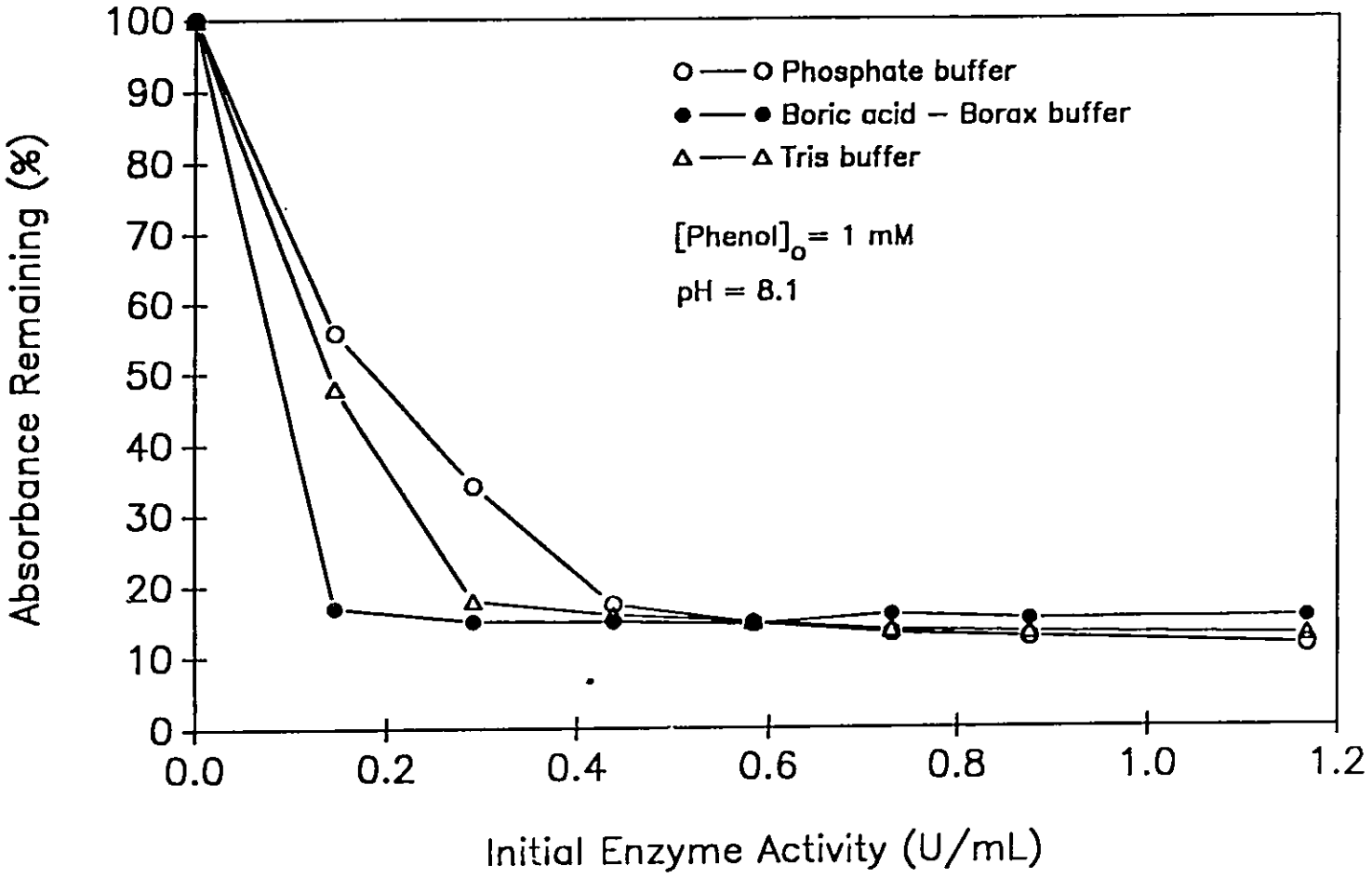


Figure 5–17: Removal of Phenol at pH 8.1 Using Phosphate, Tris and Borate Buffers at 25°C

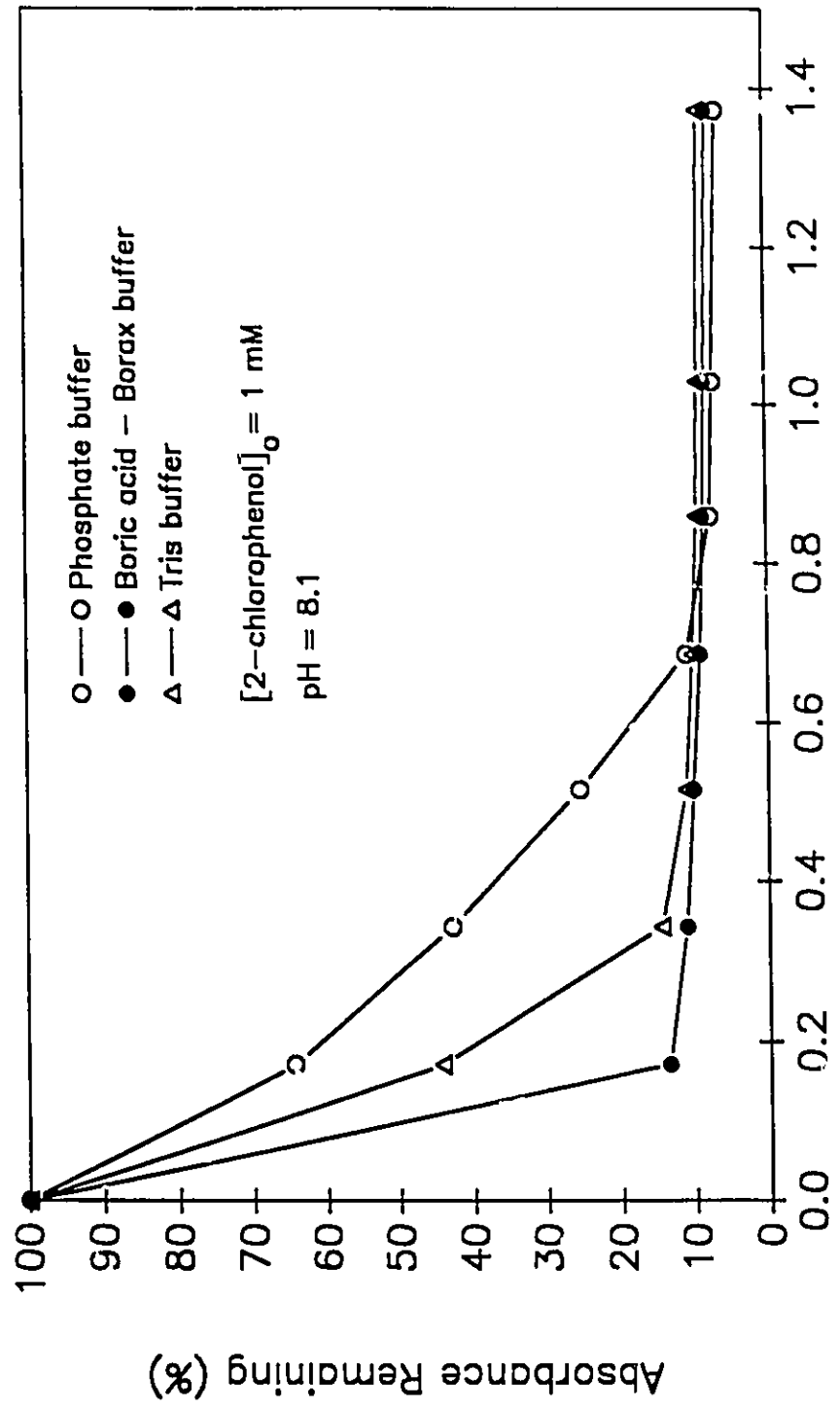


Figure 5–18: Removal of 2–Chlorophenol at pH 8.1 Using Phosphate, Tris and Borate Buffers at 25°C

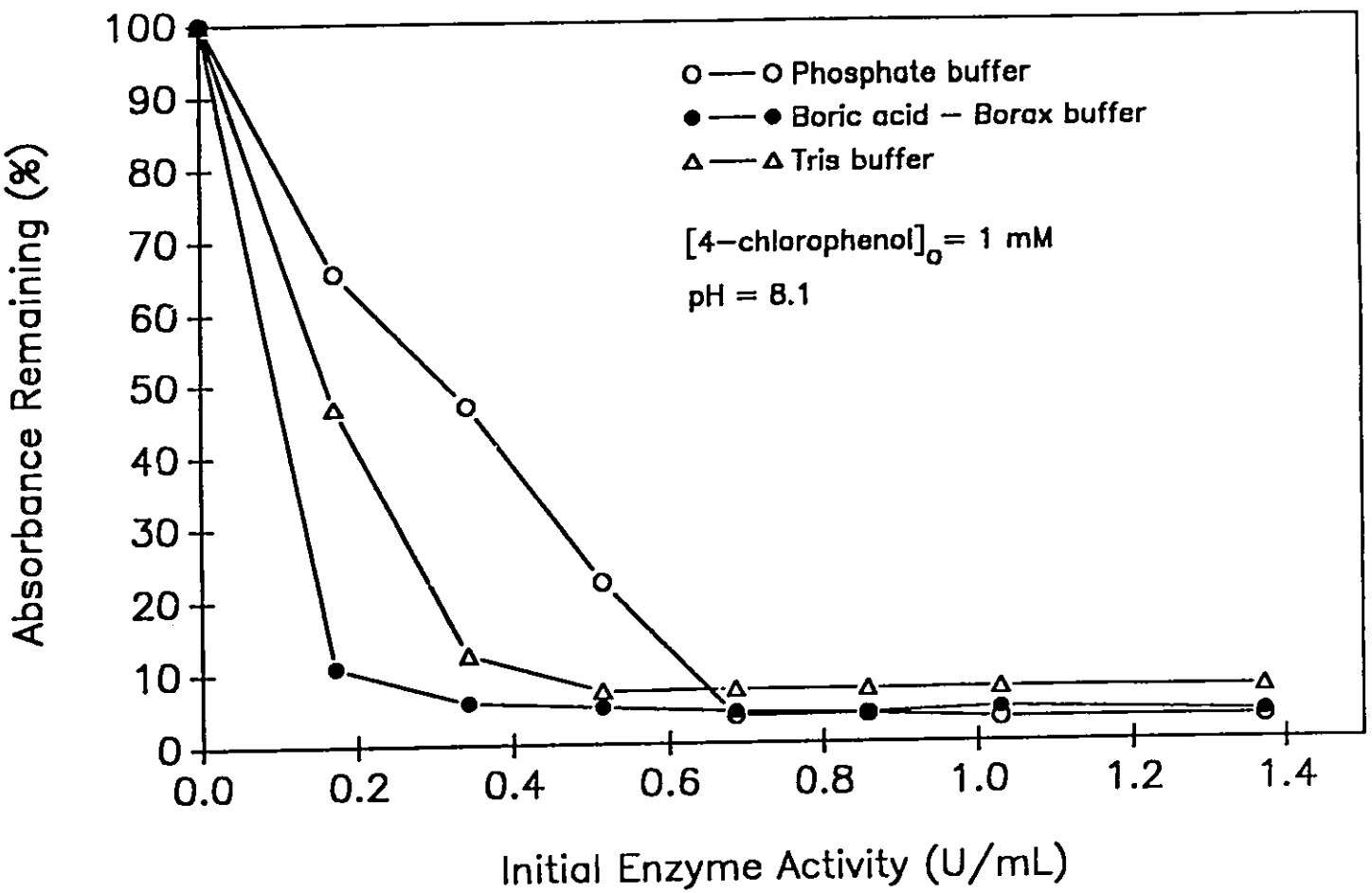


Figure 5–19: Removal of 4–Chlorophenol at pII 8.1 Using Phosphate, Tris and Borate Buffers at 25°C

precipitate.

Further investigation is required to explain the increased catalytic turnovers in the presence of borate. In addition, the possibility of using borate as a chemical additive to improve polymerization efficiency under wastewater treatment conditions should be examined.

5.8 Co–precipitation

The degree to which a compound may be removed from solution by enzyme catalyzed polymerization is dependent on its reactivity toward peroxidase and the solubility of the products formed. Due to the adverse effect of one or both of these factors, the removal of aromatic compounds from water would appear to have limited application to those compounds which can only be efficiently precipitated from solution. Klibanov *et al.* (1980) have reported a co–precipitation phenomenon in which easily removed compounds (those with low residuals) aid in the precipitation of other compounds which cannot be removed to the same extent. Co–precipitation may result from the formation of mixed polymers which have the hydrophobic characteristics of the polymers formed by the more efficiently removed compound.

Experiments were performed to determine if the compounds which were not efficiently removed from solution, 2,4–dichlorophenol and 4–methylphenol, could be co–precipitated with more easily removed compounds such as phenol, 3–chlorophenol and 4–chlorophenol to enhance removal. In addition, experiments were performed in which 2,4–dichlorophenol and 4–methylphenol were treated together to observe any co–precipitation effects. These experiments were conducted using excess peroxide (2 to 1 molar ratio of H_2O_2 to phenolic), HRP (3 U/mL) and reaction time to drive the reaction to completion. All treatments were conducted at neutral pH.

The results shown in Table 5–2 demonstrate that co–precipitation of 4–methylphenol and 2,4–dichlorophenol with phenol, 3–chlorophenol or 4–chlorophenol produced a lower residual absorbance in the treated water than when the compounds were treated separately. In addition, when 4–methylphenol and 2,4–dichlorophenol were treated together the clearance of both compounds improved substantially. Therefore, co–precipitation is effective not only when a hard–to–remove compound is treated with a readily–removed compound but even between two hard–to–remove compounds.

The enhanced removal of poorly removed compounds in mixtures of pollutants is of practical importance since real industrial wastewaters contain a mixture of pollutants. Therefore, mixtures of pollutants will facilitate the removal of the contaminants by the enzyme and hydrogen peroxide.

5.9 Treatment in a Semi–Batch Reactor

It has been demonstrated that the rate of enzyme inactivation in a batch reactor increases with enzyme concentration. This results from an increased probability of a phenoxy radical finding and reentering the active site of an enzyme when the enzyme is present in a high concentration. In order to minimize this probability, two alternatives are possible: (1) reduce free radical concentration; and/or (2) reduce the amount of enzyme available for inactivation at any one instant.

Reduction of the free radical concentration can be accomplished by limiting the amount of hydrogen peroxide available at any time resulting in a slower rate of formation of the phenoxy radicals. While this would make it more difficult for a free radical to find an enzyme to inactivate, it also decreases the rate at which the radical finds another radical with which to polymerize. Therefore, the phenoxy radical would remain in solution for a longer period until it either finds a second free

Table 5–2: Co–precipitation of Hard–to–Remove Aromatic Substrates

Hard–to–Remove Compound	Co–precipitator Compound	Absorbance Reduction (%)
4–methylphenol	none (at optimal pH)	83
	phenol	99
	3–chlorophenol	93
	4–chlorophenol	98
	2,4–dichlorophenol	87
2,4–dichlorophenol	none (at optimal pH)	83
	phenol	89
	3–chlorophenol	89
	4–chlorophenol	92
	4–methylphenol	95

[HRP] = 3 U/mL [Aromatic] = 0.5 mM (each) [H_2O_2] = 2 mM

pH = 7.0 (unless otherwise stated) T = 25°C

Note: Reduction (%) is based on absorbance at the peak wavelength corresponding to the hard–to–remove compound at the 1 mM level

radical or it finds an enzyme to inactivate. The lifetime of the enzyme would only be extended provided the probability of a radical inactivating an enzyme is substantially lower than the probability of it reacting with another radical. This possibility was tested by operating a semi–batch reactor in which the peroxide was added over time to the batch system containing phenol and peroxidase. The addition rate of the peroxide was varied from instantaneous (batch reactor) to addition over a 20 hour period. The amount of enzyme in the reactor was also varied. In all addition rates studied there was no improvement in the removal of phenol as a function of enzyme dose. Therefore, the lifetime of the enzyme was not extended by minimizing the instantaneous peroxide concentration.

The second alternative, of reducing the enzyme available for inactivation, can be accomplished by adding the enzyme to the reaction mixture over an extended period. In this situation where the substrates (peroxide and aromatic compound) are not limiting, the enzyme would undergo its catalytic cycle without delays associated with finding a substrate. While this catalytic cycle is operating, the probability of a free radical entering the active site of the enzyme would be reduced because it is likely that the site would already be occupied by another aromatic molecule since the aromatic substrate is present in very high concentration with respect to the free radicals. Therefore, free radical inactivation of the enzyme would be reduced. This theory was tested by adding the enzyme over time to a batch reactor containing peroxide and phenol. The addition rate varied from a spike (batch reactor) to addition over a 20 hour period. The amount of enzyme added was varied from none to 3 U/mL for each flowrate. The results of these semi–batch reactor runs are summarized in Figure 5–20.

The curves of Figure 5–20 demonstrate that as the rate of enzyme addition to the reaction mixture is decreased the resulting clearance of phenol from solution is increased for a given dose of enzyme. Therefore, the number of reactions catalyzed

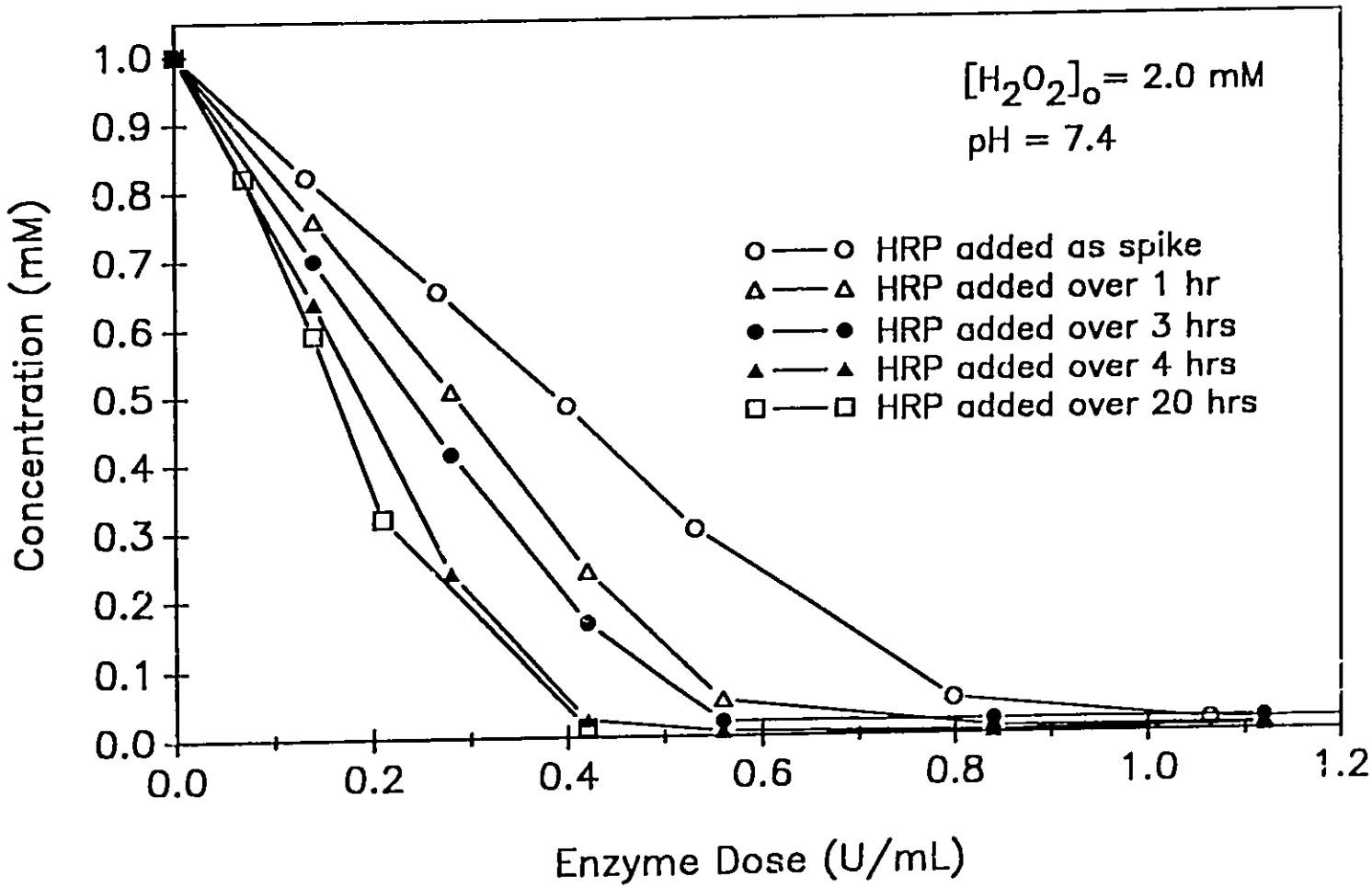


Figure 5–20: Phenol Removal as a Function of HRP Addition Rate and Dose in a Semi–Batch Reactor at 25°C

per enzyme is extended by maintaining a low instantaneous enzyme concentration in the reactor. This improvement is limited by other inactivation mechanisms as can be seen by the diminishing improvements in enzyme lifetime as the the addition rate is reduced from addition over 4 hours to 20 hours. However, sufficient improvement was made to indicate that the instantaneous concentration of enzyme in solution is an important parameter in the design of a waste treatment system. Note that there was no improvement in the residual amount of phenol remaining in solution following treatment in the semi-batch reactor. This indicates that the same soluble byproducts are formed regardless of the rate of addition of enzyme to the reactor.

6. KINETIC MODELLING

The selection of a reactor system which operates in the safest and most efficient manner can be the key to the economic success or failure of a chemical process. Mathematical models based on physical and chemical laws are frequently employed in the development of such systems. These models may be used to evaluate the relative importance of process parameters and will aid in the selection and design of an appropriate reactor system. As such, mathematical modelling would assist in the selection, design and optimization of a system for treatment of wastewater by enzyme catalyzed polymerization.

Traditionally, steady–state kinetic models have been developed to evaluate rate constants for enzyme systems. However, these models have limited applications in reactor development since they describe only the initial stages of a reaction once a steady–state has been achieved. The conditions under which a steady-state model holds true are not usually the conditions which are encountered in the milieu of a reactor. The progress of a reaction may be modelled inaccurately if the time dependence of the concentrations of reactants and enzymes are neglected (Barshop *et al.*, 1983). The alternative to deriving a steady–state model is to explicitly establish the differential equations governing a reaction mechanism and numerically integrate over the desired time interval. This allows for the simulation of reactions of much greater complexity than is possible by applying an overall steady–state approach.

Both steady–state and unsteady–state (transient) models of the horseradish peroxidase system have been developed. The steady–state model was used to estimate the effects of imposing pseudo–steady state assumptions on model development. Model validation was performed by comparing experimental data with model predictions.

6.1 The Horseradish Peroxidase System

The mechanism of the horseradish peroxidase system is illustrated in Figure 6–1. When divided into its individual segments, this mechanism consists of the following reactions:

$$E + H_2O_2 \xrightarrow{k_1} E_i + H_2O \qquad (6\text{–}1)$$

$$E_i + AH_2 \xrightarrow{k_8} E_{ii} + \cdot AH \qquad (6\text{–}2)$$

$$E_{ii} + AH_2 \xrightarrow{k_9} E + \cdot AH + H_2O \qquad (6\text{–}3)$$

$$2 \cdot AH \xrightarrow{k_p} \text{Polymer Products} \qquad (6\text{–}4)$$

$$E \,/\, E_i \,/\, E_{ii} + \cdot AH \xrightarrow{k_r} E_{inact} \qquad (6\text{–}5)$$

$$E_i + H_2O_2 \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} (E_i \cdot H_2O_2) \qquad (6\text{–}6)$$

$$(E_i \cdot H_2O_2) \xrightarrow{k_3} E + O_2 + H_2O \qquad (6\text{–}7)$$

$$(E_i \cdot H_2O_2) \xrightarrow{k_i} E_{670} \qquad (6\text{–}8)$$

$$(E_i \cdot H_2O_2) \xrightarrow{k_4} E_{ii} + O_2^- \qquad (6\text{–}9)$$

$$E_{ii} + H_2O_2 \underset{k_{-5}}{\overset{k_5}{\rightleftharpoons}} (E_{ii} \cdot H_2O_2) \qquad (6\text{–}10)$$

$$(E_{ii} \cdot H_2O_2) \xrightarrow{k_6} E_{iii} + H_2O \qquad (6\text{–}11)$$

$$E_{iii} \xrightarrow{k_7} E + O_2^- \qquad (6\text{–}12)$$

The rate equations for the above reactions are a mixture of first and second order. Kinetic rate constants for many of these rate equations are available in

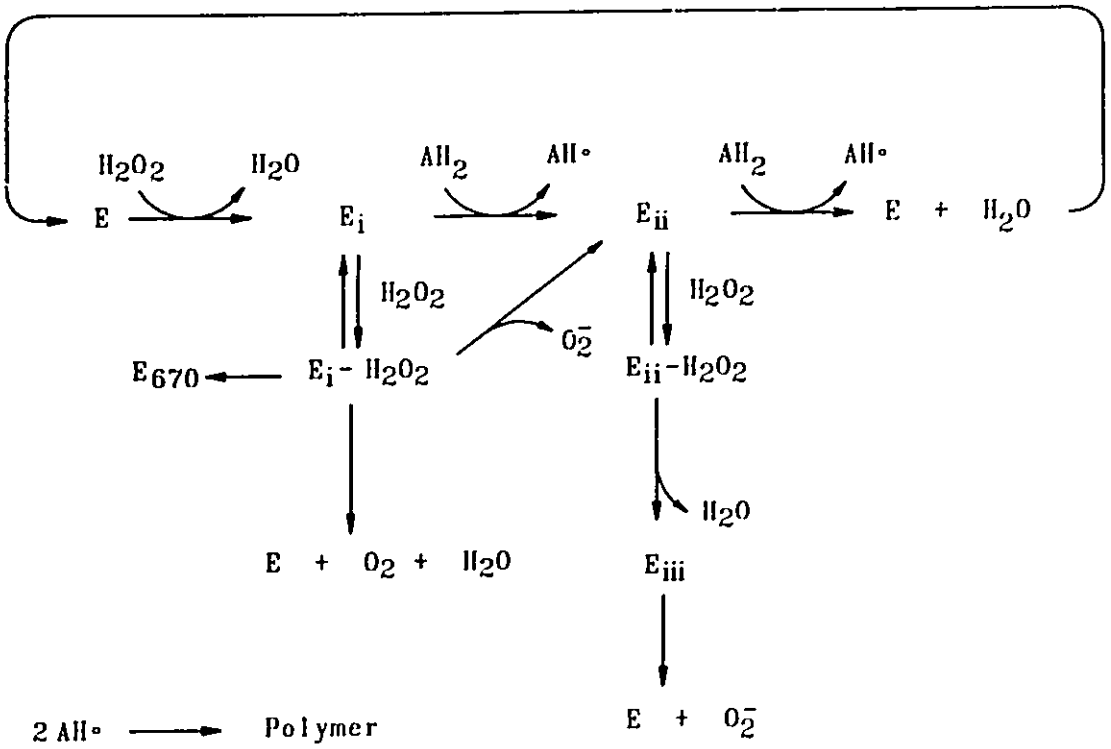


Figure 6–1: Mechanism of Horseradish Peroxidase Catalyzed Polymerization of Aromatic Compounds

recent literature. Rate constants, their literature sources, and the pH and temperature at which they were evaluated are listed in Tables 6–1 and 6–2.

The values for rate constants k_2, k_{-2} and k_3 are not directly known but the overall rate constant, k_c, for the catalase reaction,

$$E_i + H_2O_2 \xrightarrow{k_c} E + O_2 + H_2O \qquad (6\text{–}13)$$

was measured by Nakajima and Yamazaki (1987) over a range of temperatures between 5°C and 20°C at neutral pH. Arnao *et al.* (1990b) assumed that the rate of the catalase reaction was limited by the decomposition of $E_i \cdot H_2O_2$ according to Reaction 6–7 and that the rate of the reverse step was negligible ($k_{-2} \simeq 0$). Therefore, they concluded that the overall rate constant, k_c, is a measure of k_2. They used this assumption in their evaluation of the rate constant for the second step of the catalase reaction, k_3.

No data are currently available for the rate constants k_5, k_{-5} and k_6. In addition, the existence of the intermediate complex, $E_{ii} \cdot H_2O_2$, has not been demonstrated. However, Noble and Gibson (1970), Nakajima and Yamazaki (1987) and Adediran and Lambeir (1989) report apparent rate constants, k_{app}, for the formation of Compound III from Compound II according to

$$E_{ii} + H_2O_2 \xrightarrow{k_{app}} E_{iii} + H_2O \qquad (6\text{–}14)$$

at neutral pH and various temperatures. Therefore, Reaction 6–14 will be used to express the formation of Compound III from Compound II. Rate constant evaluations performed by Nakajima and Yamazaki (1987) have the greatest amount of supporting data; therefore, their evaluation of k_{app} was used in this study.

Table 6–1: Kinetic Rate Constants Cited from Literature

Constant	Temperature	Value	Literature source
k_1	25°C	2×10^7 $M^{-1} s^{-1}$	Yamazaki and Nakajima, 1986
k_2	25°C	5×10^3 $M^{-1} s^{-1}$	Arnao *et al.*, 1990b
k_{-2}	–	$\simeq 0$	Arnao *et al.*, 1990b
k_3	25°C	1.76 s^{-1}	Arnao *et al.*, 1990b
k_4	25°C	7.85×10^{-3} s^{-1}	Arnao *et al.*, 1990b
k_5	–	–	not available
k_{-5}	–	–	not available
k_6	–	–	not available
k_7	20°C 25°C	2.2×10^{-3} s^{-1} 4.18×10^{-3} s^{-1}	Nakajima and Yamazaki, 1987 extrapolated from above
k_8	–	–	see Table 6–2
k_9	–	–	see Table 6–2
k_{app}	20°C 25°C	25 $M^{-1} s^{-1}$ 31 $M^{-1} s^{-1}$	Nakajima and Yamazaki, 1987 extrapolated from above
	20°C	40 $M^{-1} s^{-1}$	Noble and Gibson, 1970
	25°C	20 $M^{-1} s^{-1}$	Adediran and Lambeir, 1989
k_c	10°C 25°C	5×10^2 $M^{-1} s^{-1}$ 1×10^3 $M^{-1} s^{-1}$	Nakajima and Yamazaki, 1987 extrapolated from above
k_i	25°C	3.92×10^{-3} s^{-1}	Arnao *et al.*, 1990b
k_p	–	–	not available
k_r	–	–	not available

Note: All constants were evaluated at neutral pH. Extrapolations to evaluate rate constants at 25°C were made using the Arrhenius Equation and activation energies supplied by the authors.

(s^{-1} = 1st order rate constant; $M^{-1} s^{-1}$ = 2nd order rate constant)

Table 6–2: Rate Constants Specific to Several Phenols

Compound	Constant	Value ($M^{-1} s^{-1}$)	Literature Source
phenol	k_8	2.76×10^6	Job and Dunford, 1976
	k_9	3.15×10^5	Sakurada *et al.*, 1990
		2.86×10^5	Dunford and Adediran, 1986
4–methylphenol	k_8	4.20×10^7	Job and Dunford, 1976
	k_9	1.00×10^6	Dunford and Adediran, 1986
		1.06×10^6	Sakurada *et al.*, 1990
4–chlorophenol	k_8	1.13×10^7	Job and Dunford, 1976
	k_9	1.10×10^6	Sakurada *et al.*, 1990

Note: Rate constants are 2nd order and were evaluated at pH 7.0 and 25°C

No data are currently available concerning the rate of polymerization (k_p) of the free radical products of the catalytic cycle or the rate of inactivation of peroxidase by free radicals (k_r).

6.1.1 The Basis for Model Development

Mathematical expressions have been derived for the horseradish peroxidase mechanism based on the Law of Mass Action which states that the rate of a reaction is proportional to the product of the concentrations of the reactants raised to the power of the number of molecules reacting according to the stoichiometric equation. The variables and reactions which are used in the development of kinetic models are defined in Table 6–3 and Figure 6–2, respectively.

All kinetic measurements and modelling were performed at neutral pH and 25°C. Not all reaction rate constants cited from the literature were evaluated at 25°C but they were adjusted, as needed, using the Arrhenius Equation with activation energies provided by the respective authors. Model simplifications are made when the values of rate constants are unavailable.

The enzyme concentrations (E, E_0, E_i, etc.) used in the model development are expressed as molar quantities. However, enzyme concentrations are determined using an activity assay which is based on the rate of conversion of a substrate of HRP under fixed conditions. The molar concentration of active HRP is directly proportional to the enzyme activity; therefore, the initial molar concentration of active enzyme can be calculated using:

$$E_0 = C_a \times \text{Activity (U/mL)}$$

where the constant of proportionality, C_a, is 0.153 μM $(U/mL)^{-1}$ for HRP.

Table 6–3: Variable Definitions

Variable Name	Variable Description
$[AH_2]$	aromatic substrate concentration
$[AH_2]_0$	initial aromatic substrate concentration
E	concentration of HRP in native form
E_0	total enzyme concentration (initial enzyme)
E_i	concentration of Compound I
E_{ii}	concentration of Compound II
E_{iii}	concentration of Compound III
E_{670}	concentration of HRP in P–670 form
E_{inact}	concentration of HRP inactivated by free radicals
$[E_i \cdot H_2O_2]$	concentration of Compound I complex with H_2O_2
$[H_2O_2]$	peroxide concentration
$[H_2O_2]_0$	initial peroxide concentration
t	reaction time

Note: All concentrations are expressed as molar quantities.

Catalytic cycle of horseradish peroxidase	
$E + H_2O_2 \xrightarrow{k_1} E_i + H_2O$	(6–1)
$E_i + AH_2 \xrightarrow{k_8} E_{ii} + \cdot AH$	(6–2)
$E_{ii} + AH_2 \xrightarrow{k_9} E + \cdot AH + H_2O$	(6–3)
Reactions of Compound I without aromatic substrate	
$E_i + H_2O_2 \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} (E_i \cdot H_2O_2)$	(6–6)
$(E_i \cdot H_2O_2) \xrightarrow{k_4} E_{ii} + O_2^-$	(6–9)
$(E_i \cdot H_2O_2) \xrightarrow{k_3} E + O_2 + H_2O$	(6–7)
or	
$E_i + H_2O_2 \xrightarrow{k_c} E + O_2 + H_2O$	(6–13)
Inactivation of horseradish peroxidase	
$(E_i \cdot H_2O_2) \xrightarrow{k_i} E_{670}$	(6–8)
$E / E_i / E_{ii} + \cdot AH \xrightarrow{k_r} E_{inact}$	(6–5)
Compound III formation and decomposition	
$E_{ii} + H_2O_2 \xrightarrow{k_{app}} E_{iii} + H_2O$	(6–14)
$E_{iii} \xrightarrow{k_7} E + O_2^-$	(6–12)

Figure 6–2: Reactions Used in Model Development

6.1.2 Method of Numerical Integration

The differential equations derived in the development of kinetic models are not usually amenable to analytical solutions unless a great deal of simplification is made. The alternative is to solve the equations using one of the multitude of numerical integration techniques available; the technique chosen depends on the form and behavior of the equations being solved. For example, it has been found that the equations describing the horseradish peroxidase system are extremely stiff. Stiffness arises in a set of differential equations when the values of its time constants are widely different in magnitude. In chemical systems, this situation is typical when species react as quickly as they are formed (Barshop *et al.*, 1983). This complicates the numerical solution since very small time steps are required to solve a stiff set of differential equations so that the propagated discretization error is minimized and the solution remains numerically stable.

The equations describing the horseradish peroxidase system are inherently stiff due to:

(i) reaction rate constants which vary by several orders of magnitude within the equations ($k_1 > k_8 > k_9 >>> k_2, k_3, k_4$, etc.)

(ii) reaction of native enzyme with hydrogen peroxide almost as quickly as it is reformed in the catalytic cycle ($k_1 = 2 \times 10^7\ M^{-1}\ s^{-1}$).

Since the native enzyme is continuously reformed by the catalytic cycle, the differential equations describing the cycle remain stiff during the full time domain of the reaction.

Barshop *et al.* (1983) recommend the implementation of solution techniques which automatically adjust the time step at each iteration according to the prevailing stiffness. Such methods were found to be inappropriate for the HRP system since the step-size could not be increased significantly above the interval required to maintain numerical stability in the initial rapid stages of the reaction.

The additional functional evaluations (twice as many per step), required to predict an appropriate choice for the integration interval, slowed the program execution time to a greater extent than when using a method with a fixed step–size. Since very small integration intervals must be used with a fixed step–size and, as a consequence, a large number of time steps must be evaluated, an integration technique was sought which would minimize the propagated discretization error. For this reason, the Runge-Kutta-Fehlberg method was chosen.

The Runge–Kutta–Fehlberg Integration Technique uses a Runge–Kutta method with a local truncation error of order five (Burden *et al.*, 1981) to approximate the solution, y(t), to the initial–value problem,

$$\frac{dy}{dt} = f(t, y)$$

with $a \leq t \leq b$ and $y(a) = \alpha$. The solution may be determined using the Runge–Kutta–Fehlberg algorithm with an integration time interval, h;

$$y_{i+1} = y_i + \frac{16}{135} K_1 + \frac{6656}{12825} K_3 + \frac{28540}{56430} K_4 + \frac{9}{50} K_5 + \frac{2}{55} K_6$$

$$t_i = a + i \times h$$

with $y_0 = \alpha$ and where y_i is the approximate solution of y(t) at time t_i. The intermediate approximations of the solution required by this algorithm are determined using:

$$K_1 = h\, f(t_i, y_i)$$

$$K_2 = h\, f(t_i + \frac{1}{4} h, y_i + \frac{1}{4} K_1)$$

$$K_3 = h\, f(t_i + \frac{3}{8} h,\ y_i + \frac{3}{32} K_1 + \frac{9}{32} K_2)$$

$$K_4 = h\, f(t_i + \frac{12}{13} h,\ y_i + \frac{1932}{2197} K_1 - \frac{7200}{2197} K_2 + \frac{7296}{2197} K_3)$$

$$K_5 = h\, f(t_i + h,\ y_i + \frac{439}{216} K_1 - 8\, K_2 + \frac{3680}{513} K_3 - \frac{845}{4104} K_4)$$

$$K_6 = h\, f(t_i + \frac{1}{2} h,\ y_i - \frac{8}{27} K_1 + 2\, K_2 - \frac{3544}{2565} K_3 + \frac{1859}{4104} K_4 - \frac{11}{40} K_5)$$

6.2 Development of a Steady–State Ping–Pong Model

Enzyme catalyzed reactions in which there is a transfer of a group from a donor to the enzyme followed by a second transfer from enzyme to accepter is known as a substituted–enzyme or ping–pong mechanism (Cornish–Bowden and Wharton, 1988). A conventional ping–pong mechanism is an ordered two–substrate, two–product scheme in which the enzyme passes through a single intermediate form before returning to its native state. In contrast, the HRP cycle involves the production of three products (H_2O and 2 $\cdot$AH's) from two substrates (H_2O_2 and AH_2) while the enzyme passes through two intermediates (E_i and E_{ii}). Therefore, the HRP catalytic cycle is a modified ping–pong mechanism (Everse *et al.*, 1990).

A steady–state ping–pong model is developed in which the following assumptions are made:

(i) Inactivation reactions are insignificant with respect to the catalytic cycle due to the relative magnitude of inactivation rate constants with respect to the cycle rate constants.

(ii) Compounds I and II are present at steady–state levels since the formation of Compound I from native enzyme and peroxide and subsequent production of Compound II are extremely rapid.

(iii) Aromatic substrate and peroxide are consumed with a one–to–one stoichiometry which results from the formation of large polymers. Other mechanisms, discussed previously, which explain the one–to–one

stoichiometry cannot be implemented without rate constants describing the rate of polymerization of free radicals and reaction of intermediate compounds with HRP. Until these constants are measured, the one–to–one stoichiometry must be artificially imposed on the model.

The differential equations describing the transient behavior of Reactions 6--1 to 6–3 are:

$$\frac{d[H_2O_2]}{dt} = -k_1 E [H_2O_2] \tag{6–1}$$

$$\frac{dE_i}{dt} = k_1 E [H_2O_2] - k_8 E_i [AH_2] \tag{6–2}$$

$$\frac{dE_{ii}}{dt} = k_8 E_i [AH_2] - k_9 E_{ii} [AH_2] \tag{6–3}$$

A material balance on the horseradish peroxidase enzyme produces:

$$E = E_0 - E_i - E_{ii} \tag{6–4}$$

where E_0 represents the initial amount of enzyme provided to the reaction mixture.

Since the production of Compound I is very quick and the rate of return of enzyme to the native state is limited by the rate of reaction of Compound II catalysis, it may be assumed that the formation of Compounds I and II reaches a steady state rapidly. Therefore, Equations 6–2 and 6–3 may be simplified using:

$$\frac{dE_i}{dt} \approx 0 \text{ and } \frac{dE_{ii}}{dt} \approx 0$$

to produce.

$$E_i = \frac{k_1}{k_8} \frac{[H_2O_2]}{[AH_2]} E \tag{6–5}$$

$$E_{ii} = \frac{k_1}{k_9} \frac{[H_2O_2]}{[AH_2]} E \qquad (6\text{–}6)$$

Substitution of Equations 6–5 and 6–6 into the enzyme balance and solving the resulting expression for the native enzyme concentration, E, produces:

$$E = \frac{E_0}{\left[1 + \left(\frac{k_1}{k_8} + \frac{k_1}{k_9}\right) \frac{[H_2O_2]}{[AH_2]} \right]} \qquad (6\text{–}7)$$

Substitution of Equation 6–7 into equation 6–1 produces an expression for the rate of disappearance of peroxide from solution in terms of the concentrations of aromatic substrate, hydrogen peroxide and native enzyme supplied to the reaction mixture, E_0:

$$\frac{d[H_2O_2]}{dt} = \frac{-E_0}{\left[\frac{1}{k_1[H_2O_2]} + \left(\frac{k_8 + k_9}{k_8 k_9}\right) \frac{1}{[AH_2]} \right]} \qquad (6\text{–}8)$$

A one–to–one stoichiometry can be imposed on the model without altering the Chance–George mechanism by assuming that the rate of disappearance of aromatic compound is half of that predicted by the system of reactions. This assumption is equivalent to stating that, following polymerization, half of the products remain in solution and return to the catalytic cycle resulting in the formation of larger polymers. Also inherent in this assumption is the premise that the reaction rates of the polymers remaining in solution are not substantially lower than the reaction rate of the monomer. This assumption must be made in the absence of rate constants for the formation and reaction of intermediate products with HRP.

Therefore, the amount of aromatic substrate present in solution at any instant, expressed as the concentration of the monomer, is given by:

$$[AH_2] = [AH_2]_0 - ([H_2O_2]_0 - [H_2O_2]) \qquad (6\text{–}9)$$

Solving Equation 6–9 for peroxide concentration and substituting into Equation 6–8 produces an expression for the rate of disappearance of aromatic compound from solution with time:

$$\frac{d[AH_2]}{dt} = \frac{-E_0}{\left[\frac{1}{k_1([H_2O_2]_0 - [AH_2]_0 + [AH_2])} + \left(\frac{k_8 + k_9}{k_8 k_9}\right)\frac{1}{[AH_2]}\right]} \qquad (6\text{–}10)$$

Equation 6–10 can be solved analytically. Integration gives:

$$t = -\frac{1}{k_1 E_0}\ln\left(\frac{[H_2O_2]_0 - [AH_2]_0 + [AH_2]}{[H_2O_2]_0}\right) - \frac{1}{E_0}\left(\frac{k_8 + k_9}{k_8 k_9}\right)\ln\left(\frac{[AH_2]}{[AH_2]_0}\right) \qquad (6\text{–}11)$$

If the initial concentrations of peroxide and aromatic substrate are equal ($[H_2O_2]_0 = [AH_2]_0$) then Equation 6–11 may be simplified to produce:

$$t = -\frac{1}{E_0}\left(\frac{1}{k_1} + \left(\frac{k_8 + k_9}{k_8 k_9}\right)\right)\ln\left(\frac{[AH_2]}{[AH_2]_0}\right) \qquad (6\text{–}12)$$

which may be solved explicitly for the aromatic substrate concentration as a function of time. According to this equation, changes in the logarithm of $[AH_2]/[AH_2]_0$ should be linear with respect to time for equimolar concentrations of peroxide and aromatic substrate. The validity of Equation 6–12 was tested using

the data collected for 4–chlorophenol and illustrated previously in Figure 5–15. The resulting plots of experimental data, shown in Figure 6–3, do not demonstrate linear behavior as was predicted by the model. Since substrate depletion is accounted for in the model, the deteriorating slopes of the curves of Figure 6–3 must result from enzyme inactivation and not depletion of aromatic substrate and peroxide. Therefore, the steady–state ping–pong model is inadequate for the purpose of reactor design.

6.2.1 Effect of the Steady–State Assumption

Equation 6–11 represents the analytical solution to a set of equations which have been simplified through use of a steady–state assumption. As an alternative, the original differential equations, Equations 6–1, 6–2 and 6–3, and the enzyme balance, Equation 6–4, can be solved simultaneously through numerical integration without imposing the steady–state limitation on the model.

Equations 6–1 through 6–4 were integrated using the Runge-Kutta-Fehlberg algorithm with a step size of 0.0001 seconds. This minute step size was required to maintain numerical stability in the solution. Figure 6–4 contains the results of the numerical solution overlayed on the analytical (exact) solution of the steady–state model. Enzyme concentrations in the range of 0.01 to 0.1 μM were used to correspond with typical concentrations used in clearance studies for the treatment of 1 mM aromatic substrate and peroxide. Results indicate virtually no difference ($< 0.01\%$) in model predictions with or without the steady–state assumption. The numerical solution predicted that steady–state is achieved with respect to Compounds I and II within the first millisecond. Therefore, the assumption that the enzyme drives to a steady–state very quickly is justified.

Numerical solution of the differential equation describing the steady–state model (Equation 6–10) can be accomplished using a step size of 2 seconds as

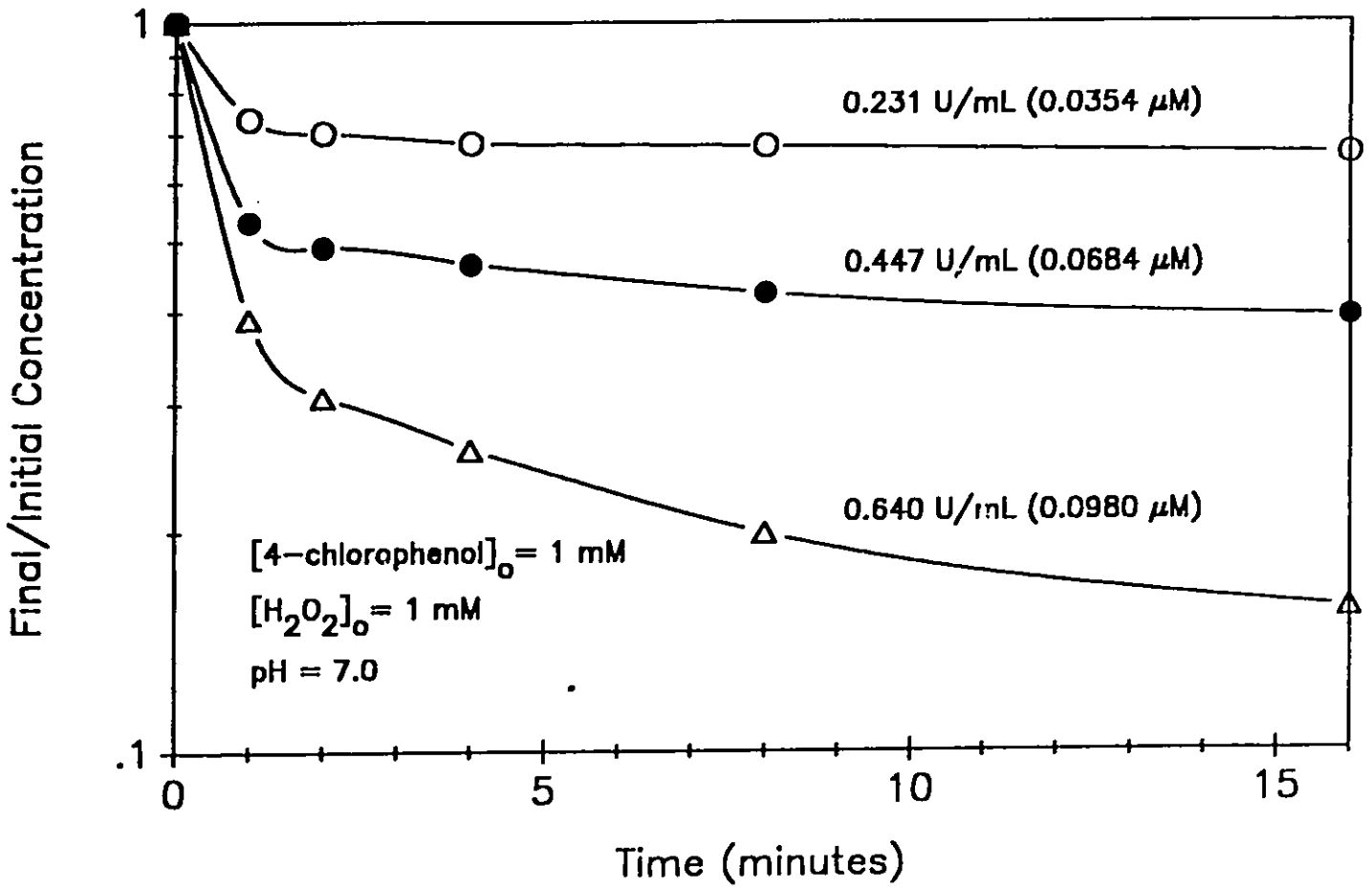


Figure 6–3: Plot of Logarithm of $[AH_2]/[AH_2]_0$ Versus Time for 4–Chlorophenol in a Batch Reactor

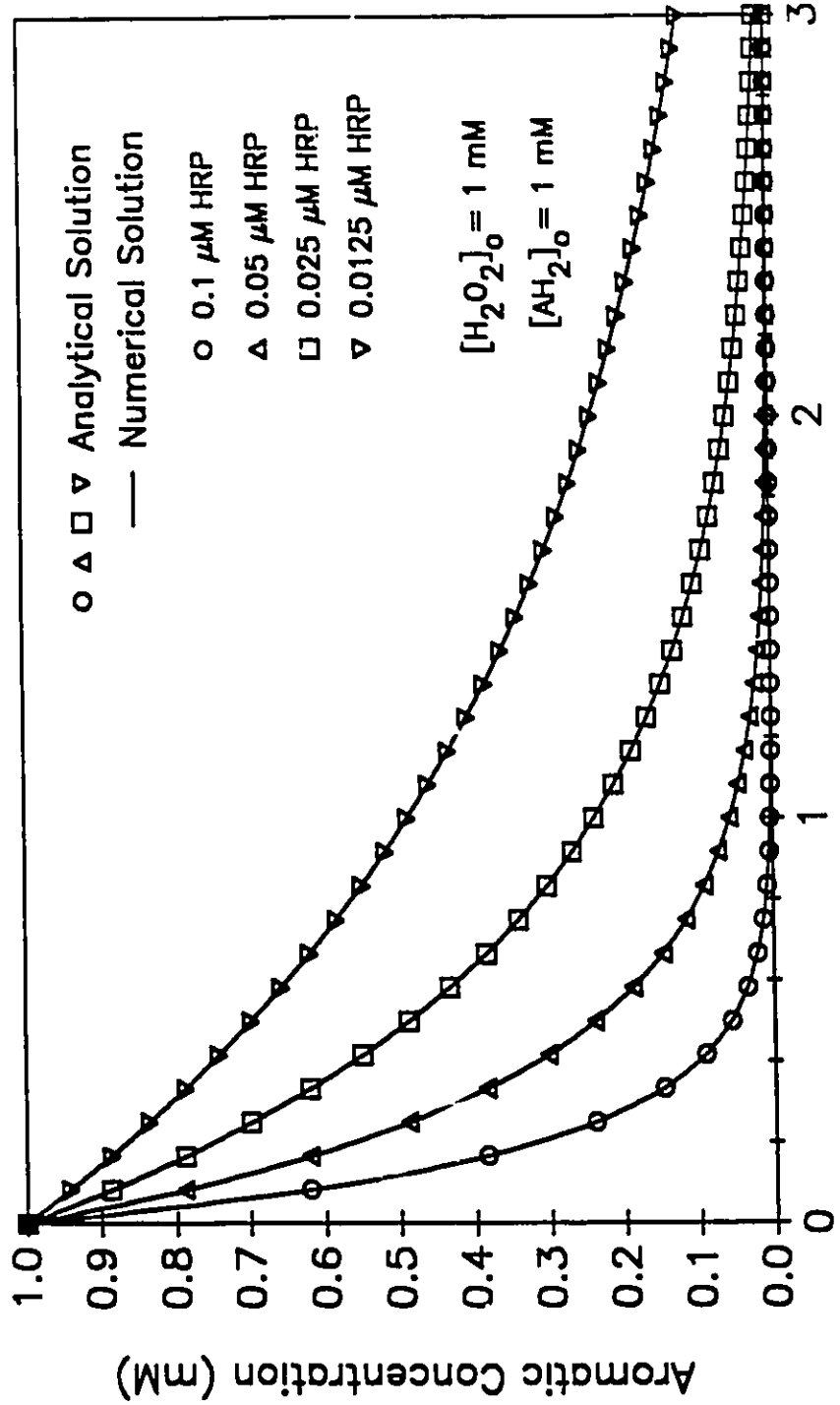


Figure 6–4: Comparison of Analytical Solution of Steady–State Ping-Pong Model with Numerical Solution of Unsteady–State Ping–Pong Model Using Rate Constants for 4–Chlorophenol

opposed to the 0.0001 second step size required for the unsteady–state model. This increase in step size by a factor of 2×10^4 produced little change in the numerical solution ($< 0.01\%$). Therefore, the steady–state assumption dramatically reduced the stiffness of the system of equations with a corresponding reduction in computation time. In addition, with the increased step size, the solution method was changed to the Fourth Order Runge–Kutta Integration Technique which required fewer functional evaluations than the Runge–Kutta–Fehlberg method and produced the same solution.

6.3 Development of a Transient–State Model

Transient–state models are developed based on the equations listed in Figure 6–1. Initially, all reaction mechanisms are included in the development except for free radical inactivation for which rate constants are unavailable. The model is tested to determine whether Compound III and P–670 formation can account for the degree of inactivation observed in Figure 5–15. Simplifications in the model are implemented and tested to determine their effects on the model's predictive abilities.

A transient model can be developed from the reactions listed in Figure 6–2 without making steady–state assumptions. Assuming k_{-2} is negligible, the differential equations describing the system are:

$$\frac{dE_i}{dt} = k_1 E [H_2O_2] - k_8 E_i [AH_2] - k_2 E_i [H_2O_2] \qquad (6\text{–}13)$$

$$\frac{dE_{ii}}{dt} = k_8 E_i [AH_2] - k_9 E_{ii} [AH_2] + k_4 [E_i \cdot H_2O_2] - k_{app} E_{ii} [H_2O_2] \qquad (6\text{–}14)$$

$$\frac{dE_{iii}}{dt} = k_{app} E_{ii} [H_2O_2] - k_7 E_{iii} \qquad (6\text{–}15)$$

$$\frac{dE_{670}}{dt} = k_i [E_i \cdot H_2O_2] \qquad (6\text{–}16)$$

$$\frac{d[E_i \cdot H_2O_2]}{dt} = k_2 E_i [H_2O_2] - k_3 [E_i \cdot H_2O_2] - k_i [E_i \cdot H_2O_2] - k_4 [E_i \cdot H_2O_2] \qquad (6\text{–}17)$$

$$\frac{d[H_2O_2]}{dt} = -k_1 E [H_2O_2] - k_2 E_i [H_2O_2] - k_{app} E_{ii} [H_2O_2] \qquad (6\text{–}18)$$

For a one–to–one stoichiometry between aromatic compound and peroxide, the rate of disappearance of aromatic substrate is:

$$\frac{d[AH_2]}{dt} = (- k_8 E_i [AH_2] - k_9 E_{ii} [AH_2]) \times \frac{1}{2} \qquad (6\text{–}19)$$

where the factor of $^1/_2$ accounts for the halved removal of aromatic compound observed with respect to consumption of peroxide as predicted by the catalytic cycle.

A material balance on HRP produces an expression for the concentration of native enzyme:

$$E = E_0 - E_i - E_{ii} - E_{iii} - E_{670} - [E_i \cdot H_2O_2] \qquad (6\text{–}20)$$

6.3.1 Solution of the Transient–State Model

Equations 6–13 through 6–20 were solved using the Runge–Kutta–Fehlberg algorithm with a step size of 0.0001 seconds. Step sizes larger than 0.0002 seconds produced numerical instability in the solution. Step sizes less than 0.0001 seconds produced the same solution within 0.001%. An example of model output is shown in Figure 6–5 using the rate constants cited from literature for 4–chlorophenol. Comparison of model output with the experimental data of Figure 5–15 shows little agreement between predicted and actual removal of 4–chlorophenol with time.

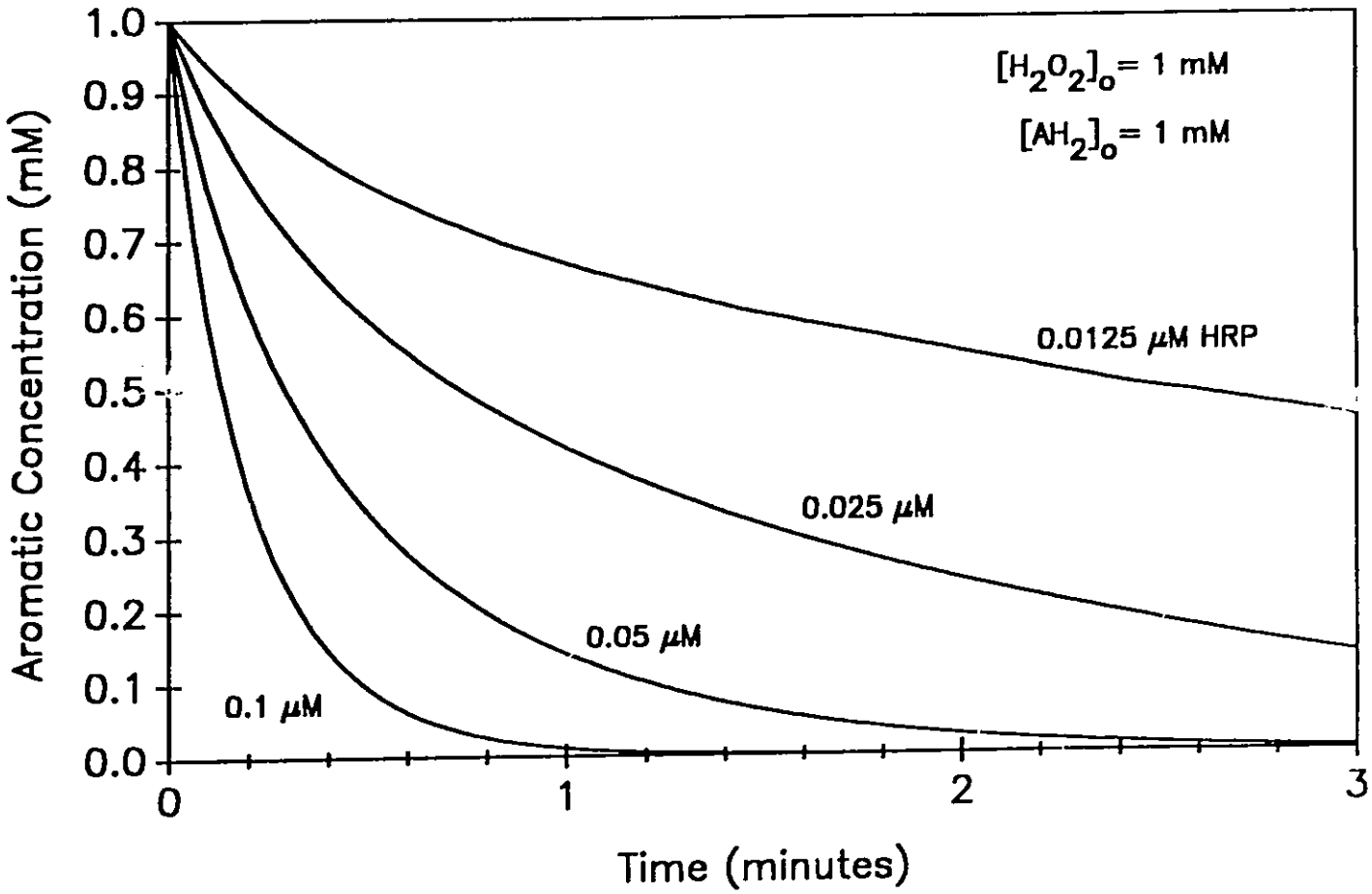


Figure 6–5: Predicted 4–Chlorophenol Concentration Versus Time Incorporating Only P–670 and Compound III Inactivation Mechanisms

Contrary to actual observations, the model predicts eventual full conversion of aromatic substrate. In addition, it fails to predict the initial fast rate followed by a more gradual rate of removal of organic from solution. These differences indicate that Compound III and P–670 formations do not account for the inactivation observed in the actual reaction mixture. Therefore, other mechanisms for the inactivation of HRP must be incorporated into the model before it can reflect the true reaction conditions.

6.3.2 Inactivation of HRP by Other Mechanisms

Modelling of Compound III and P–670 formations over the course of the reaction has demonstrated that these inactive forms of HRP do not account for the large-scale inactivation that has been demonstrated experimentally. Therefore, it is likely that other mechanisms, such as free-radical inactivation, are predominantly responsible for the finite lifetime of the enzyme.

In the absence of a rate constant for free radical inactivation, simplification is required to implement this mechanism in the kinetic model. Previously, it was shown that the number of catalytic turnovers for 4–chlorophenol is constant and is independent of 4–chlorophenol, HRP, and peroxide concentrations. This would indicate that inactivation is proportional to the removal of 4–chlorophenol. Such behaviour suggests that HRP can be treated as a pseudo–substrate of the reaction; that is, each enzyme molecule has a fixed number of catalytic turnovers that it can accomplish before it is inactivated. This consumption of HRP can be represented by the following reaction:

$$HRP + K_s\, AH_2 \longrightarrow E_{inact} + Products \qquad (6\text{–}15)$$

where K_s is the number of molecules of AH_2 removed per enzyme provided to the

reaction mixture (turnovers). This apparent stoichiometry allows the amount of inactive enzyme to be calculated based on the amount of aromatic substrate removed from the solution. Therefore, assuming that peroxidase can be treated as a pseudo-substrate with a fixed number of catalytic turnovers, K_s, the amount of inactivated enzyme can be calculated using:

$$E_{inact} = \frac{1}{K_s}([AH_2]_0 - [AH_2]) \qquad (6\text{–}21)$$

E_{inact} includes enzyme inactivated through the formation of P–670 since the two cannot be distinguished experimentally. Therefore, the enzyme balance must be revised accordingly:

$$E = E_0 - E_i - E_{ii} - E_{iii} - E_{inact} - [E_i \cdot H_2O_2] \qquad (6\text{–}22)$$

The number of catalytic turnovers achieved by HRP for 4–chlorophenol was measured to be 9 000 at pH 7.0 and 25°C under non–limiting conditions of peroxide, aromatic and reaction time. Therefore, K_s was set equal to 9 000 in the model of the 4–chlorophenol system.

Figure 6–6 compares the transient–state model output with the experimental data collected for 4–chlorophenol removal in a batch reactor at neutral pH and 25°C. There is excellent agreement between predicted and measured results.

6.4 Simplification of the Transient–State Model

The transient–state model comprises seven differential and two material balance equations defined by ten constants. Since the values of the constants change according to reaction conditions, and must therefore be measured, the elimination of several constants from the model would simplify its application.

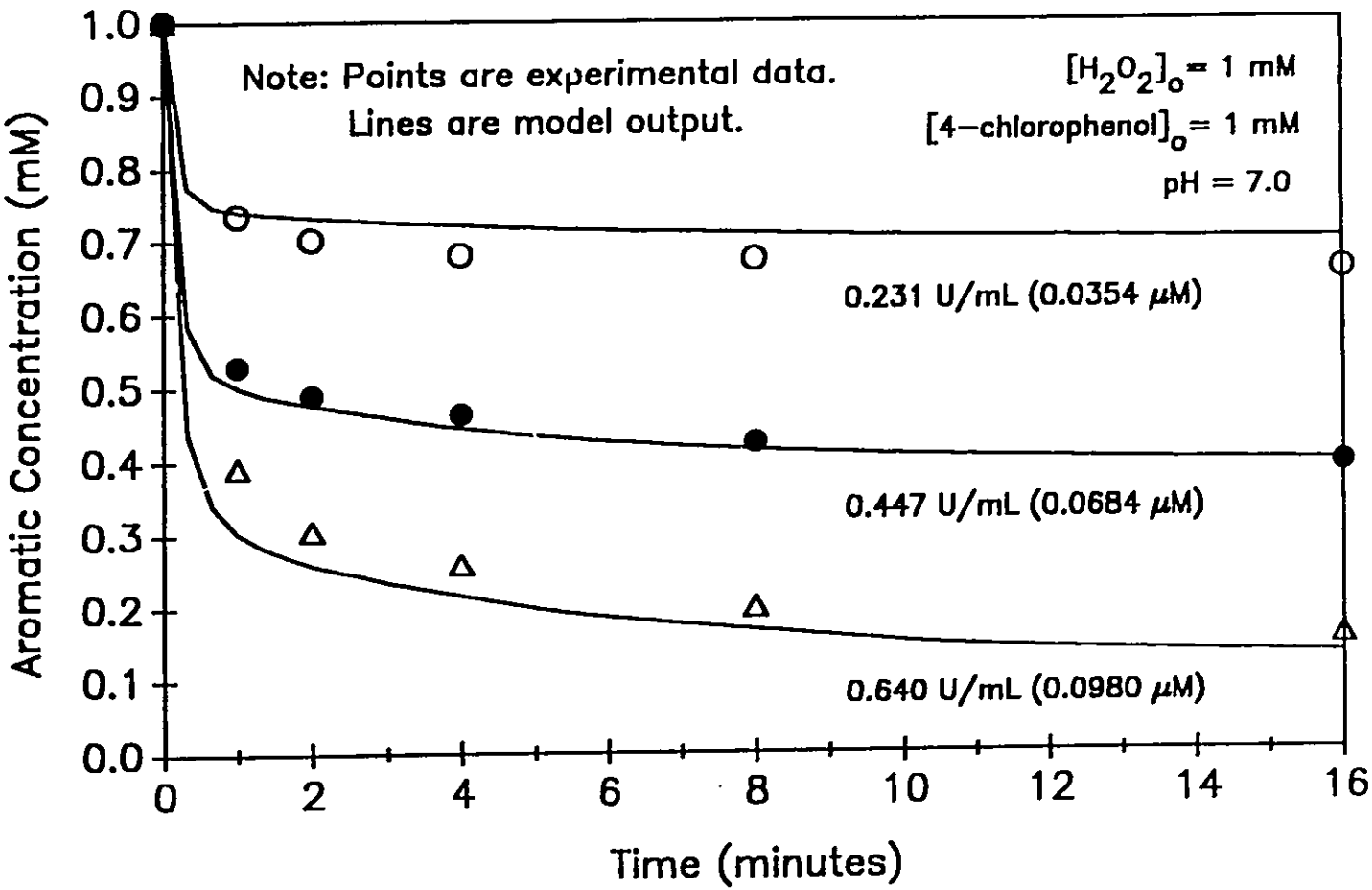


Figure 6–6: Comparison of Transient Model Results with Experimental Data for 4–Chlorophenol Treatment in a Batch Reactor at 25°C and pH 7.0

6.4.1 Elimination of Minor Mechanisms

Figure 6–7 predicts the distribution of enzyme forms for the upper and lower concentrations of enzyme used in the batch reactor experiments. Over the time interval plotted, less than 0.1% of the enzyme was converted to the P–670 form. In addition, less than 0.02% of the total peroxide was consumed by the catalase reaction of HRP for both concentrations of enzyme. The concentration of enzyme–peroxide complex peaked at a level of 2% of the total enzyme provided initially to the mixture. Both graphs demonstrate that the majority of the reaction occurs in the first 30 seconds of reaction time. The rate at which the remaining proportion of aromatic substrate is removed is dependent on the rate of release of native enzyme from the Compound III state.

In the absence of aromatic substrate, the proportion of enzyme converted to the P–670 and Compound III forms is significant as shown in Figure 6–8 for 0.025 μM HRP exposed to a range of peroxide concentrations. The enzyme concentration was chosen to match the concentration in Figure 4–4. Unfortunately, the extent of inactivation predicted by the model fails to match the inactivation noted in Figure 4–4. Also, the model does not fit the data generated by Arnao *et al.* (1990b) even though these data were used to evaluate rate constants for inactivation of HRP through P–670 and Compound III formation. Therefore, further research is required to determine the mechanism and accurate rate constants for the formation of inactive HRP in the presence of peroxide.

Since P–670 formation and conversion of peroxide through the catalase reaction are minimal with respect to other reactions of horseradish peroxidase, elimination of these mechanisms from the model should cause little change in the solution. This simplification will reduce the number of equations to be numerically integrated with a corresponding savings in computation time. The simplified model is only applicable when an aromatic substrate is present since P–670 formation is

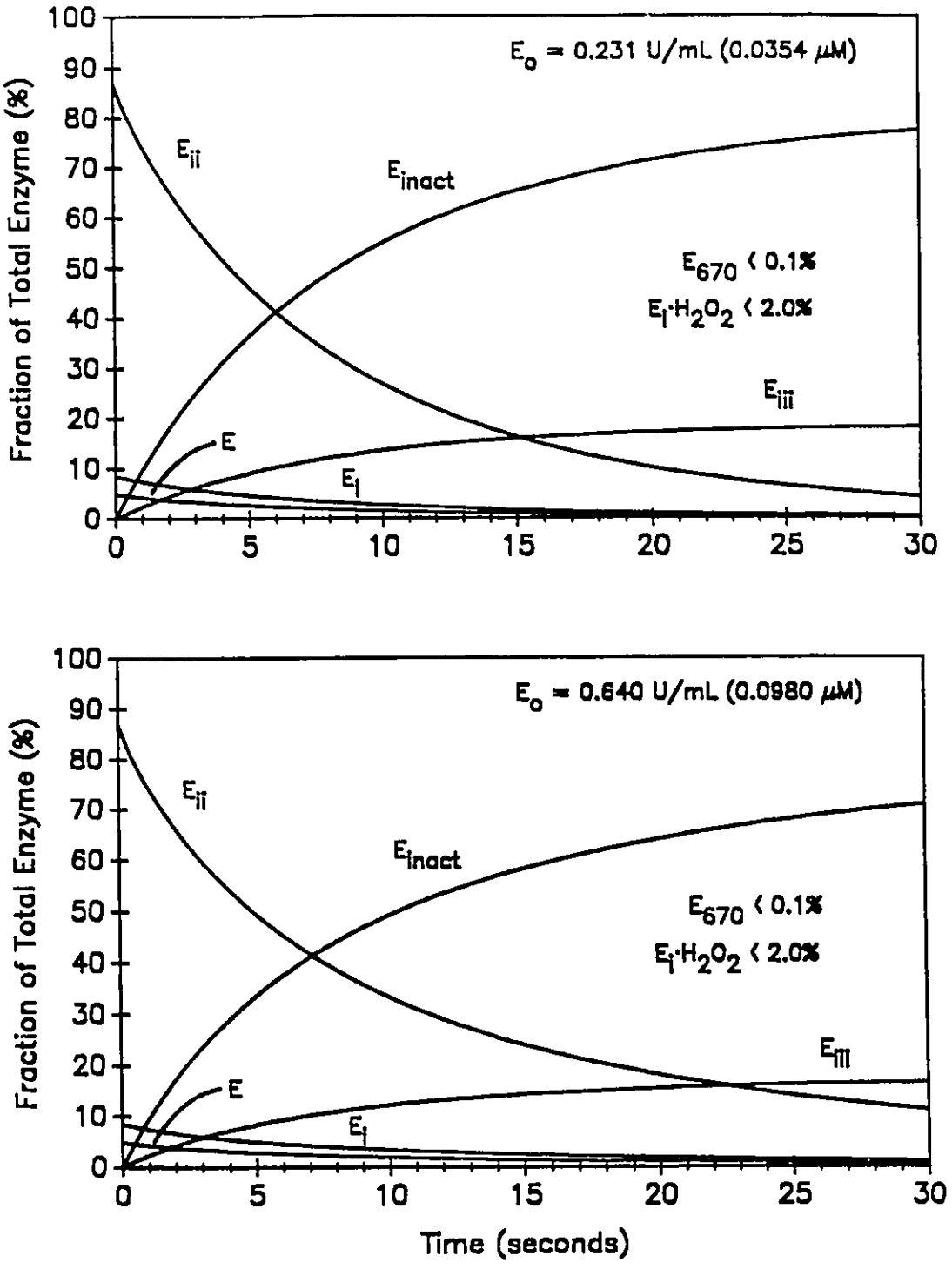


Figure 6–7: Predicted Distribution of Enzyme Forms Using 4–Chlorophenol Rate Constants with 1 mM H_2O_2, 1 mM 4–Chlorophenol, pH 7.0 and 25°C

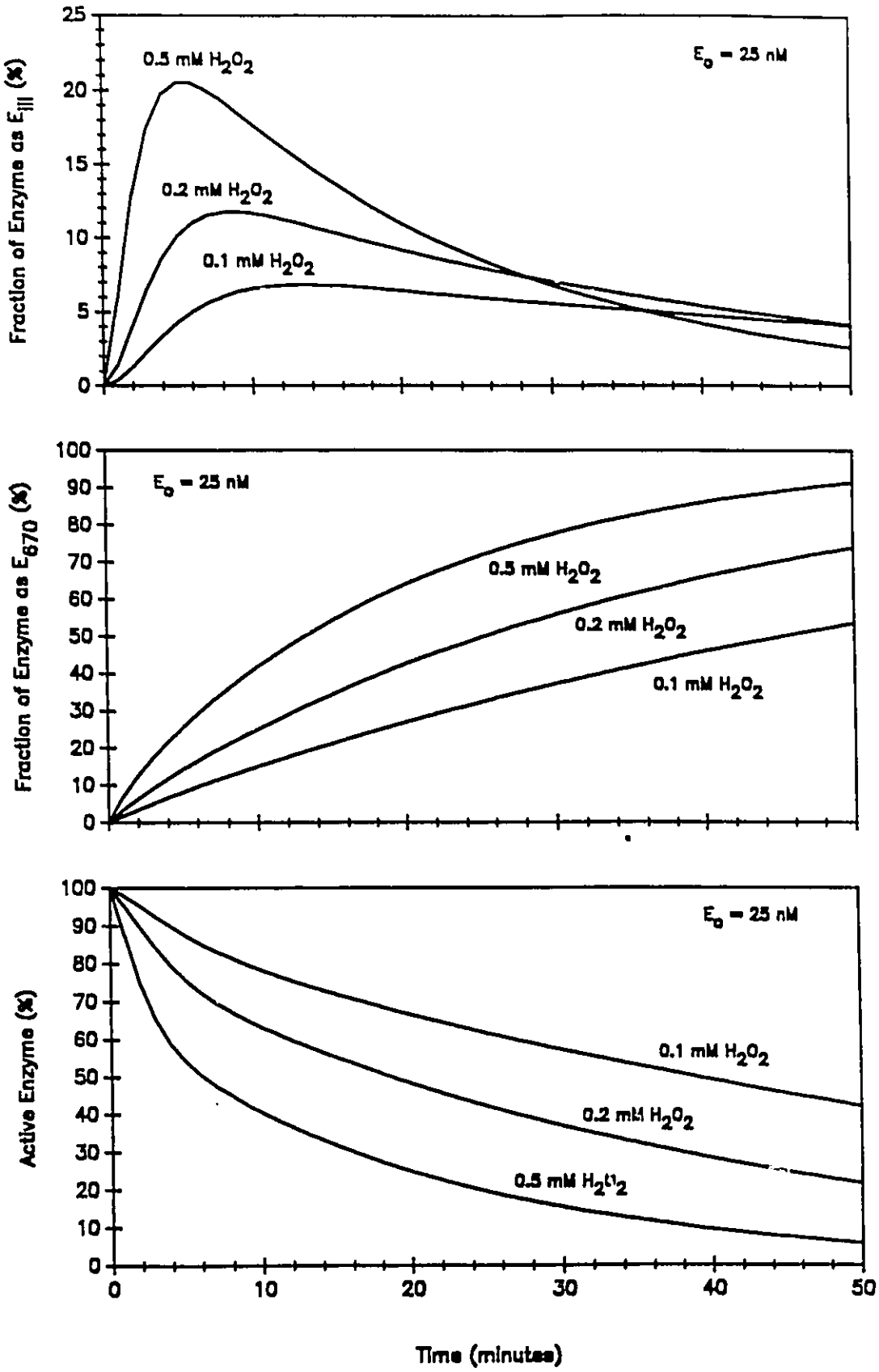


Figure 6–8: Model Prediction of Compound III and P–670 Formation in the Absence of an Aromatic Substrate

significant in the absence of an aromatic substrate.

Figure 6–7 shows that the enzyme is distributed between Compound I, Compound II and native enzyme forms immediately following the start of the reaction. Model predictions indicate that the maximum levels of Compounds I and II concentrations are achieved within the first two milliseconds under the reaction conditions stated in Figure 6–7. Previously, it was demonstrated that the steady–state simplification in the ping–pong model reduced much of the stiffness in the system of differential equations without significant change in the numerical solution. This simplification is implemented and tested in a modification of the transient–state model.

6.4.2 Development of a Pseudo–Steady–State Model

The steady–state assumption used in the development of the ping–pong model may also be incorporated into the transient–state model since the reaction rate constants for the formation of Compounds I and II are several magnitudes higher than the rate constants for other segments of the overall mechanism. This difference in magnitudes suggests that the enzyme will respond very quickly to changes in reaction conditions with respect to other variables. Therefore, Compounds I and II formation is extremely fast with respect to all other enzyme forms thus allowing a steady–state approximation for the formation of Compounds I and II at any instant in time. This steady–state approximation in the unsteady-state is referred to as a "pseudo–steady–state" assumption.

The following assumptions are made in the development of the pseudo-steady-state model:

(i) The amount of enzyme inactivated is directly proportional to the amount of aromatic substrate removed from solution (i.e. K_s = constant).

(ii) The catalase reaction and its corresponding peroxide consumption are insignificant with respect to other reaction mechanisms when an aromatic substrate is present.

(iii) Compound II formation occurs only through the reaction of Compound I with an aromatic molecule.

(iv) A pseudo–steady state exists with respect to the formation of Compounds I and II.

(v) Aromatic substrate and peroxide are consumed with a one–to–one stoichiometry.

Therefore, the system to be modelled includes:

$$E + H_2O_2 \xrightarrow{k_1} E_i + H_2O \qquad (6\text{–}1)$$

$$E_i + AH_2 \xrightarrow{k_8} E_{ii} + \cdot AH \qquad (6\text{–}2)$$

$$E_{ii} + AH_2 \xrightarrow{k_9} E + \cdot AH + H_2O \qquad (6\text{–}3)$$

$$E_{ii} + H_2O_2 \xrightarrow{k_{app}} E_{iii} + H_2O \qquad (6\text{–}14)$$

$$E_{iii} \xrightarrow{k_7} E + O_2^- \qquad (6\text{–}12)$$

$$HRP + K_s\, AH_2 \longrightarrow E_{inact} + \text{Products} \qquad (6\text{–}15)$$

The differential equations describing this system of reactions are:

$$\frac{dE_i}{dt} = k_1\, E\, [H_2O_2] - k_8\, E_i\, [AH_2] \qquad (6\text{–}23)$$

$$\frac{dE_{ii}}{dt} = k_8\, E_i\, [AH_2] - k_9\, E_{ii}\, [AH_2] - k_{app}\, E_{ii}\, [H_2O_2] \qquad (6\text{–}24)$$

$$\frac{dE_{iii}}{dt} = k_{app}\, E_{ii}\, [H_2O_2] - k_7\, E_{iii} \qquad (6\text{–}25)$$

$$\frac{d[H_2O_2]}{dt} = -\, k_1\, E\, [H_2O_2] - k_{app}\, E_{ii}\, [H_2O_2] \qquad (6\text{–}26)$$

Equation 6–26 may be simplified assuming that very little peroxide is consumed in the formation of Compound III with respect to consumption in the catalytic cycle (i.e. $k_1\, E\, [H_2O_2] >> k_{app}\, E_{ii}\, [H_2O_2]$). Therefore, Equation 6–26 becomes:

$$\frac{d[H_2O_2]}{dt} \approx -\, k_1\, E\, [H_2O_2] \qquad (6\text{–}27)$$

Assuming a one–to–one stoichiometry, the aromatic substrate concentration may be calculated according to:

$$[AH_2] = [AH_2]_0 - ([H_2O_2]_0 - [H_2O_2]) \qquad (6\text{–}28)$$

and the concentration of inactive enzyme is:

$$E_{inact} = \frac{1}{K_s} ([AH_2]_0 - [AH_2]) \qquad (6\text{–}21)$$

A material balance on the total enzyme in solution produces:

$$E = E_0 - E_i - E_{ii} - E_{iii} - E_{inact} \qquad (6\text{–}29)$$

Since the formation of Compounds I and II are very rapid, they are effectively at steady-state with respect to other variables;

$$\frac{dE_i}{dt} \approx 0 \text{ and } \frac{dE_{ii}}{dt} \approx 0$$

Therefore, Equation 6–23 can be solved for Compound I production in the steady-state:

$$E_i = \frac{k_1}{k_8} \frac{[H_2O_2]}{[AH_2]} E \qquad (6\text{–}30)$$

Setting Equation 6–24 equal to zero, simplifying with Equation 6–30 and solving for Compound II concentration gives:

$$E_{ii} = \frac{k_1[H_2O_2]\ E}{k_9[AH_2] + k_{app}[H_2O_2]} \qquad (6\text{–}31)$$

Substitution of Equations 6–30 and 6–31 into the enzyme material balance and solving for the native enzyme concentration gives:

$$E = \frac{E_0 - E_{iii} - E_{inact}}{\left[1 + \frac{k_1[H_2O_2]}{k_8[AH_2]} + \frac{k_1[H_2O_2]}{k_9[AH_2] + k_{app}[H_2O_2]}\right]} \qquad (6\text{–}32)$$

Equation 6–32 may be substituted into Equation 6–27 resulting in an expression for the rate of disappearance of peroxide as a function of initial peroxidase dose and enzyme concentration in Compound III form:

$$\frac{d[H_2O_2]}{dt} = \frac{-(E_0 - E_{iii} - E_{inact})}{\left[\frac{1}{k_1[H_2O_2]} + \frac{1}{k_8[AH_2]} + \frac{1}{k_9[AH_2] + k_{app}[H_2O_2]}\right]} \qquad (6\text{–}33)$$

Similarly, substitution of Equations 6–31 and 6–32 into Equation 6–25 gives the rate of formation of Compound III according to:

$$\frac{dE_{iii}}{dt} = \frac{k_{app}\ (E_0 - E_{iii} - E_{inact})[H_2O_2]}{1 + (k_9[AH_2] + k_{app}[H_2O_2])\left[\ \frac{1}{k_1[H_2O_2]} + \frac{1}{k_8[AH_2]}\ \right]} - k_7 E_{iii} \quad (6\text{–}34)$$

Equations 6–33 and 6–34 may be simplified by noting that the rate of reaction of Compound II with aromatic substrate is much greater than the rate of reaction with peroxide to form Compound III (i.e. $k_9[AH_2] >> k_{app}[H_2O_2]$). In addition, the rate of disappearance of aromatic compound is directly proportional to the rate of disappearance of peroxide. Therefore, Equation 6–33 becomes:

$$\frac{d[AH_2]}{dt} = \frac{-\ (E_0 - E_{iii} - E_{inact})}{\left[\ \frac{1}{k_1[H_2O_2]} + \left(\frac{k_8 + k_9}{k_8\ k_9}\right)\frac{1}{[AH_2]}\ \right]} \quad (6\text{–}35)$$

which is of the same form as Equation 6–8 of the steady–state model except that the numerator is modified to include inactive enzyme forms. Similarly, Equation 6–34 becomes:

$$\frac{dE_{iii}}{dt} = \frac{k_{app}\ (E_0 - E_{iii} - E_{inact})}{\left[\ 1 + \frac{k_9}{k_8} + \frac{k_9}{k_1}\frac{[AH_2]}{[H_2O_2]}\ \right]}[H_2O_2] - k_7 E_{iii} \quad (6\text{–}36)$$

Simplification of the transient–state equations has reduced the model to a system consisting of only two differential equations (Equations 6–35 and 6–36) and two material–balance equations (Equations 6–21 and 6–28) which must be solved simultaneously using six constants (k_1, k_7, k_8, k_9, k_{app} and K_s).

6.4.3 Verification of the Pseudo–Steady–State Model

The pseudo–steady–state model was tested for its ability to replicate the predictive capabilities of the unsteady–state model and match experimental data collected under a variety of conditions. The kinetic data corresponding to the removal of both phenol and 4–chlorophenol as a function of time under a variety of aromatic compound, hydrogen peroxide and peroxidase concentrations are presented in Appendix E.

Comparisons of output from the unsteady–state and pseudo–steady–state models are contained in Figures 6–9 and 6–10. Figure 6–9 indicates that the simplifications made in the development of the pseudo–steady–state model produce very little deviation in the simulation of the horseradish peroxidase system. Figure 6–10 demonstrates that making a steady–state assumption and neglecting the formation of P–670 and intermediate $E_i \cdot H_2O_2$ complex results in an insignificant effect on the predicted distribution of enzyme forms in the reaction mixture. Thus, the pseudo–steady–state model demonstrates the advantages of reduced complexity and computation time without introducing significant error into the model's solution.

The accuracy of the pseudo–steady–state model was validated under a variety of experimental conditions. The number of catalytic turnovers (K_s) for 4–chlorophenol at pH 7.0 and 25°C was found to be 9 000. Figure 6–11 compares model output with the experimental data generated using various HRP doses and fixed 4–chlorophenol and peroxide concentrations. As with the unsteady–state model, the pseudo–steady–state model demonstrates excellent agreement with these experimental data. Figure 6–12 depicts the model application to batch reactions in which the amount of 4–chlorophenol in the reaction mixture is varied but peroxidase and peroxide concentrations are fixed. There is excellent agreement over a wide range of 4–chlorophenol concentrations. Figure 6–13 compares model and

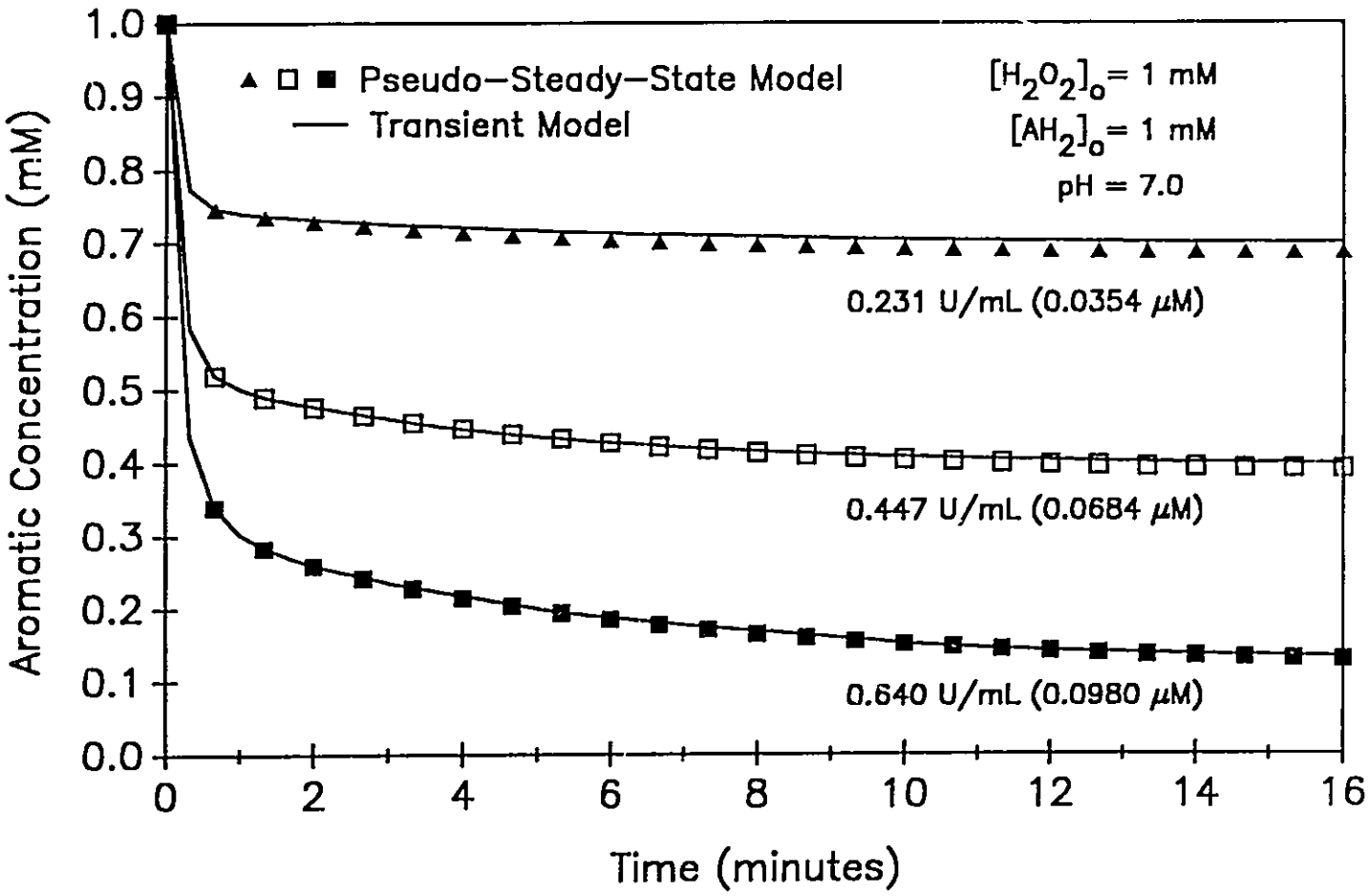


Figure 6–9: Comparison of Pseudo–Steady State Model Output with Transient Model Results Using 4–Chlorophenol Rate Constants

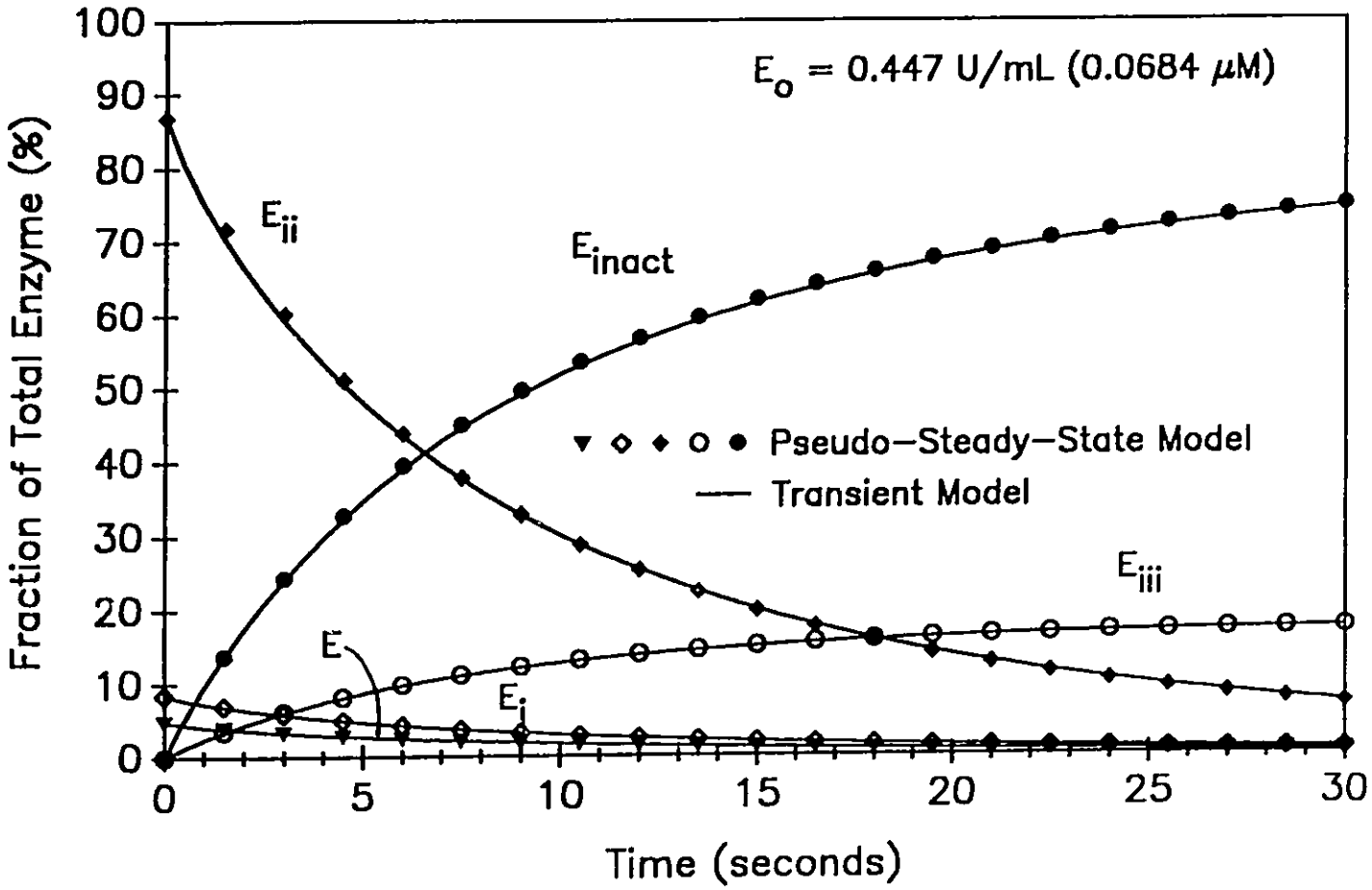


Figure 6–10: Comparison of Distribution of Enzyme Forms with Time Using the Pseudo–Steady State Model and the Transient Model (1 mM H_2O_2, 1 mM 4–Chlorophenol, pH 7.0, 25°C)

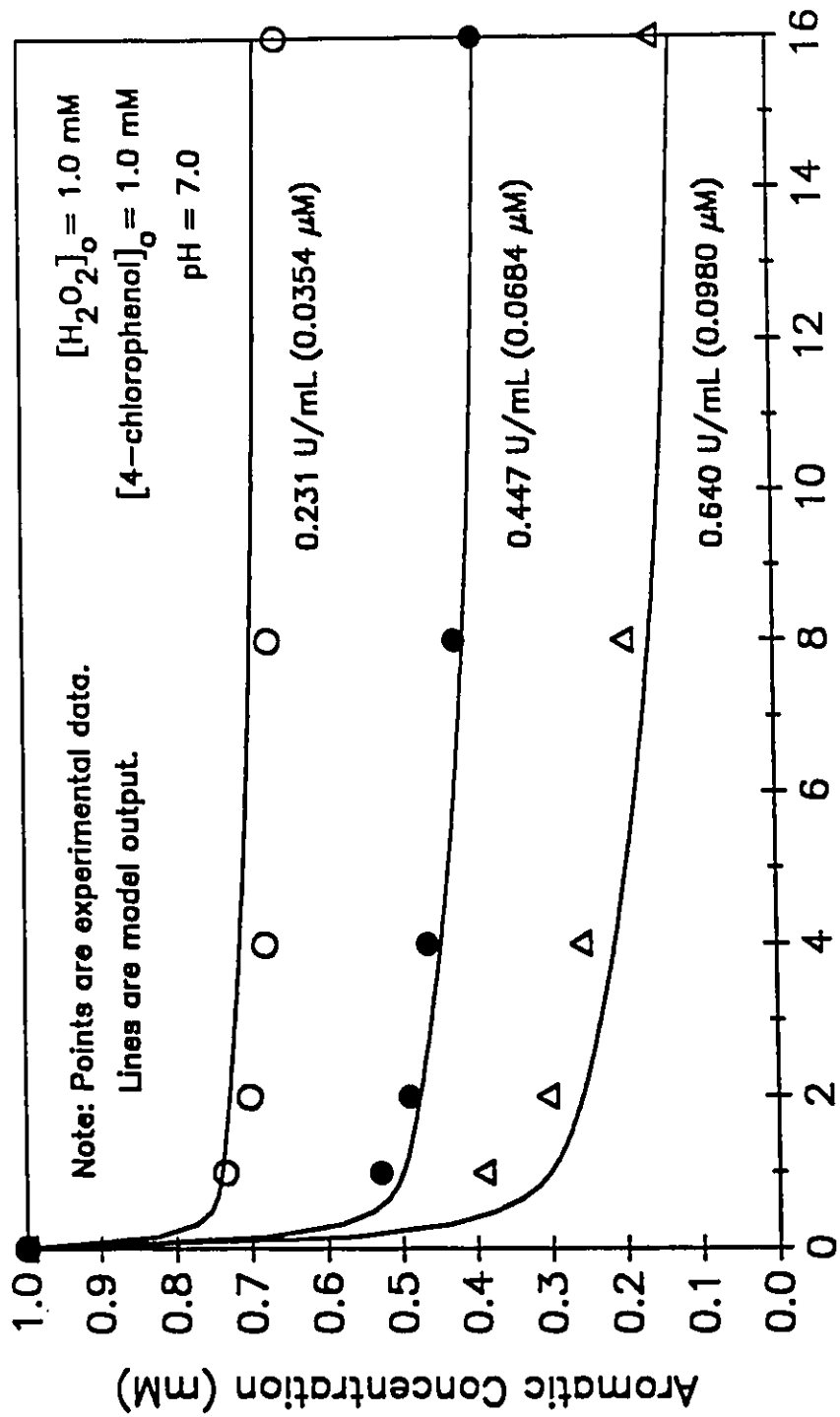


Figure 6–11: Comparison of Pseudo–Steady State Model Output with Experimental Data for Batch Reactions of 4–Chlorophenol With Varied HRP Doses (pH 7.0, 25°C)

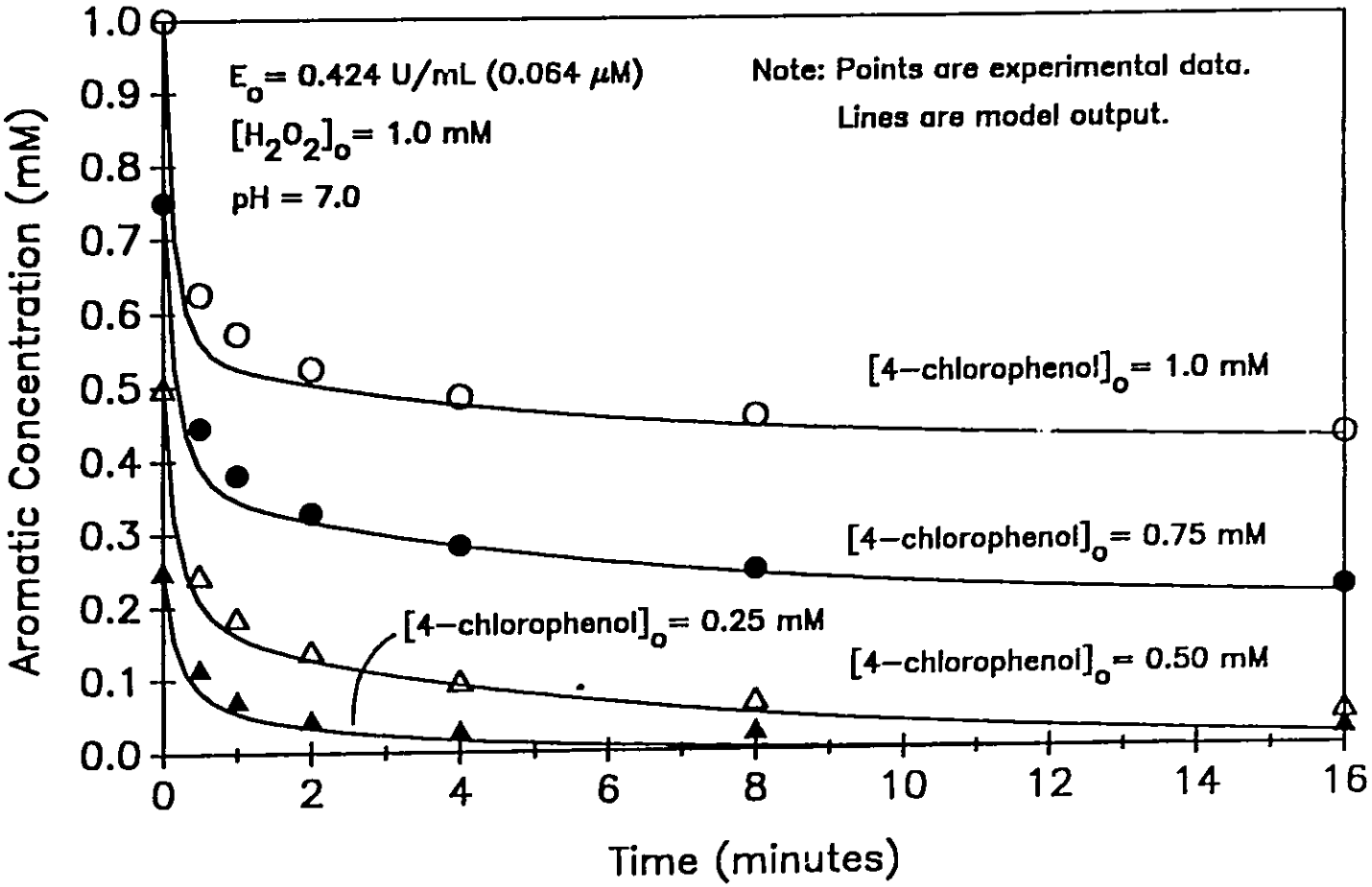


Figure 6–12: Comparison of Pseudo–Steady State Model Output With Experimental Data for Batch Reactions With Varied Initial 4–Chlorophenol Concentrations (pH 7.0, 25°C)

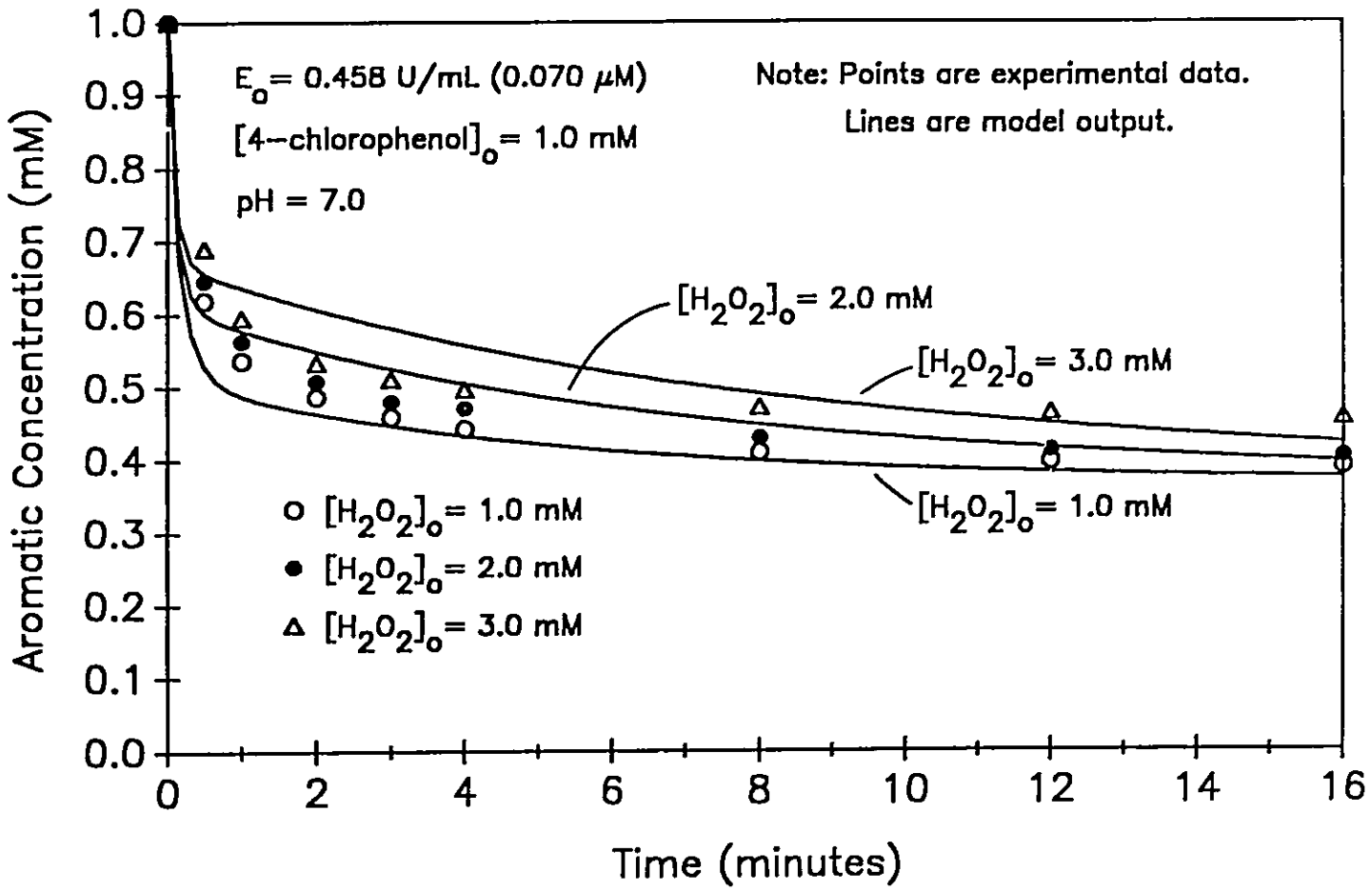


Figure 6–13: Comparison of Pseudo–Steady State Model Output With Experimental Data for Batch Reactions of 4–Chlorophenol With Varied Initial Peroxide Concentrations (pH 7.0, 25°C)

experimental results when the amount of peroxide is varied and 4–chlorophenol and peroxidase are held constant. This figure shows the greatest discrepancy between model and experimental results but the curve trends are consistent and the two never deviate more than 8%.

Complications arise with the application of the pseudo–steady–state model to the reaction of phenol in a batch reactor. These complications result from simplifications made in the development of the model. For example, the degree of clearance of phenol, unlike 4–chlorophenol, was not directly proportional to peroxide dose as was shown in Figure 5–6. However, the model assumes that aromatic compound precipitates from solution at a rate which is proportional to the rate of peroxide consumption. In addition, the number of catalytic turnovers achieved by HRP in the removal of phenol was found to be a function of phenol and peroxidase concentrations. The model was developed with the assumption that catalytic turnovers are constant and independent of aromatic substrate and peroxidase concentrations. The effect of these assumptions was tested by applying the pseudo–steady–state model to the removal of phenol from solution in a batch reactor under the same conditions as modelled for 4–chlorophenol. The results are shown in Figures 6–14, 6–15 and 6–16. The value of K_s used in the model runs was not fixed as it was for 4–chlorophenol. K_s was changed in each model run based on the turnovers achieved under each set of conditions once the reaction had gone to completion. These catalytic turnovers varied between 4900 and 6600 with the lowest turnovers achieved while at highest enzyme concentration and lowest phenol concentration. Turnovers were constant under varying peroxide concentrations when HRP and phenol concentrations were fixed.

In Figures 6–14 to 6–16, substantially higher deviations were noted between model and experimental data than encountered in the case of the 4–chlorophenol system. In general, the model overpredicted initial reaction rates and the degree of

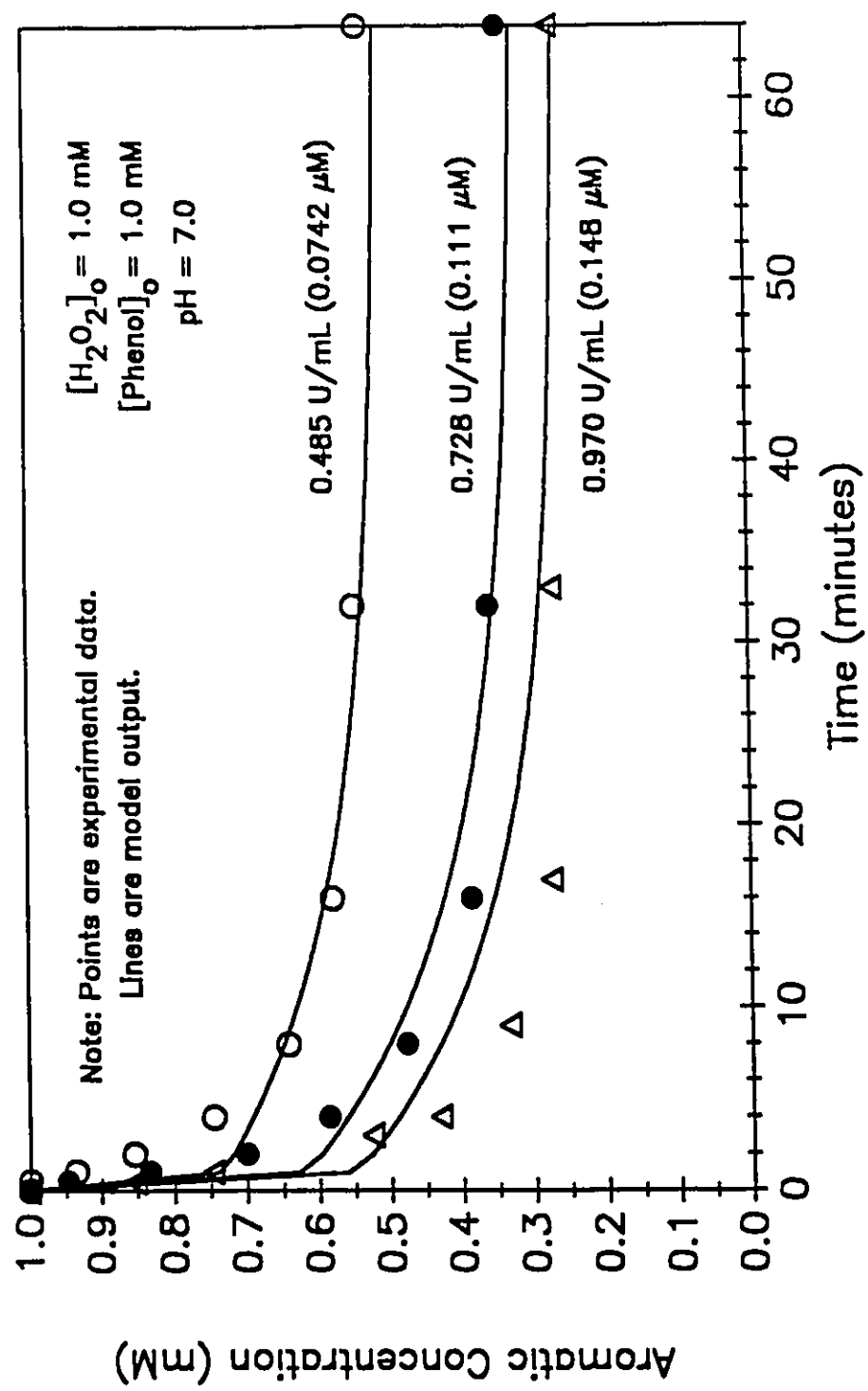


Figure 6–14: Comparison of Pseudo–Steady State Model Output with Experimental Data for Batch Reactions of Phenol With Varied HRP Doses (pH 7.0, 25°C)

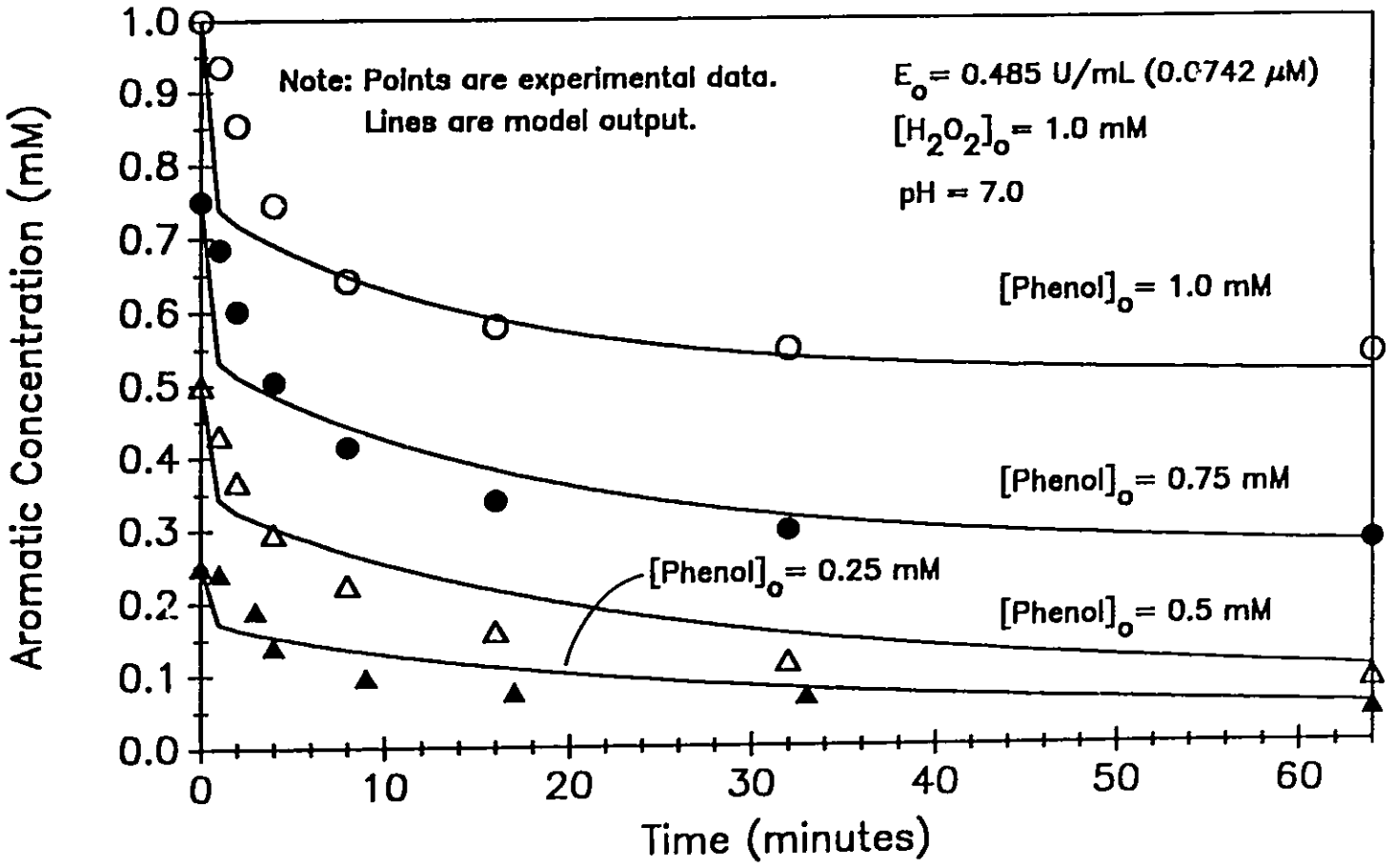


Figure 6–15: Comparison of Pseudo–Steady State Model Output With Experimental Data for Batch Reactions With Varied Initial Phenol Concentrations (pH 7.0, 25°C)

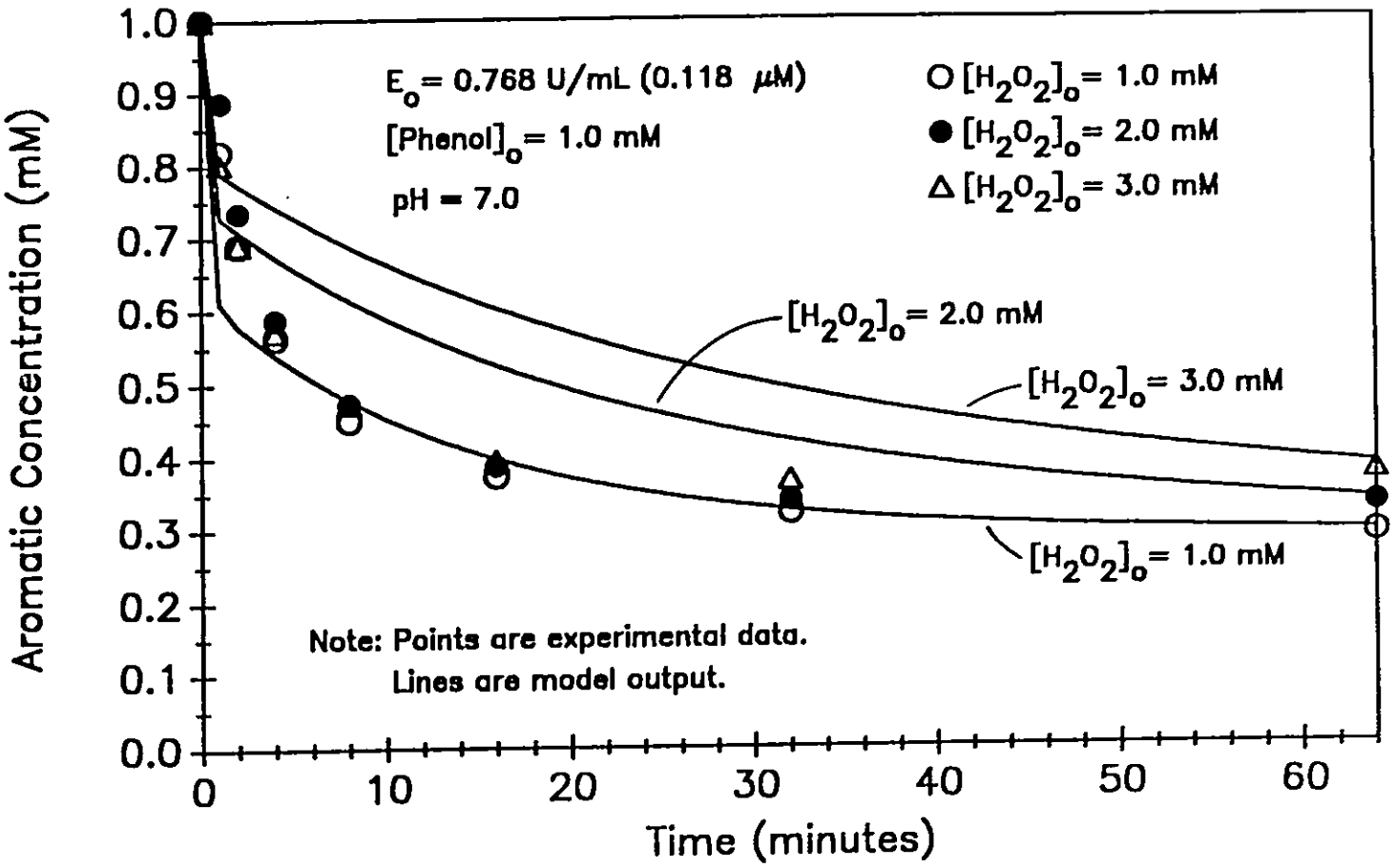


Figure 6–16: Comparison of Pseudo–Steady State Model Output With Experimental Data for Batch Reactions of Phenol With Varied Initial Peroxide Concentrations (pH 7.0, 25°C)

Compound III formation. Since batch experiments demonstrated that HRP inactivation was greater at higher enzyme concentrations, it is likely that the degree of inactivation in the initial stage of the reaction (when HRP concentration is highest) is underestimated resulting in a corresponding overestimation of the reaction rate. In addition, the model assumes that the amount of phenol removed from solution is proportional to the amount of peroxide consumed. However, the stoichiometry tests have shown that removal does not begin until a soluble product has been formed first; hence, the actual initial removal rate should be slower than the predicted rate.

The degree of Compound III formation was overpredicted by the model for both 4–chlorophenol and phenol as seen in Figures 6–13 and 6–16, respectively. This overprediction is indicated by the large effects that peroxide concentration has on model output with respect to the actual effects observed in experimental data. It was expected that the formation of Compound III would be much greater in the case of phenol than 4–chlorophenol due to much slower reaction rate constants for phenol. These lower constants should create a greater opportunity for Compound III formation over the time course of the reaction. However, very little Compound III was formed as seen in Figure 6–16 where removal rates are virtually independent of peroxide concentration. This discrepancy can be explained by the protection of enzyme from Compound III formation through the formation of intermediate enzyme forms which make the enzyme unavailable for inactivation. For example, many enzymes react according to:

$$E + A \underset{}{\overset{k_a}{\rightleftharpoons}} EA \xrightarrow{k_b} E + P \qquad (6\text{–}37)$$

where E is the enzyme, A is a reactant, P is a product and EA is an enzyme-

substrate complex (Cornish–Bowden and Wharton, 1988). This reaction scheme is used in the development of the standard Michaelis–Menten rate equation. If the Compound I and II forms of horseradish peroxidase react in a similar manner, then much of the enzyme may be bound in a form similar to EA shown above. If the second step of Reaction 6–37 is rate limiting (i.e. $k_b << k_a$) then at any instant a significant portion of the enzyme may be held in the enzyme–substrate complex resulting in significant protection of the enzyme from inactivation. Therefore, if the hypothetical rate constant k_b for phenol is much smaller than the corresponding value for 4–chlorophenol, phenol would have a slower reaction rate but greater protection from Compound III formation due to a build–up of more enzyme-substrate complex. This theory is consistent with the data collected in this investigation but cannot be verified until the existence of an enzyme–substrate complex is demonstrated and rate constants are evaluated.

The accuracy of the pseudo–steady–state model is only as good as the quality of the measurements of the constants that define the system. The relatively small deviation between model results and experimental data for 4–chlorophenol is surprising since only one constant (K_s) of the six used in this model was actually measured in this laboratory. The remaining five constants (k_1, k_7, k_8, k_9 and k_{app}) were measured by other authors under conditions which were not completely identical to the those used in this investigation. Model accuracy can be improved by performing all rate constant measurements using the same enzyme stock and under fixed conditions of pH, temperature and buffer concentration.

A sensitivity analysis of the pseudo–steady–state model has been performed and is summarized in Appendix F. This analysis has shown that the model is insensitive to changes in k_1 and k_8 because these rate constants are not the rate limiting constants in the catalytic cycle. The degree of Compound III formation and its effect on the time at which the reaction goes to completion is very sensitive

to changes in k_7 and k_{app}. The value of k_9 is critical since it is the rate limiting constant in the catalytic cycle. It defines the overall rate of reaction and the amount of Compound II available for conversion to Compound III. The model is extremely sensitive to changes in K_s because it defines the degree of removal that can be achieved by a given dose of enzyme.

The major deficiency of the pseudo–steady–state model is the assumption that the number of turnovers (K_s) of horseradish peroxidase with respect to a particular aromatic compound is fixed. This is not always true as was demonstrated by batch and semi–batch experiments with phenol showing that the number of catalytic turnovers is a function of horseradish peroxidase and phenol concentrations. Since the model is very sensitive to changes in K_s, an expression must be developed which will define the function

$$K_s = f([HRP], [AH_2])$$

for all aromatic substrates. Since inactivation is likely to occur as a result of free radical inactivation, expressions must be developed which describe the formation and polymerization of free radicals in the reaction mixture. In addition, rate constants must be measured which describe the inactivation of HRP by these free radicals.

Additional improvements which can be made in the model include incorporation of the formation of intermediate enzyme–substrate complexes and a mechanistic explanation for the observed one–to–one stoichiometry of reaction between peroxide and aromatic substrates. These additions to the model should improve its accuracy; however, the complexity of the model would increase substantially. For example, these additions would require a more than doubling of the number of reaction rate constants incorporated into the model. Also, due to the

complex nature of the reaction and the many sources of enzyme inactivation, it is doubtful that the accuracy of the model can be extended to wastewaters in which there are a multitude of contaminants. Therefore, it is suggested that the model in its present form should be used as a means for describing and interpreting the general trends of the enzyme catalyzed reaction.

7. CONTINUOUS FLOW REACTOR DEVELOPMENT

Several reactor configurations can be used for the enzymatic treatment of wastewater including batch, semi–batch, continuous stirred tank and tubular reactors. These basic reactor types are pictured in Figure 7–1. The choice of the optimal reactor configuration is based on the information collected from the batch reactor studies and kinetic modelling. Ultimately, the choice of the reactor is made on the basis of cost, efficiency and reaction requirements.

7.1 Reactor Options

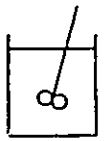
Batch reactors are used for small scale operations, for testing new processes that have not been fully developed, for the manufacture of expensive products and for processes that are difficult to implement in a continuous flow configuration (Fogler, 1986). In a batch reactor, there is neither inflow nor outflow of reactants or products. The composition of the mixture is uniform throughout the reactor but changes with time. The batch reactor has the advantage of high conversions that can be obtained by leaving the reactants in the reactor for a sufficient period of time. It has the disadvantages of high labour costs per unit production and difficult large–scale implementation.

A semi–batch reactor is a batch system in which one or more of the reactants is added continuously to a reaction mixture of spatially uniform composition. The composition of the mixture changes with time. Although a semi–batch reactor has the same disadvantages as the batch reactor, it has the capability to minimize unwanted side reactions through the maintenance of a low concentration of one or more of the reactants.

In an ideal continuous stirred tank reactor (CSTR) there are no spatial nor temporal variations in concentration or reaction rate in the vessel. Since the

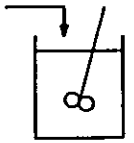
BATCH REACTOR

- uniform composition in reactor
- composition changes with time i.e. C = C(t)



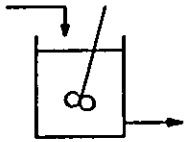
SEMI-BATCH REACTOR (SBR)

- uniform composition in reactor
- C = C(t)
- reaction rate controlled by continuous addition of one of the reactants



CONTINUOUS STIRRED TANK REACTOR (CSTR)

- uniform composition in reactor
- steady-state reactor i.e. C ≠ C(t)
- effluent has same composition as reactor contents



PLUG FLOW REACTOR (PFR)

- composition changes progressively along reactor length
- perfect radial mixing

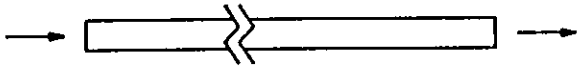


Figure 7–1: Basic Reactor Configurations

reaction mixture is homogeneous, the exit stream has the same concentration as the reactor contents. Generally, CSTR's are used when intense mixing is required. A CSTR has the advantage of a being a steady–state reactor with simple operation and control of process variables (temperature, pH, reactant and catalyst concentrations). It has the disadvantage that the conversion per unit volume of reactor is the smallest of the flow reactors (Fogler, 1986). Consequently, large reactors are usually required to achieve high conversions.

In a tubular, or plug–flow reactor (PFR), the contents react along the length of the reaction vessel but the composition and reaction rate are fixed at any given point in the reactor. The velocity and composition profiles are flat at any radial cross section. The tubular reactor is relatively easy to maintain because it has no moving parts and it usually produces the highest conversion per reactor volume of any of the flow reactors (Fogler, 1986). Control of conditions within the reactor is difficult since reaction rate and composition change along the length of the reactor.

7.2 Choice of Reactor

The choice of a suitable reactor configuration may be made based on the following criteria: reasonable cost, ability to scale–up, efficient use of enzyme, high enzyme loading per unit volume, and good catalyst stability (Pitcher, 1979). Batch and semi–batch reactor studies have demonstrated that enzyme lifetime can be extended by maintaining a low HRP concentration in the reaction mixture at any instant. In addition, kinetic modelling and batch experiments have shown that Compound III formation is significant and can be minimized by maintaining a low concentration of hydrogen peroxide in the mixture. These techniques for improving enzyme efficiency should be incorporated into the reactor design.

Batch reactors were used in this study to evaluate the effects of process conditions on the catalytic efficiency of horseradish peroxidase. While good removal

of aromatic pollutants was achieved in a batch reactor, it is unrealistic to implement a batch waste treatment system due to high operating costs. Similarly, the use of semi-batch reactors is limited even though in this type of reactor the enzyme's catalytic lifetime can be extended by controlling the instantaneous concentration of enzyme in the reaction mixture. Instead, the waste treatment must be implemented in a continuous flow mode.

Howaldt *et al.* (1983) maintain that for substrate–inhibited reactions, a CSTR generally requires less enzyme than a tubular reactor. Also, when product inhibition occurs, the PFR always out–performs the CSTR. In the HRP system substrate–inhibition appears in the form of Compound III production at high peroxide concentrations. In addition, product inhibition is present in the form of free radical inactivation of horseradish peroxidase.

Under ideal conditions, a tubular flow reactor has no mixing in the direction of flow and complete mixing perpendicular to the direction of flow. As the flow passes along the length of the reactor tube, the reactants combine and are depleted. Under ideal conditions, the composition of the contents of a batch reactor and effluent from a PFR would be the same provided initial conditions and retention time are identical (Levenspiel, 1972). Therefore, the PFR is expected to produce the same results as the batch reactor provided ideal conditions are maintained. In the PFR pictured in Figure 7–2(a), the highest concentration of the enzyme occurs at the inlet where the reactants are mixed and reaction rate is greatest. However, it is at this point that the enzyme will be inactivated at the highest rate due to the high enzyme concentration. As was demonstrated in the semi–batch reactor study, the enzyme lifetime can be extended and costs associated with enzyme consumption can be minimized by maintaining a low instantaneous concentration of enzyme. This is demonstrated in Table 7–1 for phenol removal in batch and semi–batch reactors at neutral pH and fixed HRP dose. A low HRP concentration can be

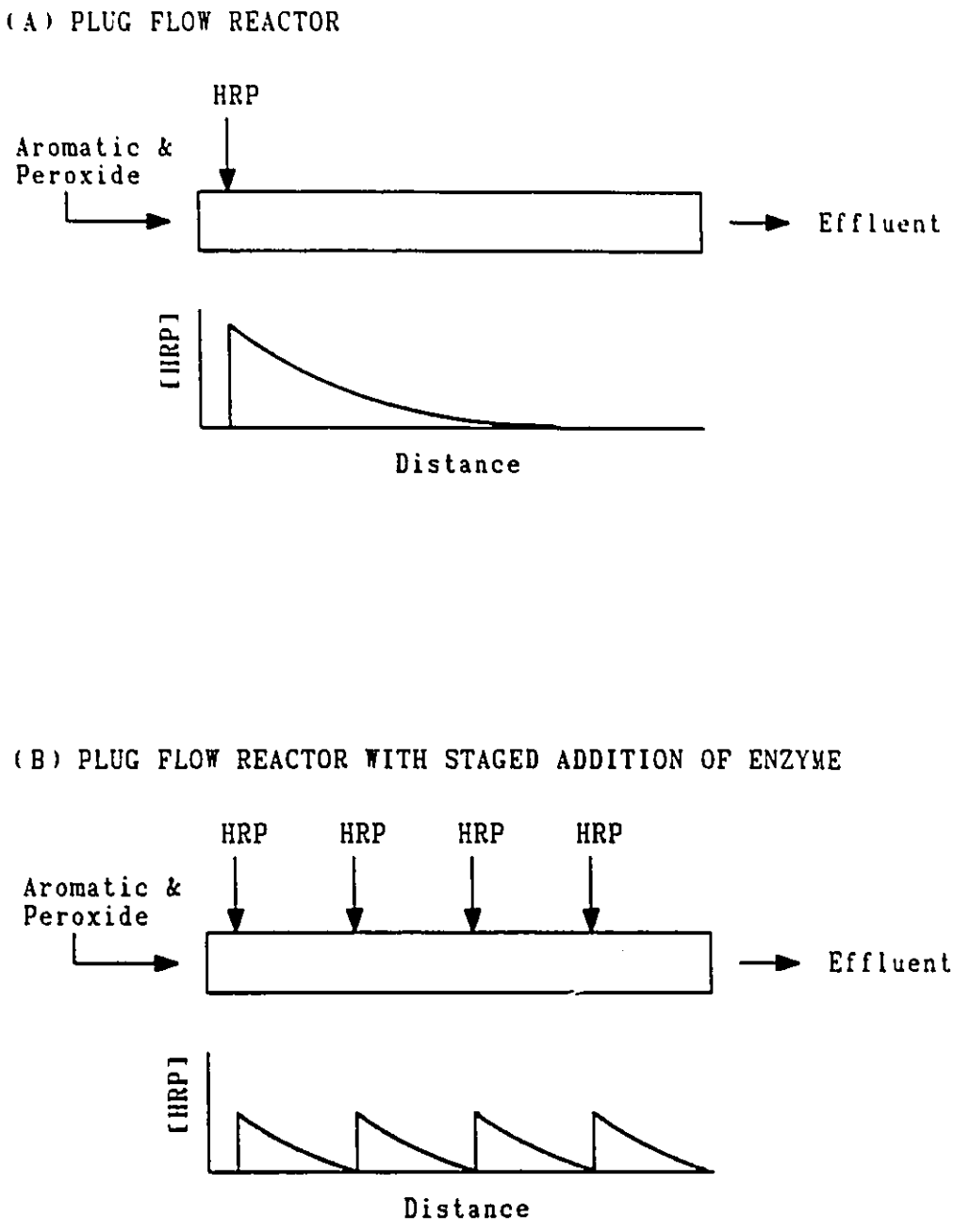


Figure 7–2: Single and Staged Addition of Enzyme to a Plug Flow Reactor

Table 7–1: Catalytic Turnovers for Phenol in Batch and Semi–Batch Reactors

Reactor Type	HRP Addition Period	Catalytic Turnovers	Turnovers Normalized w.r.t. Batch Reactor
Batch	Spike	8 400	–
Semi–Batch	1 hour	11 500	1.4
	3 hours	13 700	1.6
	4 hours	17 800	2.1
	20 hours	21 000	2.5

$[\text{Phenol}]_0 = 1.0$ mM $\quad [H_2O_2]_0 = 1.0$ mM $\quad [\text{HRP}]_0 = 0.30$ U/mL

pH = 7.0 $\quad$ T = 25°C

maintained in the PFR through staged addition of the enzyme to the reactor as shown in Figure 7–2(b). The PFR should begin to approach the efficiency of the semi–batch reactor as the number of injection points along the length of the reactor increases. However, the injection of enzyme in a staged manner also implies that the overall conversion achieved for a given reactor volume will decrease with a greater number of injection points since the enzyme concentration and, hence, the reaction rate at any point in the reactor is reduced. The design of a PFR system would involve the optimization of the number of injection points and the reactor volume required to achieve a required level of treatment.

The CSTR reactor configuration has the advantage of being a steady–state reactor in which the contents are homogeneous. Upon entering the reactor, the concentration of the influent is immediately reduced to the steady–state level dictated by the volume of the reactor, provided complete mixing is achieved. Therefore, enzyme and peroxide concentrations are maintained at low levels, thus minimizing free radical inactivation at high catalyst concentrations and the degree of formation of Compound III. Reactant concentrations can be maintained at the desired level by controlling the flowrate of the streams entering the reactor. High conversion can still be achieved by providing sufficient reactor volume. However, it was shown in Figure 5–4 that lower turnovers result when the aromatic compound concentration is low. Therefore, the gains associated with an immediate reduction in HRP and peroxide concentrations must be balanced with the losses associated with a reduced aromatic compound concentration in the CSTR. Some of this problem can be offset by operating a series of CSTR's in which the effluent from one reactor is fed to a subsequent reactor. Enzyme or peroxide can be added to the waste stream at either the entrance to the series of reactors or at each reactor stage. It should be noted that as the number of stages increases the multiple CSTR system begins to approximate the behavior of a PFR.

As in the design of a PFR system, the design of a CSTR system will involve optimization of the number of reactor stages and the reactor volume. It is likely that some improvement can be gained in system performance if the volume of each reactor in the system is optimized. However, Levenspiel (1972) indicates that the degree of improvement in reactor performance is usually quite small, only a few percent at most, so that overall economic considerations would nearly always recommend using equal–size reactors.

A kinetic model would assist in the selection of the best reactor configuration. However, such a model must incorporate the mechanisms and kinetics of enzyme inactivation. The approximations used in the development of the pseudo–steady state model are not applicable anywhere but in a batch reactor. Therefore, in the absence of a complete kinetic model, reactor systems must be tested to determine the optimal reactor configuration. Batch results and kinetic modelling have indicated that a CSTR configuration has several significant advantages. Therefore, reactor systems consisting of a single CSTR and multiple CSTR's in series with injection of HRP at each stage have been examined. While a PFR with staged injection of HRP should have some value and warrants investigation, the CSTR system has the advantage of simpler operation, lower pressure drop and mechanically induced turbulence (complete mixing). Therefore, this investigation was limited to CSTR reactor configurations.

7.3 Development of a Bench–Scale CSTR System

The operating procedures for the single and multiple CSTR systems developed in this study are discussed in Appendix C. In the single CSTR system, pictured in Figure 3–4, a mixture of aromatic and peroxide were combined in the reaction vessel with a stream of horseradish peroxidase. Peroxide and HRP were not mixed prior to addition to the reactor due to the rapid inactivation of HRP in the presence of

peroxide alone. The reactor contents were stirred vigorously by a magnetic stirrer and stir bar. Effluent flowrate was the same as the influent flowrate. The reactor volume, enzyme flowrate, and synthetic wastewater flowrate were varied so that a large range of retention times could be studied.

A multiple reactor system consisting of four CSTR's in series was constructed as shown in Figure 7–3. It was operated in the same manner as the single CSTR except that the enzyme was added continuously at each reactor stage.

7.3.1 Performance of a Single CSTR

A continuous stirred tank reactor (CSTR) configuration was chosen for the application of the enzyme catalyzed polymerization process because reactant and enzyme concentrations are lowered immediately upon entering the reactor; therefore, inactivation of HRP is reduced automatically by the low steady-state concentration of HRP in the reacting mixture. In addition, peroxide concentration is maintained at a low level, reducing Compound III formation. High conversion can still be achieved by providing sufficient reactor volume. Results for the removal of phenol and 4–chlorophenol in a single CSTR at fixed HRP dose and various retention times are shown in Figure 7–4.

Figure 7–4 indicates that the number of catalytic turnovers and, hence, the degree of removal achieved by the enzyme increases with retention time in the reactor. The retention time (τ), or mean residence time, is obtained by dividing the reactor volume (V) by the volumetric flowrate (Q) entering the reactor:

$$\tau = \frac{V}{Q}$$

This is the time necessary to process one reactor volume of fluid based on entrance conditions (Fogler, 1986).

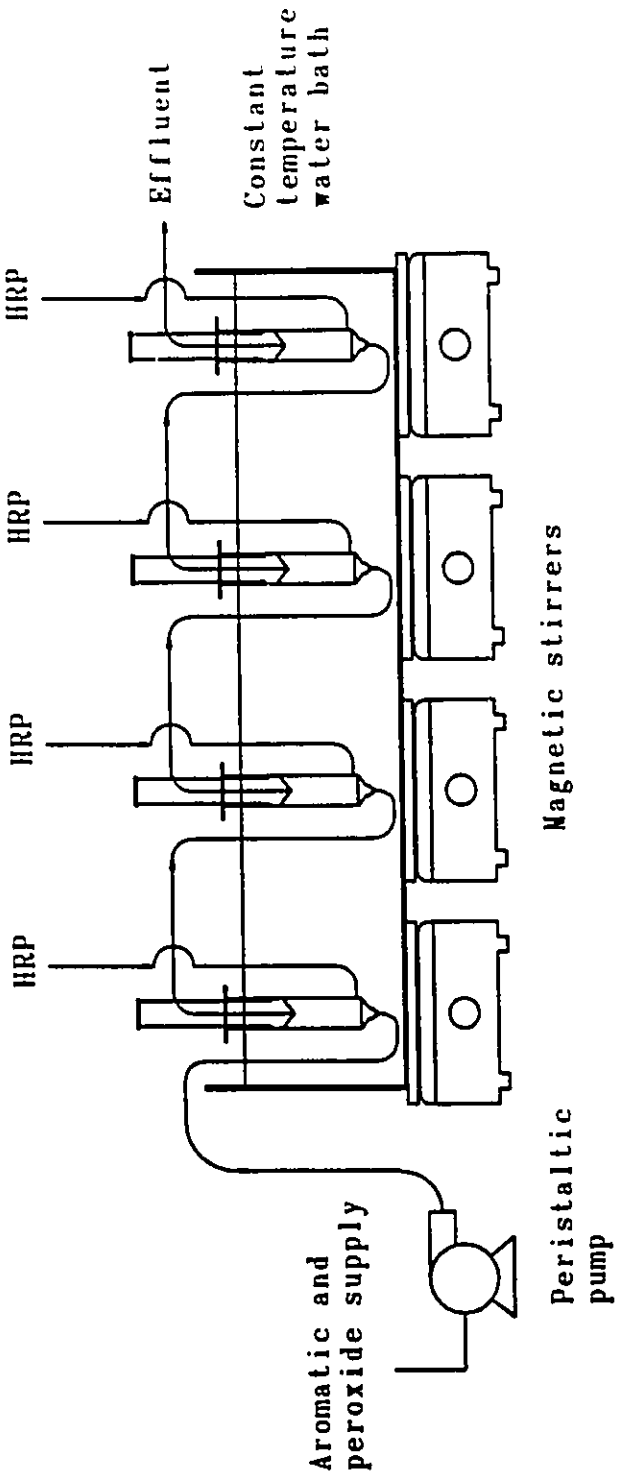


Figure 7–3: Experimental Setup for Multiple CSTR's in Series

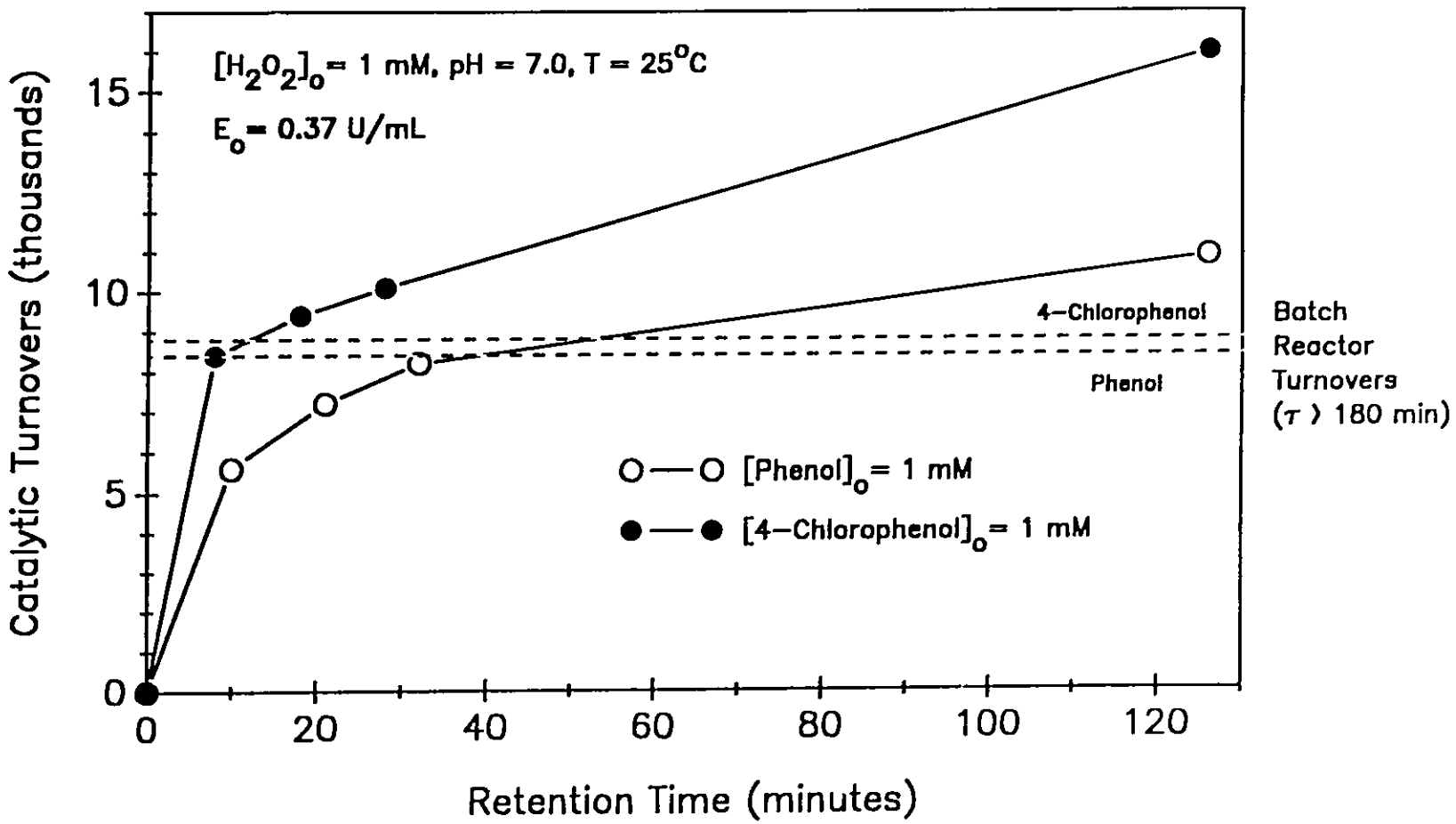


Figure 7–4: Catalytic Turnovers for Phenol and 4–Chlorophenol as a Function of Retention Time in a Single CSTR (pH 7.0, 25°C)

The initial rapid rate of change of turnovers with respect to retention time can be attributed to reactions which are mainly time limited. The gradual increase in catalytic turnovers at higher retention times results from decreased inactivation of the enzyme resulting from lower steady–state enzyme concentrations in the reactor. Under the stated conditions, the performance of the CSTR began to exceed that of a batch reactor (τ > 3 hours) with retention times of 35 and 8 minutes for phenol and 4–chlorophenol, respectively. Turnover of 4–chlorophenol improved significantly even with low retention times. Turnover of phenol increased but not to the degree demonstrated in the semi–batch reactor. This can be attributed to insufficient retention time and/or effects associated with increased inactivation at low aromatic concentrations. In a mixture of 1 mM aromatic and peroxide, the maximum number of turnovers that can be achieved by 0.37 U/mL of HRP is 17 600 at full removal of aromatic substrate. The 16 600 catalytic turnovers achieved for 4–chlorophenol with a 2 hour retention time approached this maximum indicating that substrate availability may have limited the turnovers. Therefore, it is expected that at lower enzyme concentrations the catalytic turnovers for 4–chlorophenol could be extended beyond 16 600.

Catalytic turnovers achieved in a CSTR were higher than those observed in batch reactors when sufficient reaction time was provided. The upward trend of the curves of Figure 7–4 indicates that increases in catalytic turnovers, beyond those observed here, can be achieved with longer retention times and lower enzyme concentrations.

7.3.2 Performance of Multiple CSTR's in Series

A reactor system in which CSTR's are placed in series with injection of HRP at each reactor stage has several advantages. First, as with the single CSTR system, enzyme and peroxide concentrations are reduced immediately upon injection

into each of the reactors. Secondly, unlike the single CSTR system, higher aromatic concentrations are maintained in the initial reactor stages. This should reduce enzyme inactivation arising from free radical inhibition. This hypothesis was tested by comparing reactor systems consisting of a single reactor and two, three and four reactors in series. The total volume of the systems and the flowrates of aromatic, peroxide and enzyme were constant. Total retention time was maintained at 1 hour. Each reactor in a series of CSTR's was of equal volume (total volume of 120 mL – i.e. 1 × 120 mL, 2 × 60 mL, 3 × 40 mL and 4 × 30 mL) with the horseradish peroxidase dose and flowrate divided equally among the reactors. The total flowrate of enzyme, peroxide and aromatic substrate provided to the reactor systems was 2 mL/min.

The staged CSTR systems were evaluated for the removal of phenol and 4–chlorophenol at fixed enzyme dose and inlet aromatic substrate and peroxide concentrations. Reacting mixtures were buffered at neutral pH and maintained at 25°C. A summary of the performance of the reactors in terms of effluent and intermediate reactor concentrations is located in Appendix G. Figures 7–5 and 7–6 compare the catalytic turnovers accomplished in the single and multiple CSTR systems for phenol and 4–chlorophenol respectively. Included in these figures are the turnovers obtained when removal of the aromatic is measured in the exit stream and when the still–reacting exit stream is allowed to react to completion as a batch. Tables 7–2 and 7–3 summarize the degree of removal achieved in each of the individual reactors and the reactor systems as a whole. Tables 7–4 and 7–5 show the cumulative catalytic turnovers achieved in the effluent from each reactor. All experiments were repeated to verify these trends. Results were reproducible within a range of ±1 to ±2.5% for all reactor runs. From these results, a number of observations can be made:

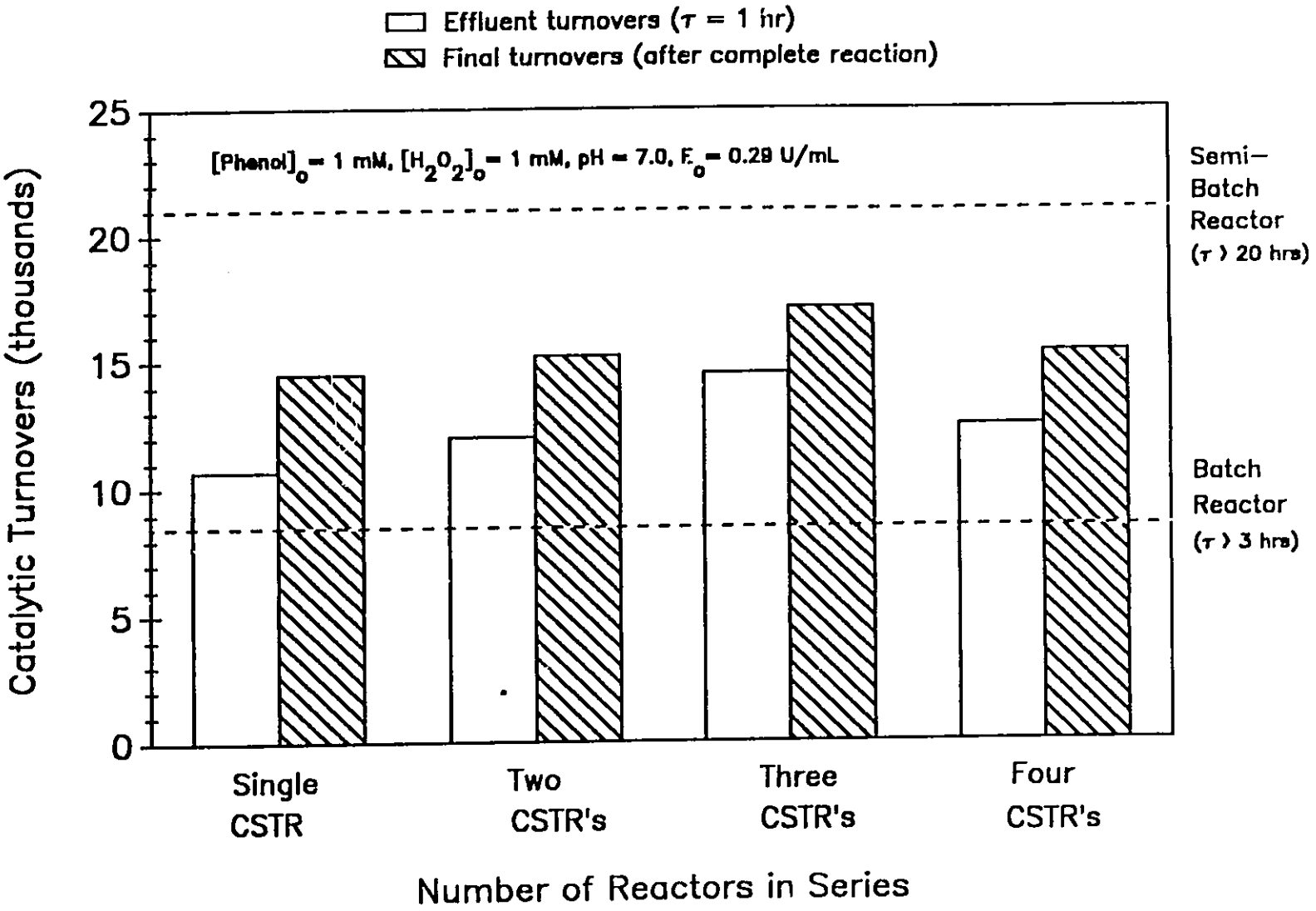


Figure 7–5: Catalytic Turnovers for Phenol in a Single CSTR and Multiple CSTR's in Series (pH 7.0, 25°C)

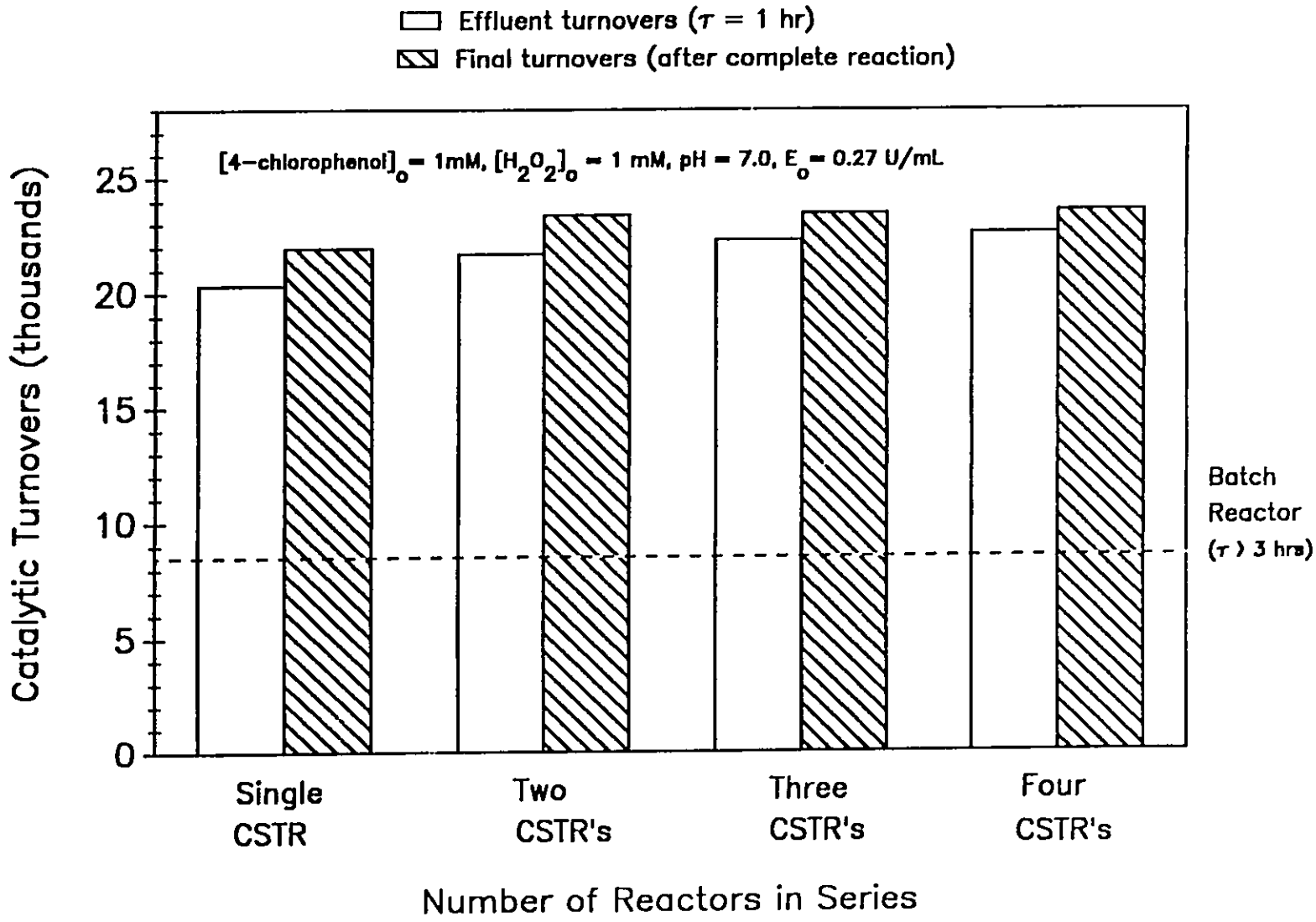


Figure 7–6: Catalytic Turnovers for 4–Chlorophenol in a Single CSTR and Multiple CSTR's in Series (pH 7.0, 25°C)

Table 7–2: Phenol Removal in Single and Multiple CSTR's in Series

No. of CSTR's in Series	Removal (%) Accomplished in Reactor				Total Removal (%)
	No. 1	No.2	No. 3	No.4	
Single CSTR	47	–	–	–	47
Two CSTR's	25	29	–	–	54
Three CSTR's	17	23	24	–	64
Four CSTR's	7	18	16	14	55

$[\text{Phenol}]_0 = 1.0$ mM $\quad [H_2O_2]_0 = 1.0$ mM $\quad [\text{HRP}]_0 = 0.29$ U/mL

pH = 7.0 $\quad T = 25^0$C $\quad \tau = 1$ hr (equal volume reactors in series)

Table 7–3: 4–Chlorophenol Removal in Single and Multiple CSTR's in Series

No. of CSTR's in Series	Removal (%) Accomplished in Reactor				Total Removal (%)
	No. 1	No.2	No. 3	No.4	
Single CSTR	83	–	–	–	83
Two CSTR's	56	33	–	–	89
Three CSTR's	38	36	18	–	92
Four CSTR's	29	30	23	11	93

$[4\text{–chlorophenol}]_0 = 1.0$ mM $\quad [H_2O_2]_0 = 1.0$ mM $\quad [HRP]_0 = 0.27$ U/mL

pH = 7.0 $\quad T = 25^0C \quad \tau = 1$ hr (equal volume reactors in series)

Table 7–4: Phenol Turnovers in Single and Multiple CSTR's in Series

No. of CSTR's in Series	Enzyme Dose per Reactor (U/mL)	Cumulative Turnovers Accomplished in Reactor			
		No. 1	No.2	No. 3	No.4
Single CSTR	0.290	10 700	–	–	–
Two CSTR's	0.145	11 300	12 000	–	–
Three CSTR's	0.097	11 500	13 500	14 500	–
Four CSTR's	0.072	6 700	11 300	12 400	12 500

$[\text{Phenol}]_0 = 1.0$ mM $\quad [H_2O_2]_0 = 1.0$ mM $\quad [\text{HRP}]_0 = 0.29$ U/mL

pH = 7.0 $\quad T = 25^{\circ}C \quad \tau = 1$ hr (equal volume reactors in series)

Table 7–5: 4–Chlorophenol Turnovers in Single and Multiple CSTR's in Series

No. of CSTR's in Series	Enzyme Dose per Reactor (U/mL)	Cumulative Turnovers Accomplished in Reactor			
		No. 1	No.2	No. 3	No.4
Single CSTR	0.270	20 400	–	–	–
Two CSTR's	0.134	27 300	21 700	–	–
Three CSTR's	0.089	27 800	27 200	22 300	–
Four CSTR's	0.067	28 300	28 700	26 700	22 600

$[4\text{–chlorophenol}]_0 = 1.0$ mM $[H_2O_2]_0 = 1.0$ mM $[HRP]_0 = 0.27$ U/mL

pH = 7.0 T = 25°C $\tau = 1$ hr (equal volume reactors in series)

(i) Single and multiple CSTR performances were superior to batch reactor performance even with a lower retention time. The catalytic turnovers for phenol approached those achieved in a semi–batch reactor with only a fraction of the retention time (1 hour in the CSTR versus 20 hours in the semi–batch reactor). For a 1 hour retention time, the turnovers achieved in a CSTR were better than those obtained in a semi–batch reactor.

(ii) Initially, the catalytic turnovers increased with the number of reactor stages. The turnovers of phenol reached a peak at three reactor stages and then began to decrease. The gains in catalytic lifetime obtained for 4–chlorophenol decreased with each additional reactor stage.

(iii) Figures 7–5 and 7–6 indicate that there was a greater difference between the catalytic turnovers in the exit stream and the final turnovers after complete reaction for phenol than for 4–chlorophenol. Therefore, the number of phenol turnovers were more limited by retention time than were 4–chlorophenol turnovers.

(iv) The maximum catalytic turnovers of phenol were 14 500 with a 1 hour retention time and 17 100 after complete reaction (using three reactor stages in series). The maximum catalytic turnovers of 4–chlorophenol were 22 600 with a 1 hour retention time and 23 600 after complete reaction (using four reactor stages in series).

The decrease in catalytic turnovers for phenol and the diminishing gains for 4–chlorophenol with higher number of reactor stages can be explained by one or both of the following reasons:

(i) As the number of reactor stages increases, the average exposure time of the enzyme to the aromatic decreases thus imposing a greater time limitation on the number of catalytic turnovers that can be achieved.

(ii) As the number of stages increases, the ratio of peroxide to enzyme concentration in each of the reactors increases. This increased availability of peroxide can result in a higher degree of Compound III formation than systems with fewer reactor stages.

As the number of reactor stages increase, the benefits associated with using a CSTR system are balanced by limiting processes such as those discussed above. These two possibilities can be tested and, perhaps, be overcome by increasing the retention time at each reactor stage and by supplying peroxide to each stage of the reactor system.

The total catalytic turnovers obtained for 4–chlorophenol approaches the maximum of 24 400 dictated by the total amount of substrate available (1 mM). Higher turnovers can be achieved using lower enzyme concentrations as shown in Table 7–5. This table demonstrates that for 4–chlorophenol the number of catalytic turnovers achieved in the first reactor increased to a maximum of 28 300 with decreasing enzyme concentration, despite the lower retention time. This verifies the importance of maintaining a low enzyme concentration in the reactor. Note that the cumulative catalytic turnovers in successive stages show a decreasing trend. In contrast, Table 7–4 shows that for phenol the cumulative turnovers achieved in successive stages increases. This difference can result from lower reaction rate constants for phenol than 4–chlorophenol (see Table 6–2). A slow reaction rate with respect to phenol causes a significant retention time limitation. For example, in the initial stages, the enzyme has insufficient time to react to its full turnover capability. The remaining active enzyme is transported to successive reactors, supplementing the turnovers accomplished in these later stages. The rapid reaction rate of 4–chlorophenol reduces the effects of retention time. The reduction in turnovers in subsequent stages results from successively lower concentrations of

aromatic resulting in increased enzyme inactivation. From these results, it is concluded that the optimal retention time and number of reactor stages in any reactor system depend on the characteristics (reaction rate constants) of the substrate.

7.3.3 Discussion

The single CSTR and multiple CSTR systems have demonstrated an ability to extend the catalytic lifetime of HRP significantly when sufficient retention time is provided. The catalytic turnovers achieved in these reactor configurations also approach the levels found in semi–batch reactors but at a much lower retention time. The higher turnovers achieved in the CSTR in comparison with batch and semi-batch reactors with equal retention times indicates, by extension, that the CSTR configuration should be superior to the plug flow reactor configuration. A plug flow reactor could, at best, be operated with the efficiency of a semi–batch reactor. A multiple CSTR configuration can be used to extend the lifetime of the enzyme beyond the single CSTR for the same retention time.

These results have demonstrated that a CSTR system is suitable for the application of enzyme catalyzed polymerization. The choice of the optimal configuration of a wastewater treatment system will depend on the economics of the process. The savings resulting from a decrease in enzyme consumption by using multiple reactors in series must be balanced against the difference in capital and operating costs between a single CSTR and a staged CSTR system. In addition, the number of stages chosen will depend on the relative gains accomplished by adding more reactor stages. This will depend on the rate constants of the substrate being removed from solution.

Additional research is required to define the operating parameters of the

reactor systems. Important variables to be examined include enzyme dose and concentration, aromatic and peroxide concentrations, pH, retention time and number of reactor stages. A full kinetic model of the system incorporating inactivation mechanisms would simplify reactor design, optimization and scale–up immensely.

8. CONCLUSIONS

Horseradish peroxidase has demonstrated a valuable potential for wastewater treatment with its capability to catalyze the polymerization and precipitation of aromatic compounds from water. It can act on a broad range of compounds including those that are biorefractory or toxic to microbes and retains its catalytic ability over wide ranges of temperature, pH, and contaminant concentration.

8.1 Batch Reactor Studies

Horseradish peroxidase showed optimal activity over a broad pH range of 5.7 to 8.5 with a peak activity at neutral pH. Activity increased with temperature up to a maximum at 50°C and then rapidly declined at higher temperatures because the enzyme was thermally denatured. The rate of inactivation increased with temperature. Above 70°C, virtually all activity was irreversibly lost within several minutes.

Horseradish peroxidase was inactivated rapidly by hydrogen peroxide in the absence of an aromatic substrate. The presence of an aromatic substrate allowed HRP to follow through its catalytic cycle resulting in protection of the enzyme from inactivation through Compound III and P–670 formation. No inactivation resulted from exposure of the enzyme to aromatic substrates in the absence of peroxide.

The degree of removal that could be achieved by a given dose of enzyme was dependent on the nature of the aromatic substrate. Optimal catalytic lifetime was achieved in the pH range of 7 to 9 for the eight phenolic compounds used in this study. The same degree of removal of 4–chlorophenol was achieved at all temperatures between 5°C and 65°C; however, the amount of enzyme required to achieve this removal increased with temperature. Treatment should be conducted at temperatures below 35°C to prevent significant thermal inactivation of

horseradish peroxidase.

The stoichiometry of the reaction between aromatic substrate and peroxide resulting in the formation of insoluble polymers was found to be unity. This was seemingly inconsistent with the stoichiometry predicted by the Chance–George mechanism. Several explanations for the difference between the observed and theoretical stoichiometry are given; however, these explanations must be validated experimentally.

The degree of removal of an aromatic substrate from solution is a function of enzyme dose due to the finite lifetime of the biocatalyst. In addition, batch and semi–batch reactor experiments revealed that the number of catalytic turnovers of a particular substrate may be extended by maintaining a low instantaneous enzyme concentration in the reaction mixture. Sufficient improvement in catalyst lifetime was made to indicate that the instantaneous HRP concentration is an important parameter in the design of a waste treatment system.

The level to which the aromatic compound concentration could be reduced was observed to be independent of the initial concentration. The peak UV absorbance of a solution decreased to a residual level which could not be reduced any further by the addition of more enzyme or peroxide. The residual absorbance is attributed to the formation of soluble by–products of the polymerization reaction which are not good substrates of the enzyme. Co–precipitation of hard–to–remove compounds with more readily removed compounds and other hard–to–remove compounds resulted in enhanced removal of both species. This is of practical importance in terms of wastewater treatment since industrial wastewaters will likely contain a mixture of pollutants.

A mathematical model has been developed which closely matches the trends of kinetic data collected in a batch reactor under various conditions of enzyme, aromatic and peroxide concentrations. The kinetic model overpredicts the

formation of Compound III in a batch solution. Further development is required to define the dominant mechanisms and kinetics of inactivation so that the application of this model can be extended to the design of a full–scale waste treatment system.

8.2 Continuous Flow Reactor Development

Batch and semi–batch reactor experiments demonstrated the importance of maintaining a low concentration of horseradish peroxidase enzyme in a reacting mixture to prolong catalyst lifetime. The enzyme catalyzed polymerization process was implemented in a continuous stirred tank reactor (CSTR) configuration because reactant and enzyme concentrations were lowered immediately upon entering the reactor. The concentration was reduced to a steady–state level that was dependent on the retention time of the reactor. Free radical inactivation of HRP was reduced due to the low steady–state concentration of catalyst in the reacting mixture. The concentration of hydrogen peroxide was also lowered, preventing excessive Compound III formation. The catalytic turnovers achieved in a single CSTR were significantly higher than those observed in batch reactors when sufficient retention time was provided.

Catalyst lifetime was extended further in a reactor system in which CSTR's were placed in series with injection of HRP at each reactor stage. As with the single CSTR system, enzyme and peroxide concentrations were reduced immediately upon injection into each of the reactors. However, unlike the single CSTR system, higher aromatic concentrations were maintained in the initial reactor stages. This allowed the enzyme to work more efficiently and reduced inactivation. The multiple CSTR configuration extended the lifetime of the enzyme beyond the single CSTR for the same retention time. However, there was an optimal number of reactor stages for treating a particular waste. For example, under the reaction conditions tested, the number of catalytic turnovers of phenol increased to a maximum with three reactor

stages and then began to decrease. The number of catalytic turnovers of 4–chlorophenol continued to increase with the number of reactor stages but the gains in catalytic lifetime diminished with each additional reactor stage. Thus, the optimal number of stages will depend on the nature of the compound being removed from solution and on the costs associated with increasing the number of reactor stages.

8.3 Application of Enzyme Catalyzed Polymerization

For certain industrial wastewaters, it may be possible to replace or supplement conventional biological or physical/chemical treatment systems with an enzyme catalyzed polymerization process. This system can replace others which are not effective in treating a particular waste. Alternatively, it can be used in conjunction with a conventional system as a pre–treatment at the source to reduce the burden on subsequent treatment steps. In situations where a very high degree of removal is required, the HRP system can be used as a pre-treatment step to remove the bulk of the aromatic compounds prior to biological treatment which would reduce the residual aromatic concentration to an acceptable level. This combination of enzyme–based and biological treatment systems has the advantages of reduced biomass generation and prevention of shock loading effects on the biological reactor. This system can also be used in conjunction with granulated activated carbon treatment thus reducing the amount of adsorbent materials for disposal.

Additional research is required to fully develop the process and to define situations where application of the enzyme catalyzed polymerization process would be most beneficial.

9. RECOMMENDATIONS

The enzyme catalyzed polymerization process must undergo further development before it can be used in industrial applications. This includes research into methods for stabilizing enzyme activity, identification and modelling of enzyme inactivation mechanisms, development of the continuous flow system and an environmental impact assessment. The production of HRP, the capabilities of other enzymes and other potential applications of enzyme catalyzed polymerization also warrant investigation.

9.1 Enzyme Stabilization

The majority of the costs for enzymatic treatment of wastewater is likely to arise from the consumption of enzyme. Therefore, methods for stabilizing and maintaining catalytic activity will contribute to the economic viability of the process. It has been demonstrated that, in the presence of borate buffer, HRP turnovers increase dramatically. Research is required to explain the protection of the enzyme from inactivation by borate buffer and to identify other additives which may potentially perform a similar function. The environmental and economic costs of using such additives should be evaluated. Other methods of stabilization including immobilization on a solid support (Bailey and Ollis, 1986; Vieth and Venkatasubramanian, 1973) and enzyme polymerization (Hoshino *et al.*, 1987) should be investigated.

9.2 Enzyme Inactivation and Kinetic Modelling

Kinetic modelling of horseradish peroxidase catalysis in a batch reactor has been successful due to the implementation of several simplifying assumptions. These assumptions fail to incorporate the mechanisms or kinetics of free radical

inactivation. This excludes application of the model from other reactor configurations including semi–batch, continuous stirred tank and plug flow reactors with staged addition of enzyme. In order to extend the application of the model, further research is required to:

(i) identify the major mechanisms of enzyme inactivation

(ii) measure inactivation rates under various reaction conditions

(iii) develop reaction rate expressions for enzyme inactivation

(iv) implement kinetic expressions in the overall kinetic model.

This refined model would be an invaluable tool for process development, optimization and scale–up.

9.3 Development of a Continuous Flow System

Further development of the continuous flow reactor is required. Process conditions which are likely to be important in optimizing the operation of the reactor system include: pH, temperature, aromatic and peroxide concentrations, enzyme concentration and dose, retention time, number of reactor stages, mass transfer effects and influence of other contaminants (non–substrates, dissolved salts, suspended solids, etc.) The development of the process should include application to synthetic and real wastewater matrices. Eventually, the process should be implemented at the pilot–scale for detailed performance and economic analyses.

In addition to reactor development, some research is required to determine the settling characteristics of the precipitate formed as a product of enzyme catalyzed polymerization. Precipitates formed in this study were separated from the aqueous phase by centrifugation following coagulation and flocculation. Centrifugation was used only because it proved to be an efficient and rapid technique to prepare the samples for UV analysis. However, the same degree of separation was accomplished by gravity settling indicating that centrifugation is not necessarily the most

cost–effective separation technique. Therefore, further studies are required to determine optimal flocculant requirements and settling conditions for separation of the precipitates formed by the polymerization reaction.

9.4 Environmental Impact Assessment

The objective of waste treatment is to reduce the impact of waste on the environment. Since the enzymatic precipitation of aromatic compounds from solution involves the formation of polyaromatic by–products there is a possibility that these products may prove to be more toxic than their monomeric precursors. Therefore, the nature of the soluble and insoluble products formed in the polymerization process must be characterized. In addition, an environmental impact assessment must be performed. This assessment must take into account all aspects of the treatment process including;

(i) the impact of horseradish peroxidase production

(ii) the nature and toxicity of soluble and insoluble by–products

(iii) the fate of soluble and insoluble by–products

(iv) disposal alternatives for precipitated by–products.

9.5 Production of Horseradish Peroxidase

The horseradish peroxidase enzyme used in this work was purchased in a highly purified form normally intended for clinical applications. Such purity is expensive and is not necessarily required for wastewater treatment. The production of horseradish peroxidase enzyme of a quality that is intended specifically for application in a wastewater treatment process should be researched. Cost–effective preparation of enzyme should reduce process costs significantly.

9.6 Other Enzymes and Applications

In addition to horseradish peroxidase, other enzymes such as ligninase, lactoperoxidase, chloroperoxidase and laccase have demonstrated a potential for wastewater treatment. These enzymes and the conditions under which they can operate should be studied. One or more of these enzymes may be better suited for treating a particular wastewater than horseradish peroxidase.

The wood rotting fungus *phanerochaete chrysosporium* has been the focus of much attention since the discovery that it produces the ligninase enzyme extracellularly. Ligninase catalyzes the same oxidation reactions as horseradish peroxidase. Extracellular production of the enzyme leads to the potential for *in situ* production of the enzyme during wastewater treatment. Successful application of this system would eliminate the need for expensive isolation and purification of the enzyme catalyst before use. This fungus has demonstrated the ability to degrade a wide spectrum of chlorocarbons and polycyclic aromatics including organohalides such as DDT, polychlorinated biphenyls, polychlorinated dibenzo(p) dioxins, lindane and chlorinated alkanes (Lewandowski *et al.*, 1990). Therefore, its potential as a biological treatment tool should be investigated.

The application of these enzymes should not be restricted to wastewater treatment. There is potential for application in such areas as soil remediation, spill control and hazardous waste treatment. In addition, applications outside the scope of environmental engineering such as polymer production and product purification are recommended for further study.

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APPENDIX A

Substrates of Horseradish Peroxidase

APPENDIX A

Substrates of Horseradish Peroxidase

The following list was compiled from literature dealing directly with the catalytic oxidation of aromatics in water using horseradish peroxidase. The degree of removal that can be achieved depends on the nature of the compound and the conditions under which the treatment is conducted. The key to the literature sources cited is located at the end of the appendix.

Compound:	Literature Source:
Phenols and Aromatic Amines:	
phenol	a, b, c, e
2–methoxyphenol (guaiacol)	a, c
3–methoxyphenol	a, c
4–methoxyphenol	a, c
2–methylphenol	a, c
3–methylphenol	a, c
4–methylphenol	a, c
2–chlorophenol	a, b, c, f, g
3–chlorophenol	a, c
4–chlorophenol	a, c
2–aminophenol	c
3–aminophenol	c
resorcinol	c
5–methylresorcinol	c
2,3–dimethylphenol	a, c
2,6–dimethylphenol	a, c
pentachlorphenol	b, f, g
benzidine	a, d
3,3'–dimethoxybenzidine	a, d
3,3'–diaminobenzidine	a, d
3,3'–dichlorobenzidine	a, d
3,3'–dimethylbenzidine	a, d
1–naphthylamine	a, d
2–naphthylamine	a, d
5–nitro–1–naphthylamine	a, d
N,N'–dimethylnaphthylamine	a
1,3–diaminophenol	a
diphenylamine	a
2,4–dichlorophenol	f, g
4–phenylazoaniline	d
aniline	c
4–chloroaniline	c
4–bromoaniline	c
4–fluoroaniline	c
4–bromo–2–methylaniline	c
m–phenylenediamine	c
4,4'–methylenedianiline	c
n–(1–naphthyl)–ethylenediamine	c
4'–amino–2,3'–dimethylazobenzene	c

Polyaromatics:

1–napthol	c
1,3–naphthalenediol	c
?–nitroso–1–napthol	c
2,7–naphthalenediol	c
p–phenylphenol	c
5–indanol	c
8–hydroxyquinoline	c
4–aminobiphenyl	d

Reference List

a. Alberti *et al.*, 1982
b. Fiessinger *et al.*, 1984
c. Klibanov *et al.*, 1980
d. Klibanov *et al.*, 1981
e. Klibanov *et al.*, 1983
f. Maloney *et al.*, 1985
g. Maloney *et al.*, 1986

APPENDIX B

Analytical Methods

APPENDIX B

Analytical Methods

B.1 Horseradish Peroxidase Activity Assay

B.1.1 General Discussion

The purpose of this assay is to determine the amount of active enzyme that is contained in a stock solution. Under saturating conditions of substrates (HDCBS, AAP and H_2O_2) the initial rate of reaction will be proportional to the amount of active enzyme in solution. This initial rate is measured by observing the rate of color formation in a solution in which a reaction between HDCBS and H_2O_2 is catalyzed by horseradish peroxidase such that the products of the reaction react with AAP to form a pink colored solution which absorbs light at a peak wavelength of 510 nm.

B.1.2 Reagents

a. Phosphate buffer (0.1 M NaPP)

Combine 160 mL of 0.2 M monobasic sodium phosphate with 840 mL of 0.2 M dibasic sodium phosphate and bring to 2 L volume with distilled water.

b. 3,5–dichloro–2–hydroxybenzenesulfonic acid (18.0 mM HDCBS)

Place 956 mg HDCBS in a 200 mL volumetric flask and bring to 200 mL volume with phosphate buffer. Store in refrigerator.

c. 4–aminoantipyrine (9.6 mM AAP)

Place 390 mg AAP in a 200 mL volumetric flask and bring to 200 mL volume with phosphate buffer. Store in refrigerator.

d. Hydrogen peroxide (1.0 mM H_2O_2)

Place 113.3 μL of 30% (w/v) hydrogen peroxide in a 100 mL volumetric flask. Remove 10 mL and place in a second flask then dilute to 100 mL. Remake this solution daily.

B.1.3 Procedure

In a semi–micro cuvette place:

500 μL of 18 mM HDCBS
250 μL of 9.6 mM AAP
100 μL of 1.0 mM H_2O_2
0 to 130 μL of NaPP buffer
20 to 150 μL of sample

The assay sample volume must be 1 mL and the rate of color formation must be measured before substrate depletion becomes significant. Immediately after the addition of the sample, shake the cuvette and then monitor the absorbance change with time at 510 nm.

Changes in the concentration of peroxide in the cuvette due to the addition of sample containing peroxide do not appear to influence the assay significantly.

B.1.4 Calculations

Find the average slope over the linear range of the data (initial rate) in terms of absorbance units per unit time (au/min). Calculate the activity in the cuvette according to:

$$\text{Activity} = \frac{\text{slope (au/min)}}{\text{25 000 au L/mol}} \times 10^6 \frac{\mu\text{mol}}{\text{mol}} \times \frac{\text{1 L}}{\text{1000 mL}}$$

expressed as μmol/min mL or U/mL where U are units of enzyme activity in terms of micromoles of HDCBS converted per minute at 25°C and pH of 7.4 with 9 mM HDCBS, 2.4 mM AAP and 0.1 mM H_2O_2. The sample activity is then calculated according to:

$$\text{Sample Activity} = \text{Activity (U/mL)} \times \frac{\text{1000 } \mu\text{L}}{\text{sample vol. } (\mu\text{L})}$$

B.1.5 Interferences

Aromatics which are substrates of HRP interfere with this reaction because of excess color formation that is dependant on the type and quantity of the aromatic in the sample. This test should only be used on samples containing no aromatic – even in trace quantities.

B.2 Hydrogen Peroxide Assay

B.2.1 General Discussion

This is a colorimetric assay used to measure the hydrogen peroxide concentration in a sample. It makes use of horseradish peroxidase (HRP) as catalyst and 3,5–dichlorobenzenesulfonic acid (HDCBS) and 4–aminoantipyrine (AAP) as color generating substrate. In this assay the amount of hydrogen peroxide introduced into the assay sample is the only limiting reactant and therefore the degree of color developed (at 510 nm) in the reaction is proportional to the peroxide concentration.

B.2.2 Reagents

a. Phosphate buffer (0.1 M NaPP)

Combine 160 mL of 0.2 M monobasic sodium phosphate with 840 mL of 0.2 M dibasic sodium phosphate and bring to 2 L volume with distilled water.

b. 3,5–dichloro–2–hydroxybenzenesulfonic acid (18.0 mM HDCBS)

Place 956 mg HDCBS in a 200 mL volumetric flask and bring to 200 mL volume with phosphate buffer. Store in refrigerator.

c. 4–aminoantipyrine (9.6 mM AAP)

Place 390 mg AAP in a 200 mL volumetric flask and bring to 200 mL volume with phosphate buffer. Store in refrigerator.

d. Horseradish peroxidase stock (0.1 mg/mL HRP)

Place 1 mg of HRP solid per 10 mL of phosphate buffer as above. Accuracy is not required in making this solution since the only requirement is that the enzyme be in excess so that the endpoint can be reached. The stronger this solution is, the faster the endpoint will be reached.

e. Hydrogen peroxide

Place 113.3 μL of 30% hydrogen peroxide in a 100 mL volumetric flask. From this 10 mM solution create standard solutions with concentrations ranging from 0 to 1.0 mM.

B.2.3 Calibration Procedure

In a semi–micro cuvette (1.5 mL volume) place:

500 μL of 18 mM HDCBS
250 μL of 9.6 mM AAP
100 μL of HRP stock
100 μL of NaPP buffer
50 μL of standard peroxide sample

The assay sample volume must be 1 mL. Don't let the peroxide concentration in the cuvette exceed 50 μM The color formation at 510 nm should be observed until a maximum amount of color is developed. Record the maximum. Repeat the same procedure but vary the concentration of peroxide in the cuvette from 5 μM to 50 μM.

Make a plot of absorbance at 510 nm versus concentration of peroxide in the cuvette.

B.2.4 Measurement of Peroxide

In a semi–micro cuvette (1.5 mL volume) place:

500 μL of 18 mM HDCBS
250 μL of 9.6 mM AAP
100 μL of HRP stock
0 to 130 μL of NaPP buffer
20 to 150 μL of sample

The assay sample volume must be 1 mL. Don't let the peroxide concentration in the cuvette exceed 50 μM The color formation at 510 nm should be observed until a maximum amount of color is developed. Record the maximum. From the calibration curve determine the amount of peroxide that was present in the cuvette. If this value exceeds 50 μM then repeat the process but use less sample volume and make up the difference with buffer – always maintain an assay sample volume of 1 mL.

Once a satisfactory cuvette concentration has been achieved, back–calculate the amount of peroxide that was present in the original sample.

B.2.5 Calculations

Calculate the sample peroxide concentration from:

$$[H_2O_2]_{sample} = [H_2O_2]_{cuvette} \times \frac{1000\ \mu L}{\text{sample vol. } (\mu L)}$$

where $[H_2O_2]_{cuvette}$ has been determined from the calibration curve.

B.3 Aromatic Substrate Assay

B.3.1 General Discussion

This is a colorimetric assay used to measure the concentration of aromatic substrate in a sample. It makes use of horseradish peroxidase (HRP) as catalyst and 4-aminoantipyrine (AAP) as a color generating substrate in combination with the aromatic in the sample. In this assay the amount of aromatic introduced into the assay sample is the only limiting reactant and therefore the degree of color developed in the reaction at the peak wavelength is proportional to the aromatic concentration.

B.3.2 Reagents

a. Phosphate buffer (0.1 M NaPP)

Combine 160 mL of 0.2 M monobasic sodium phosphate with 840 mL of 0.2 M dibasic sodium phosphate and bring to 2 L volume with distilled water.

b. 4-aminoantipyrine (9.6 mM AAP)

Place 390 mg AAP in a 200 mL volumetric flask and bring to 200 mL volume with phosphate buffer. Store in refrigerator.

c. Horseradish peroxidase stock (0.1 mg/mL HRP)

Place 1 mg of HRP solid per 10 mL of phosphate buffer as above. Accuracy is not required in making this solution since the only requirement is that the enzyme be in excess so that the endpoint can be reached. The stronger this solution is, the faster the endpoint will be reached.

d. Hydrogen peroxide (1.0 mM H_2O_2)

Place 113.3 μL of 30% (w/v) hydrogen peroxide in a 100 mL volumetric flask. Remove 10 mL and dilute to 100 mL. Remake this solution daily.

e. Aromatic substrate

Make up a stock solution of aromatic substrate with a concentration of 1 mM. From the stock solution create 25 mL solutions of aromatic substrate ranging in concentration from 0 to 0.25 mM.

B.3.3 Calibration Procedure

In a semi-micro cuvette (1.5 mL volume) place:

250 μL of 9.6 mM AAP
250 μL of 1.0 mM H_2O_2
100 μL of HRP stock
200 μL of NaPP buffer
200 μL of aromatic samples

The assay sample volume must be 1 mL. Don't let the aromatic concentration in the cuvette exceed 50 μM The color formation at the peak wavelength should be observed until a maximum amount of color is developed. Record the maximum. Repeat the same procedure but vary the concentration of aromatic in the cuvette from 5 μM to 50 μM.

Make a plot of absorbance versus concentration of aromatic in the cuvette.

B.3.4 Measurement of Aromatic Substrate

In a semi–micro cuvette (1.5 mL volume) place:

250 μL of 9.6 mM AAP
250 μL of 1.0 mM H_2O_2
100 μL of HRP stock
200 to 350 μL of NaPP buffer
50 to 200 μL of unknown sample

The assay sample volume must be 1 mL. Don't let the aromatic concentration in the cuvette exceed 50 μM The color formation at 510 nm should be observed until a maximum amount of color is developed. Record the maximum. From the calibration curve determine the amount of aromatic that was present in the cuvette. If this value exceeds 50 μM then repeat the process but use less sample volume and make up the difference with buffer – always maintain an assay sample volume of 1 mL.

Once a satisfactory cuvette concentration has been achieved, back–calculate the amount of aromatic that was present in the original sample.

B.3.5 Calculations

Calculate the sample aromatic concentration from:

$$[\text{Aromatic}]_{\text{sample}} = [\text{Aromatic}]_{\text{cuvette}} \times \frac{1000\ \mu\text{L}}{\text{sample vol. } (\mu\text{L})}$$

where $[\text{Aromatic}]_{\text{cuvette}}$ has been determined from the calibration curve.

APPENDIX C

Reactor Operating Procedures

APPENDIX C

Reactor Operating Procedures

C.1 Batch Reactor Operation

C.1.1 General Discussion

The batch reactors used in this study consist of vials containing a mixture of aromatic compound, hydrogen peroxide, peroxidase enzyme, and buffer where applicable. Control of pH and temperature are provided where necessary.

C.1.2 Equipment

The assembly shown in Figure C–1 makes up the batch reactor system. The following equipment are used:

- constant temperature water reservoir
- water bath
- peristaltic pump for circulation of water from reservoir to water bath
- up to 35 35–mL vials
- thermometer
- magnetic stirrers with insulation on surface
- teflon coated stir bars (1 per vial)

C.1.3 Reagents

The following reagents are required:

- peroxide
- aromatic compound
- horseradish peroxidase enzyme
- buffer (when required)
- catalase enzyme
- sodium hydroxide
- alum

Concentrations of these reagents are not stated since they are situation specific.

C.1.4 Operation

a. Preparation of reaction mixture

Place the aromatic compound, horseradish peroxidase and water/buffer in the vial(s) in volumes such that the concentration of species in the reactor(s) will be at the desired level once the hydrogen peroxide has been added. For example, to create a 30 mL reaction mixture with X mM phenol and Y mM H_2O_2 use the following recipe:

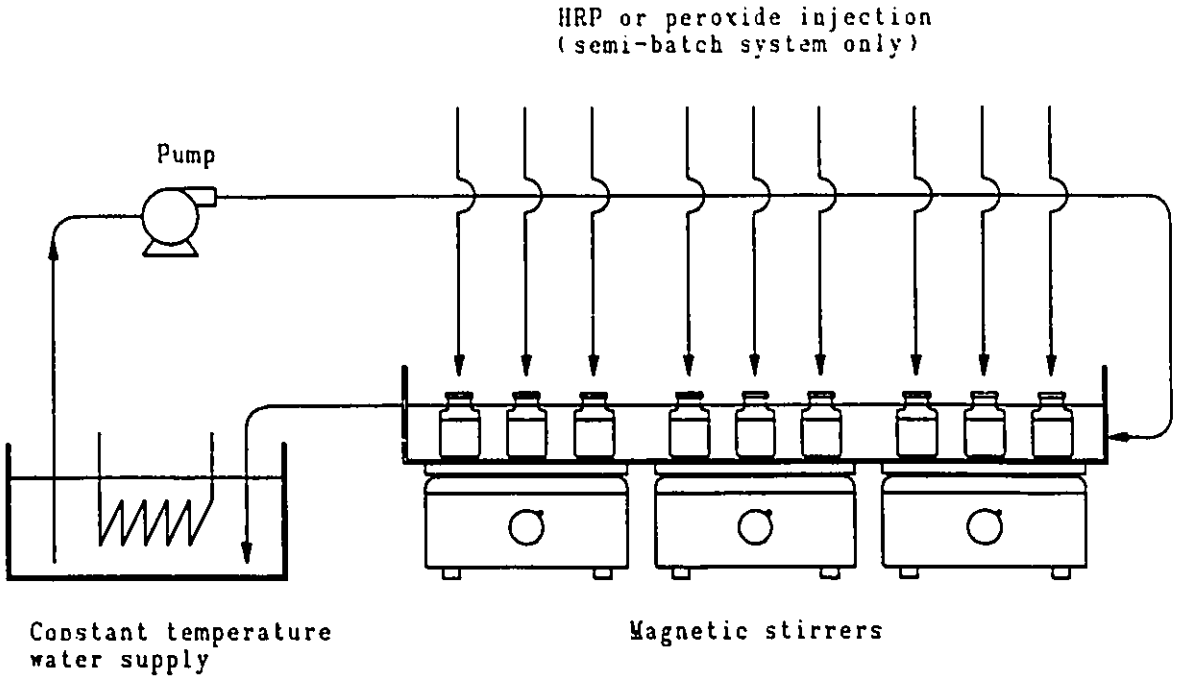

Figure C–1: Batch and Semi–Batch Reactor System Assembly

X mL of 30 mM phenol
Y mL of 30 mM H_2O_2
Z mL HRP stock
30 – X – Y – Z mL of water/buffer

Cap the samples and place them in the water bath and begin stirring. Let the temperature of the vial(s) come to equilibrium with the water bath temperature. Initiate the reaction with the addition of the hydrogen peroxide. Monitor the pH of the reaction mixture periodically to ensure that the buffering capacity of the buffer is not exceeded.

The enzyme activity of the sample is determined according to the assay technique described in Appendix A.

b. Stopping the reaction

The reaction in the vial can be halted through the addition of sufficient catalase enzyme to rapidly convert the remaining hydrogen peroxide into water and oxygen. Approximately 0.5 mL of 1 mg/mL catalase will be sufficient to stop the reaction in the 30 mL reaction mixture with up to 3 mM H_2O_2.

c. Notes

The residual amount of aromatic can be measured by direct spectrophotometric measurement or by the aromatic assay procedure described in Appendix B. Before these methods can be used each sample must be treated with alum and the pH adjusted to approximately 6.3 using sodium hydroxide (1 M) to optimize floc formation. Subsequently, the precipitate is allowed to flocculate for 10 minutes and a proportion of the reaction mixture is taken for centrifugation at 3000 G's for 40 minutes.

The residual amount of hydrogen peroxide must be measured before the addition of catalase enzyme according to the hydrogen peroxide assay described in Appendix B.

A blank sample containing peroxide and aromatic but no horseradish peroxide should be prepared and undergo all aspects of the reaction procedure for comparison with treated samples.

The volumes of catalase, alum and sodium hydroxide added to the solutions must be recorded so that the residual concentrations which existed in the reaction mixture can be back–calculated after analysis.

For kinetic studies, larger reaction mixtures (150 mL) are required. The samples are created and the reaction is initiated in the same manner as discussed above. However, samples (9.5 mL) are withdrawn at periodic intervals and quenched by their addition to test tubes containing 0.5 mL of catalase (0.4 mg/mL). The test tube contents must be vigourously stirred to allow the catalase to quickly mix with the sample.

C.2 Semi–Batch Reactor Operation

C.2.1 General Discussion

The semi–batch reactors used in this study utilize the same equipment and reagents as those used in the operation of the batch reactors with the exception of a syringe pump which is used to inject either peroxide or horseradish peroxidase to each reactor over time.

C.2.2 Equipment

Figure C–1 shows the setup of the semi–batch reactor system. The following equipment are used in addition to the equipment included in the operation of the batch reactor:

- syringe pump with multiple syringe holders
- disposable plastic syringes (1 per semi–batch reactor)
- teflon tubing (1 mm ID)

C.2.3 Reagents

The reagents are the same as those used in the batch reactor system.

C.2.4 Operation

a. Preparation of reaction mixture

Place the aromatic compound, water/buffer and either the horseradish peroxidase or peroxide in the vial(s) such that the concentration of species in the reactor(s) will be at the desired level once the remaining species has been completely added to the sample. Let the temperature of the vial(s) come to equilibrium with the water bath temperature.

Each syringe must be filled and all air bubbles removed in the both the syringe barrel and the connecting tubing to the reactor. The dose or addition rate of enzyme or peroxide to the reactors can be varied by varying the concentration of the material in the syringe.

b. Stopping the reaction

The reaction is halted in the same manner as with the batch reactor.

c. Notes

The analysis of all products is accomplished in the same way as described in the batch reactor operating procedure.

A blank sample in which the horseradish peroxidase is not supplied to the reactor must be prepared and undergo all aspects of the reaction procedure for eventual comparison with treated samples.

C.3 Continuous Stirred Tank Reactor Operation

C.3.1 General Discussion

The continuous stirred tank teactor (CSTR) used in this study consists of a syringe into which are injected a stream of horseradish peroxidase and a mixture of peroxide and an aromatic. The contents of the reactor are continuously mixed and the effluent leaves at the same flowrate as the influent.

C.3.2 Equipment

The CSTR system is pictured in Figure C–2. It consists of the following equipment:

- 140 mL plastic syringe (supplied by veternary hospitals)
- 10 mL plastic syringe
- teflon tubing (1 mm ID)
- constant temperature water bath
- magnetic stirrer
- magnetic stir bars
- peristaltic pump
- valve

C.3.3 Reagents

The following reagents are required:

- peroxide
- aromatic compound
- horseradish peroxidase enzyme
- buffer (when required)
- catalase enzyme
- sodium hydroxide
- alum

Concentrations of these reagents are not stated since they are situation specific.

C.3.4 Operation

a. Preparation of reaction mixtures

A reservoir containing a mixture of aromatic compound and hydrogen peroxide must be prepared. This mixture should not be prepared in advance of the experiment since the peroxide may slowly oxidize the aromatic without the enzyme present. The temperature of this mixture should be maintained at the reactor operating temperature.

The 10 mL syringe must be filled with horseradish peroxidase and all air bubbles removed in the both the syringe barrel and the connecting tubing to the reactor. The dose or addition rate of enzyme to the reactor can be varied by varying the concentration of the enzyme or the flowrate from the syringe pump.

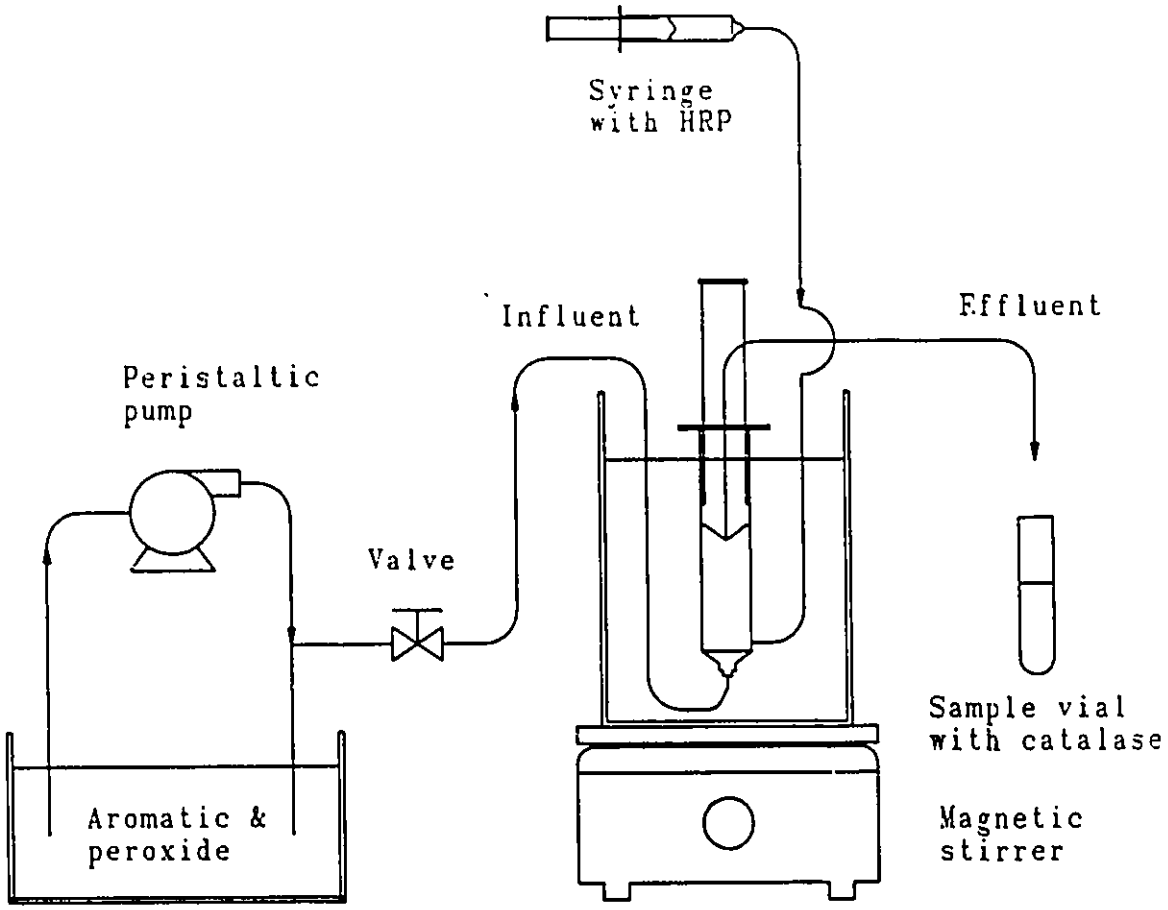

Figure C–2: Single Continuous Stirred Tank Reactor Assembly

b. Operation

Begin the procedure with the 10 mL syringe placed in the syringe pump and the CSTR (140 mL syringe) empty of liquid The reactor volume should be set by placing the syringe plunger in the appropriate position. Start up the peristaltic pump and the syringe pump simultaneously with the flows of both pumps already set to the desired rates.

Let the system sit until equilibrium is achieved. This can be determined by making repeated measurements of hydrogen peroxide concentration in the effluent until the value reaches steady state.

c. Sample collection

In order to determine the concentration of materials in the reactor at steady state a sample of the effluent must be collected. The reaction must be stopped as it leaves the reactor since conversion is dependant on residence time. This is accomplished by collecting 9.5 mL of effluent in a sample bottle which already contains a dose of 0.5 mL catalase (0.4 mg/mL). For high concentrations of peroxide, a larger dose of catalase may be required. This contents of the sample bottle should be continuously mixed as the effluent is collected. The volume of sample collected and catalase added must be recorded for later calculation of the concentration of aromatic compound in the reactor.

d. Notes

The volume of the syringe/reactor must be calibrated with influent and exit tubes and stir bar in place. The volume of the effluent tube is kept at a minimum to prevent the reaction from occuring to any significant degree after it leaves the reactor.

Residual measurement of aromatic and peroxide can be measured as described in the batch reaction operating procedure.

The peristalic pump is operated at a high rate of revolutions to minimize the effect of pulsating flow in the reactor. Flow to the reactor is regulated by a valve on the high pressure side of the peristaltic pump. The effluent flowrate is measured by collecting a sample volume over a measured period of time. While the effects associated with pulsating flow were not measured, this configuration has the advantage of providing a wide range of flowrates (through adjustment of the valve) using a fixed rate pump.

This continuous flow system can also be operated as a series of continuous flow reactors as shown in Figure C-3. The effluent from each reactor becomes the influent stream to the next reactor of the series. This system also has the option of providing a feed of enzyme to the initial influent stream or at each stage of the reactor system.

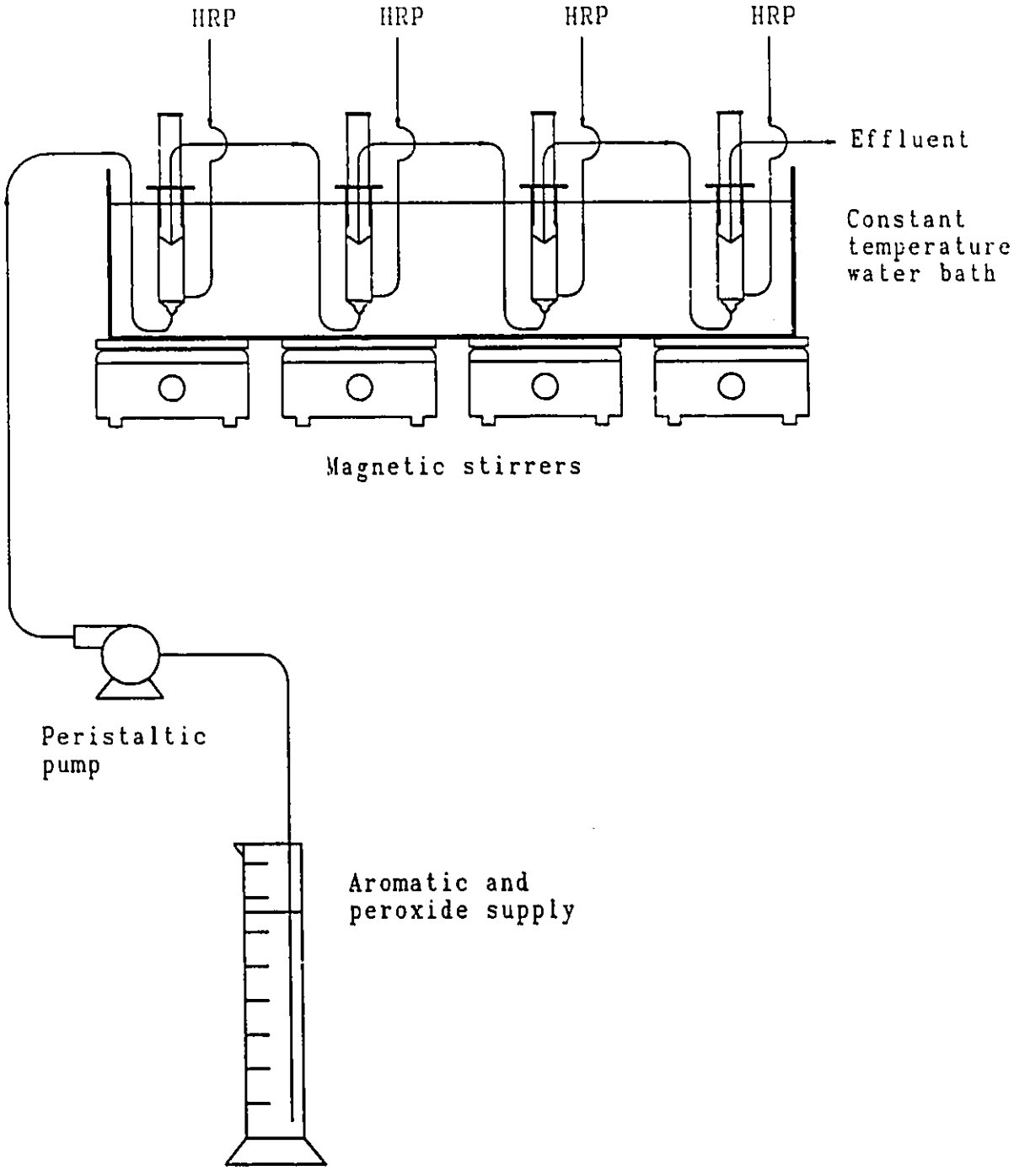

Figure C-3: Multiple Continuous Stirred Tank Reactors in Series Assembly

APPENDIX D

Clearance of Phenolic Substrates as a Function of pH and Enzyme Dose

APPENDIX D

Clearance of Phenolic Substrates as a Function of pH and Enzyme Dose

All results described in this appendix were conducted under the following conditions:

- 30 mL batch reaction mixtures were prepared in 35 mL vials using stock solutions of reaction components such as:

  30 mM aromatic
  60 mM hydrogen peroxide
  0.1 M buffers
  0.4 mg/mL HRP stock

- batch reaction mixtures were prepared according to the procedure described in Appendix C

- buffers are described in Chapter 3, "Materials and Methods"

- reaction mixtures were placed in a water bath maintained at 25°C and temperatures were allowed to equilibrate

- samples were capped and stirred continuously using a magnetic stirrer and stir bars

- reactions were initiated by the addition of the appropriate dose of horseradish peroxidase

- reactions were allowed to proceed for 5 hours before alum coagulation

- samples were centrifuged at 3000 G for 40 minutes prior to spectrophotometric analysis

- catalytic turnovers were calculated by first converting the absorbance to concentration using calibration curves and then by using the following equation:

$$\text{Turnovers} = ([AH_2]_0 - [AH_2])/\ [HRP]_0\ /\ C_a$$

with $[AH_2]_0$ and $[AH_2]$ expressed in "mM" and $[HRP]_0$ in "U/mL" and where $C_a = 0.15306 \times 10^{-3}\ \mu M\ (U/mL)^{-1}$ for HRP.

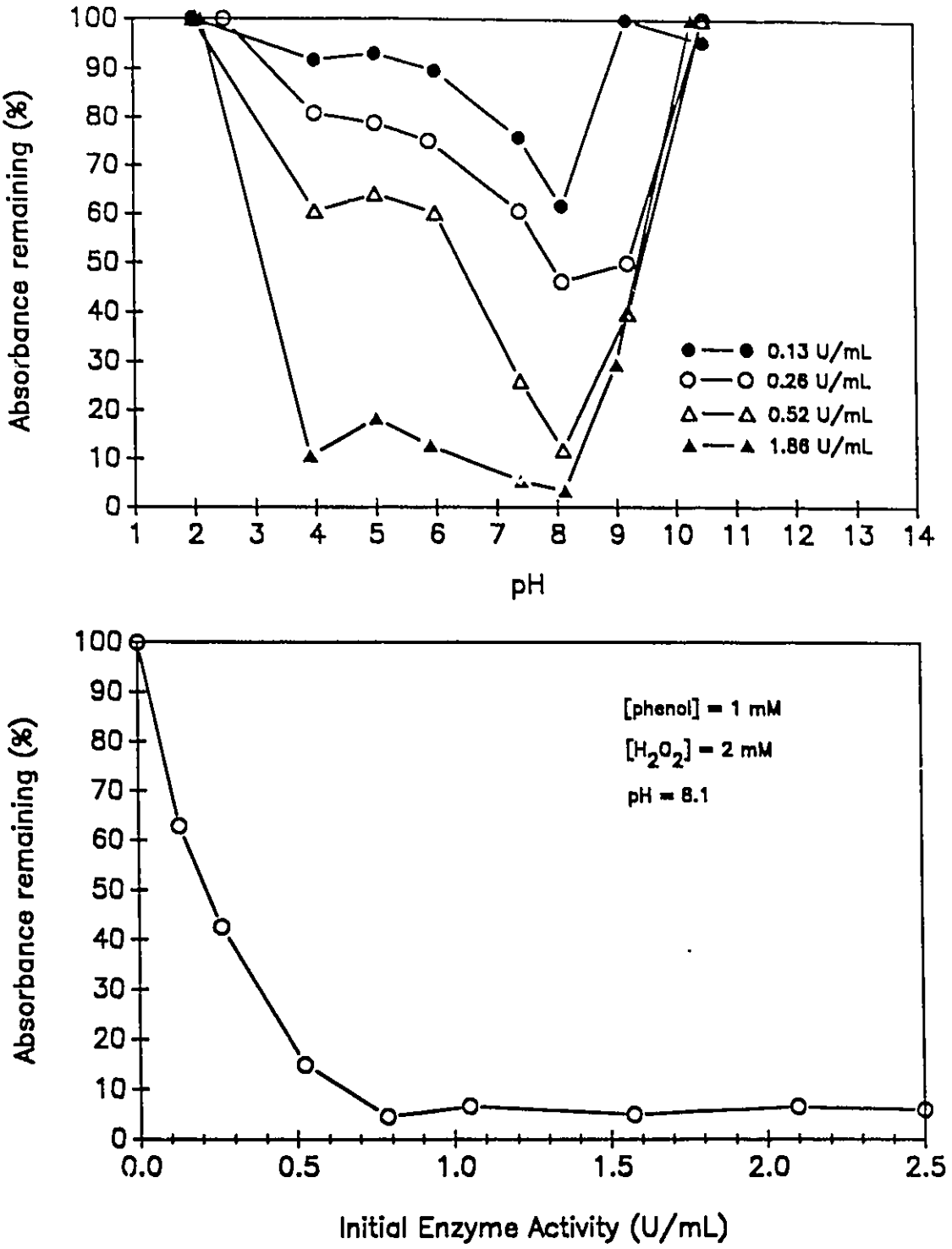

Figure D–1: Clearance of Phenol as a Function of (a) pH and (b) HRP Dose at the Optimal pH

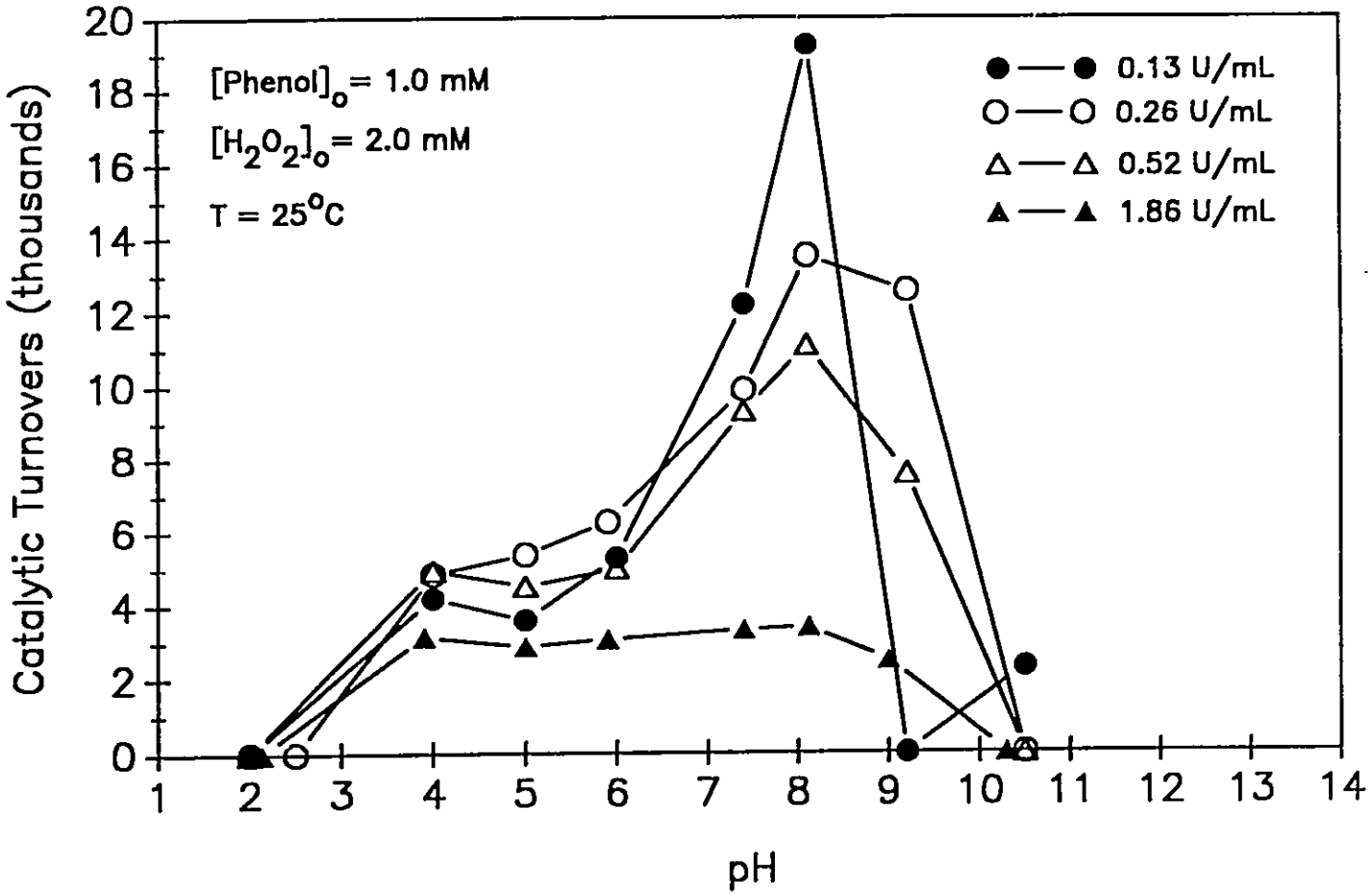

Figure D–2: Catalytic Turnovers of Phenol as a Function of pH and HRP Dose

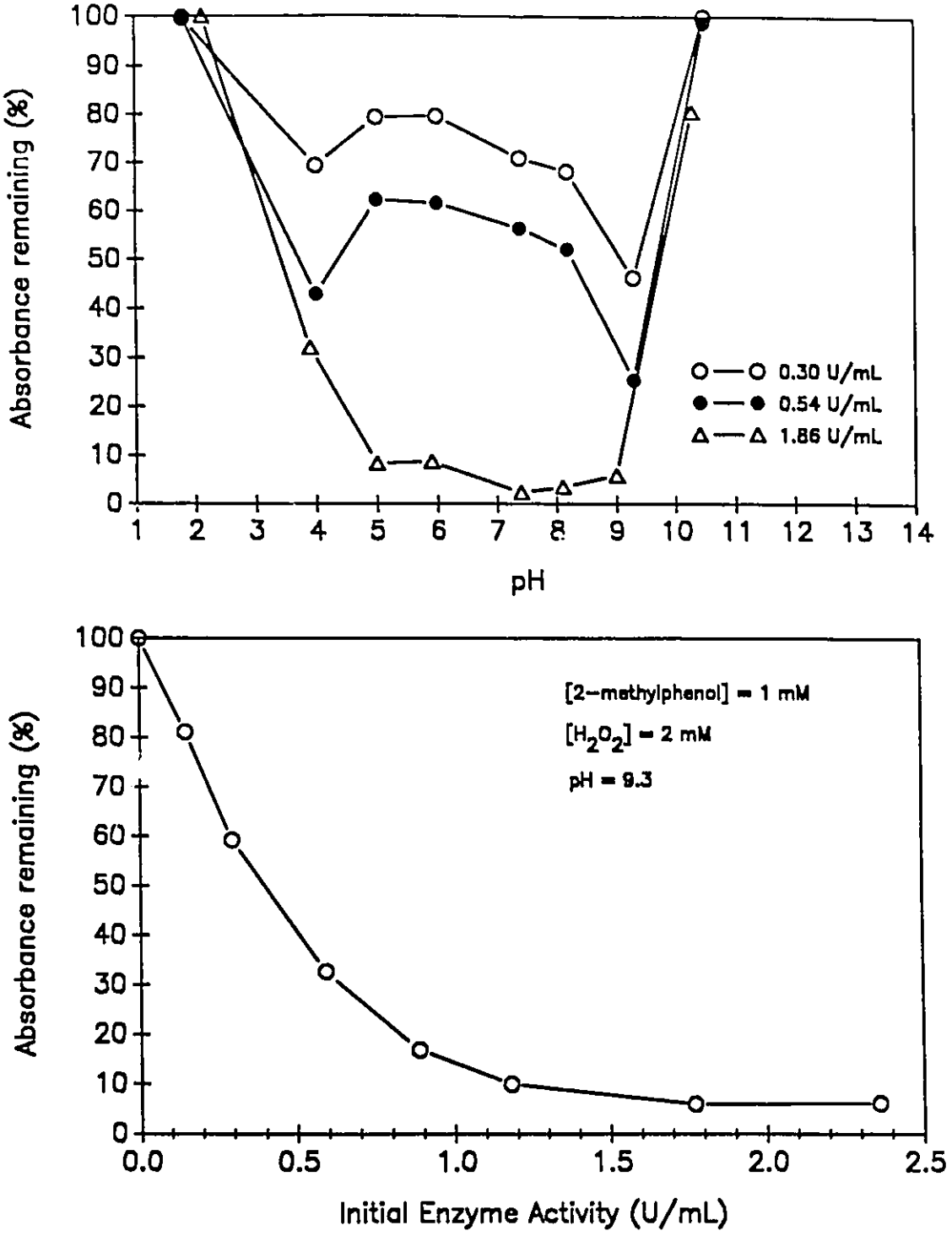

Figure D-3: Clearance of 2-Methylphenol as a Function of (a) pH and (b) HRP Dose at the Optimal pH

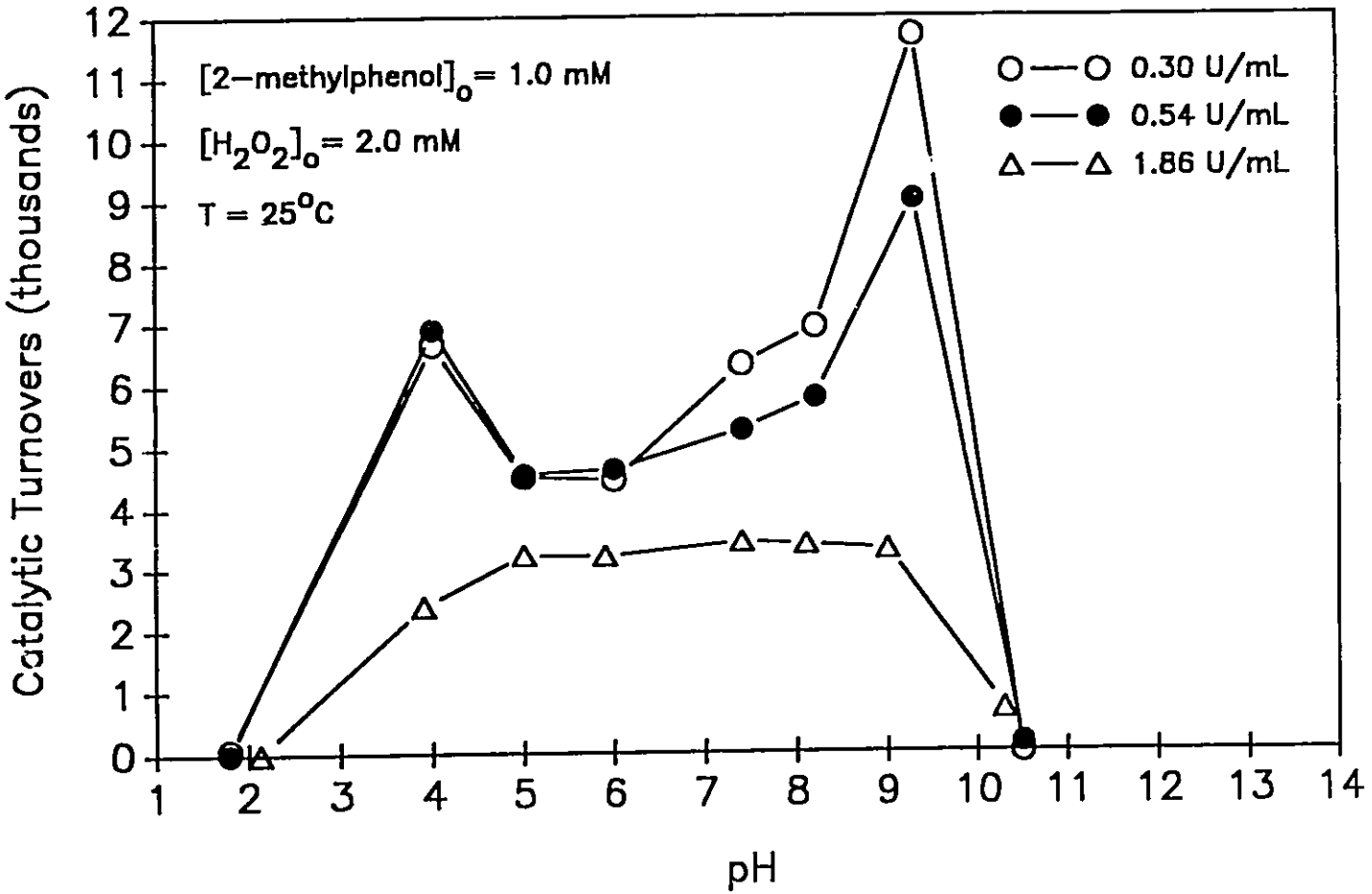

Figure D–4: Catalytic Turnovers of 2–Methylphenol as a Function of pH and HRP Dose

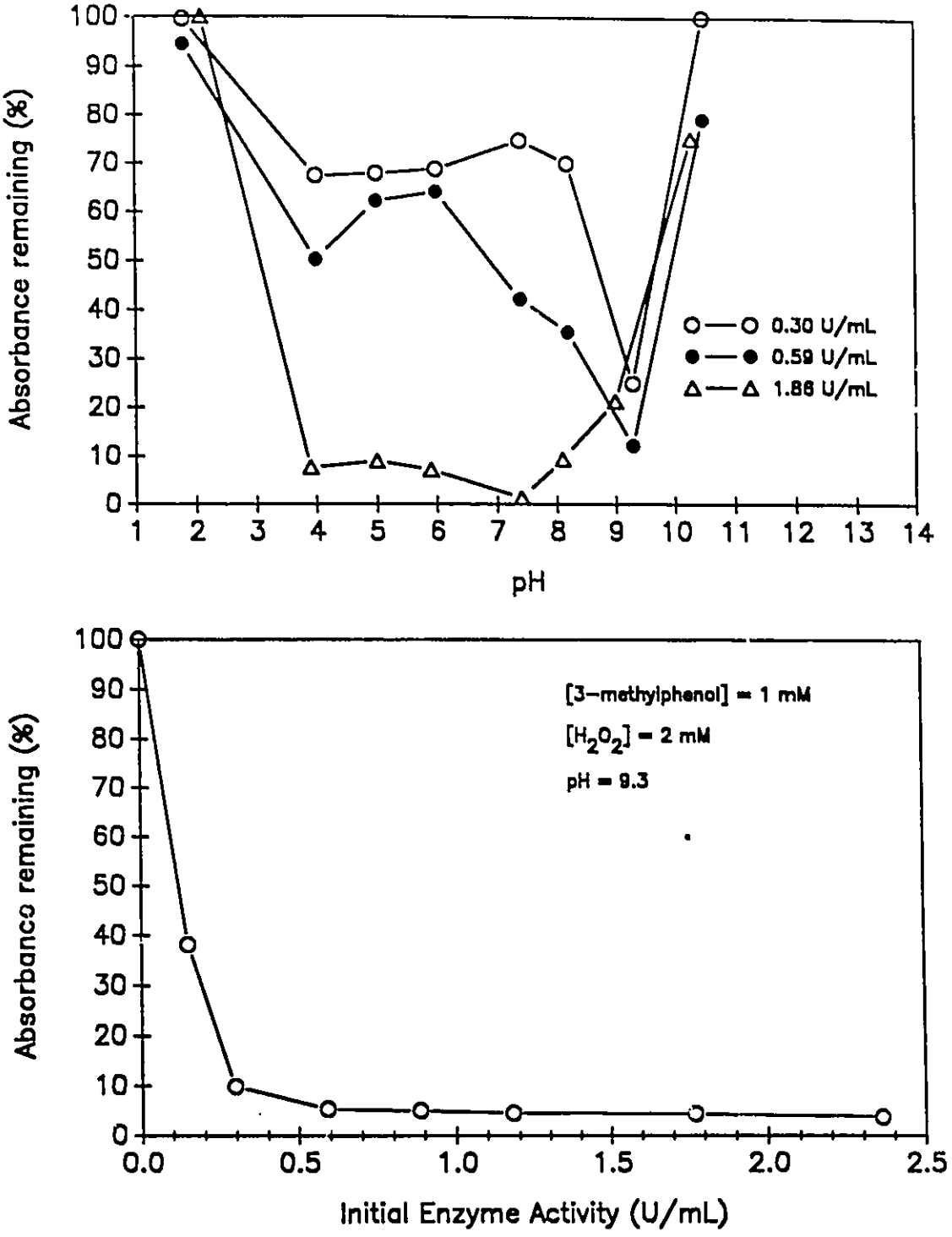

Figure D–5: Clearance of 3–Methylphenol as a Function of (a) pH and (b) HRP Dose at the Optimal pH

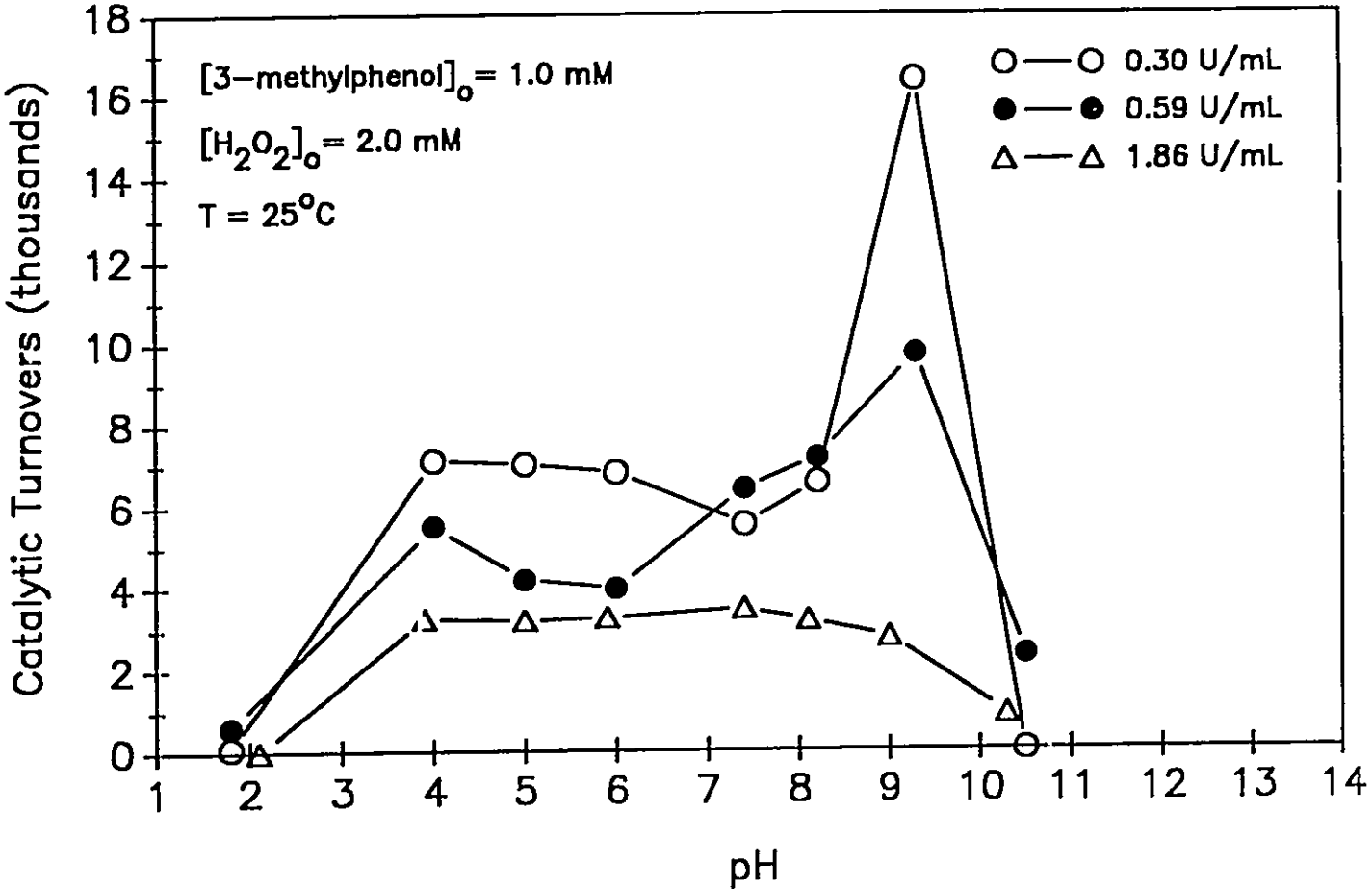

Figure D–6: Catalytic Turnovers of 3–Methylphenol as a Function of pH and HRP Dose

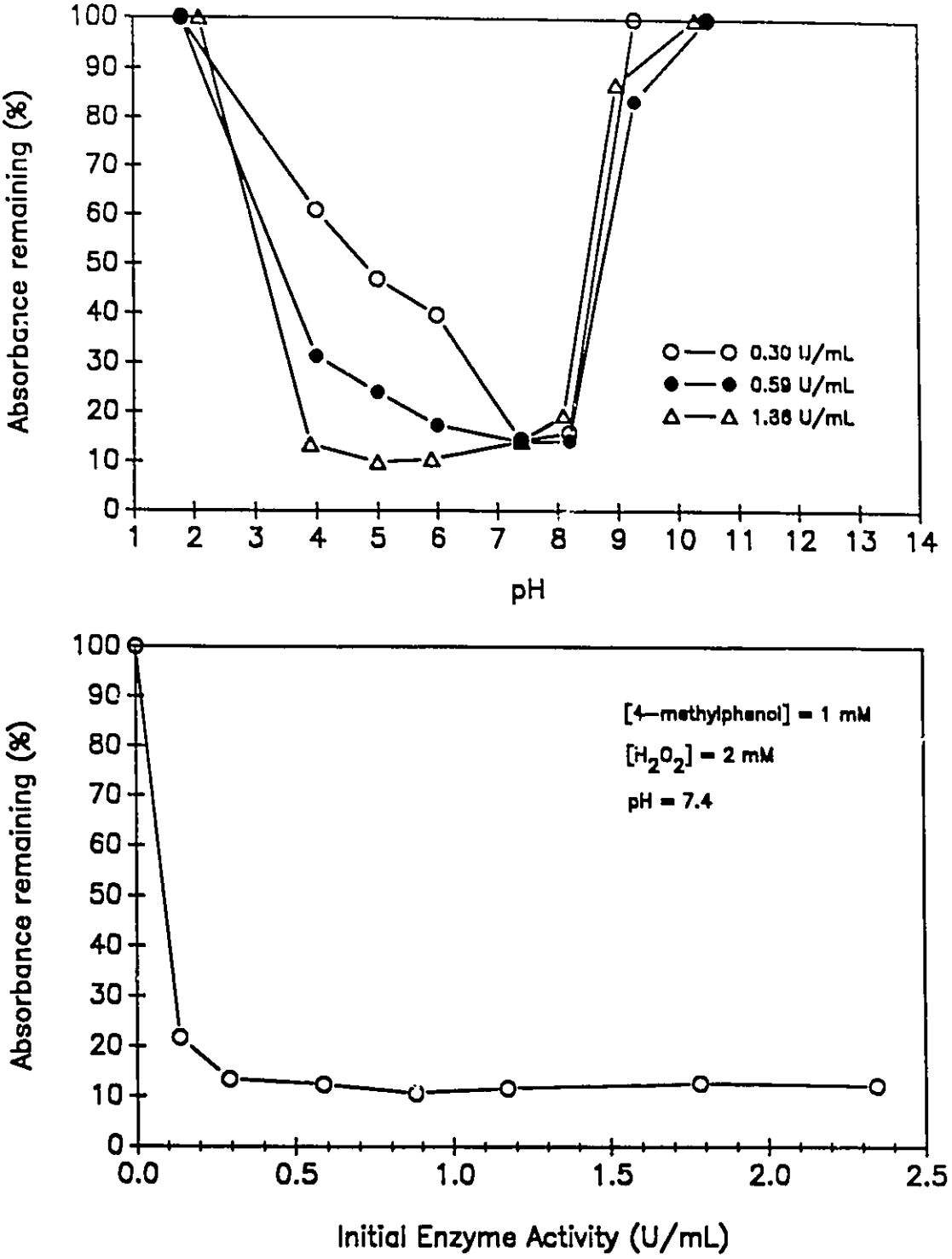

Figure D–7: Clearance of 4–Methylphenol as a Function of (a) pH and (b) HRP Dose at the Optimal pH

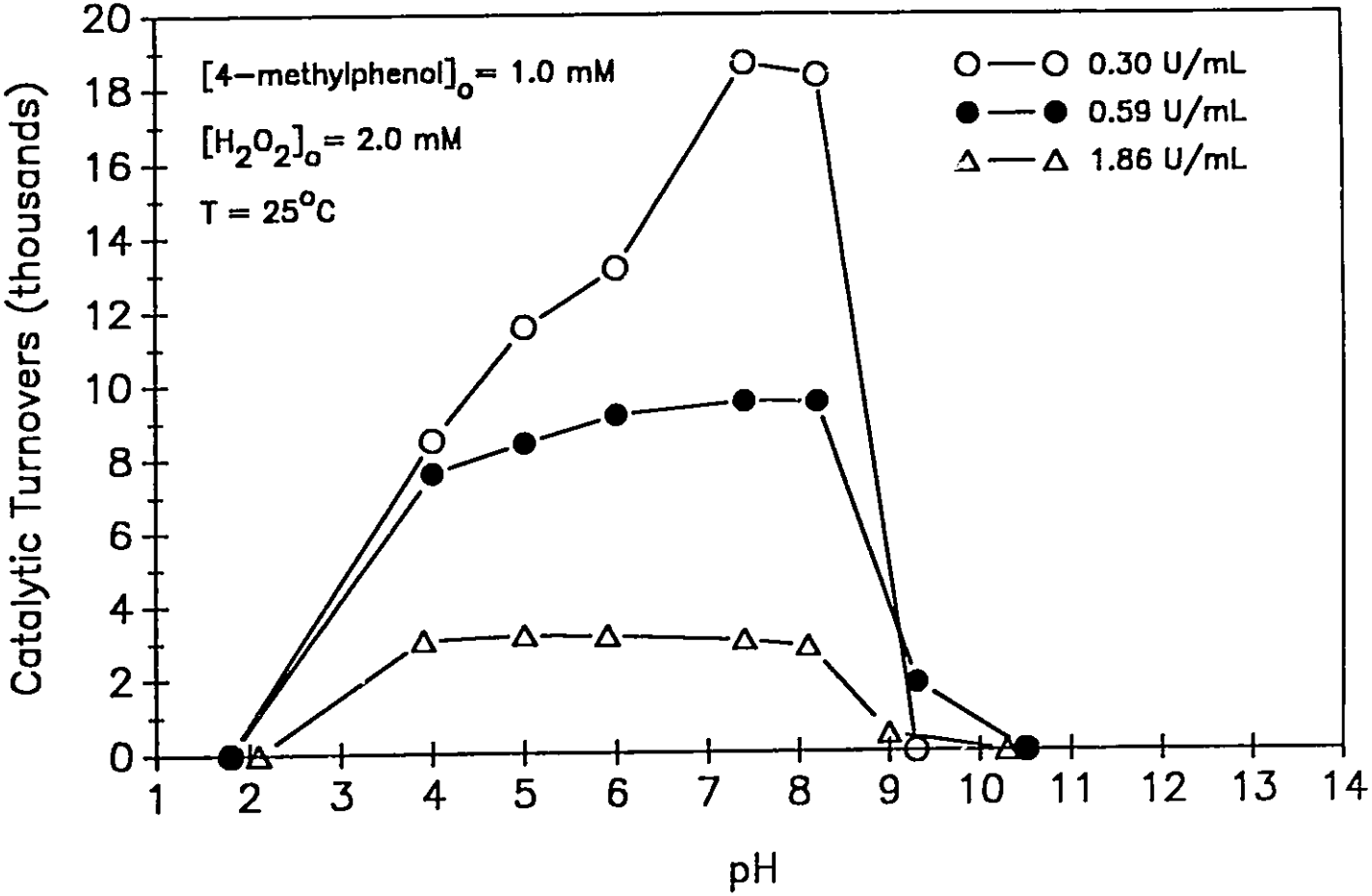

Figure D–8: Catalytic Turnovers of 4–Methylphenol as a Function of pH and HRP Dose

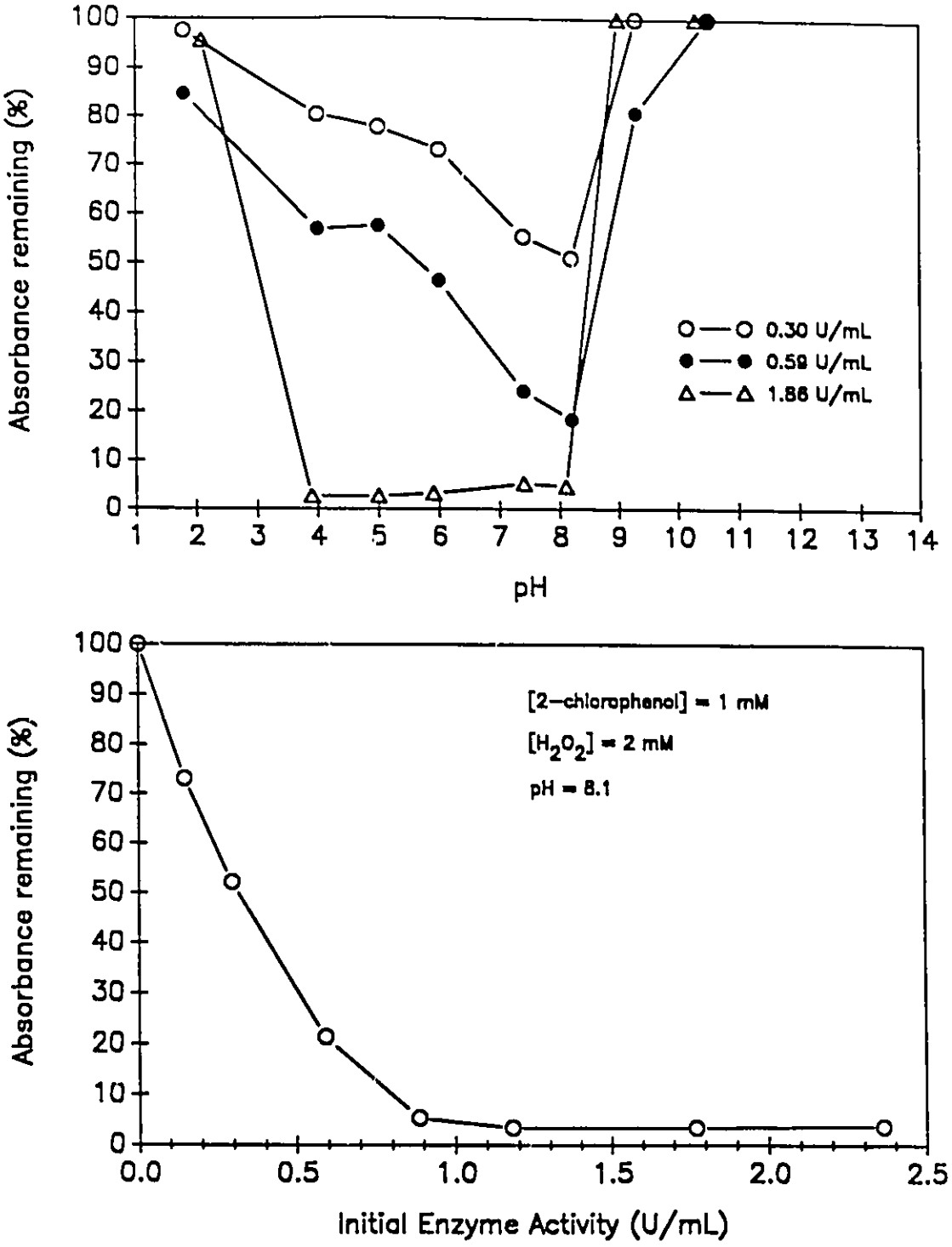

Figure D–9: Clearance of 2–Chlorophenol as a Function of (a) pH and (b) HRP Dose at the Optimal pH

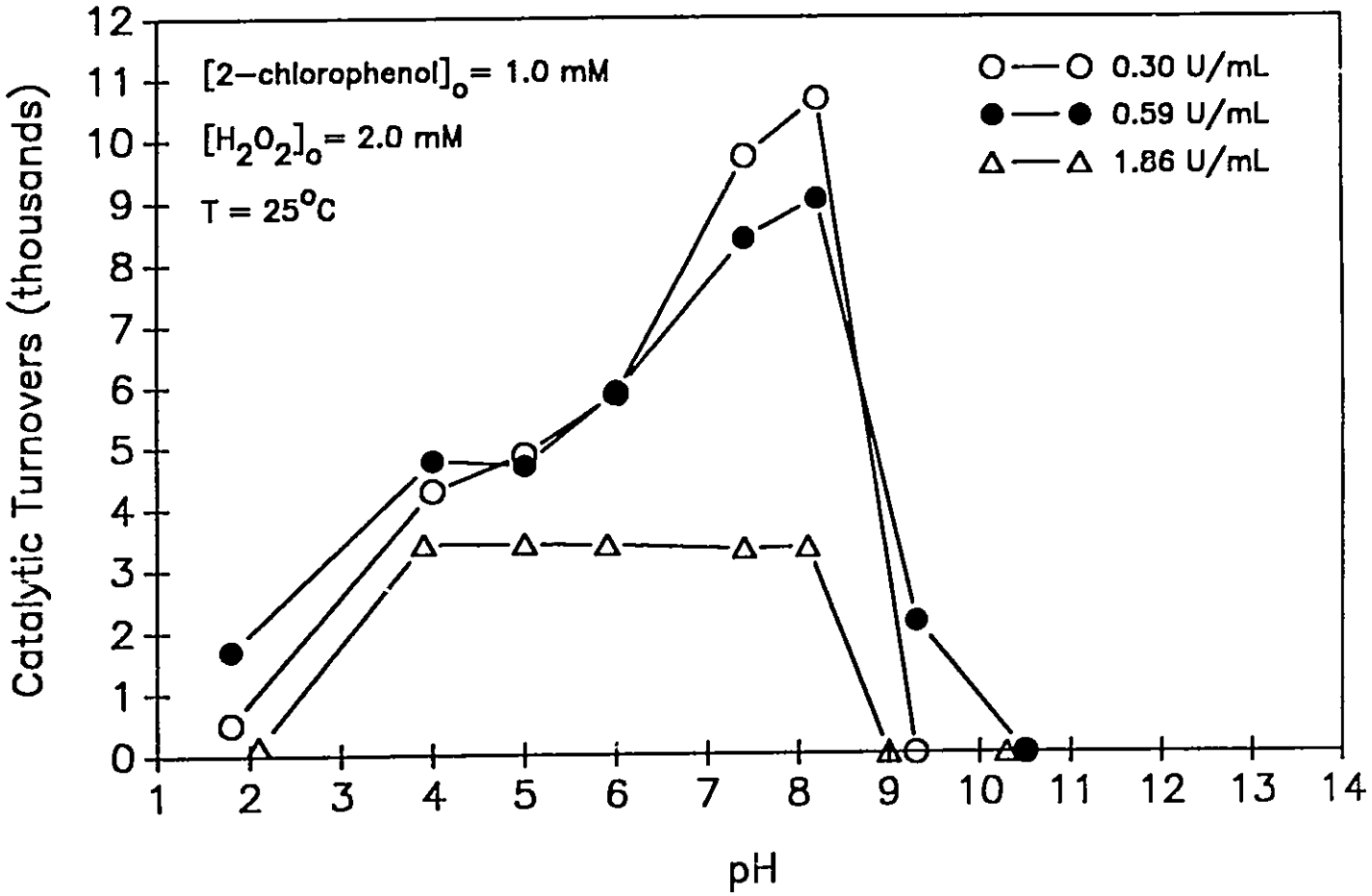

Figure D–10: Catalytic Turnovers of 2–Chlorophenol as a Function of pH and HRP Dose

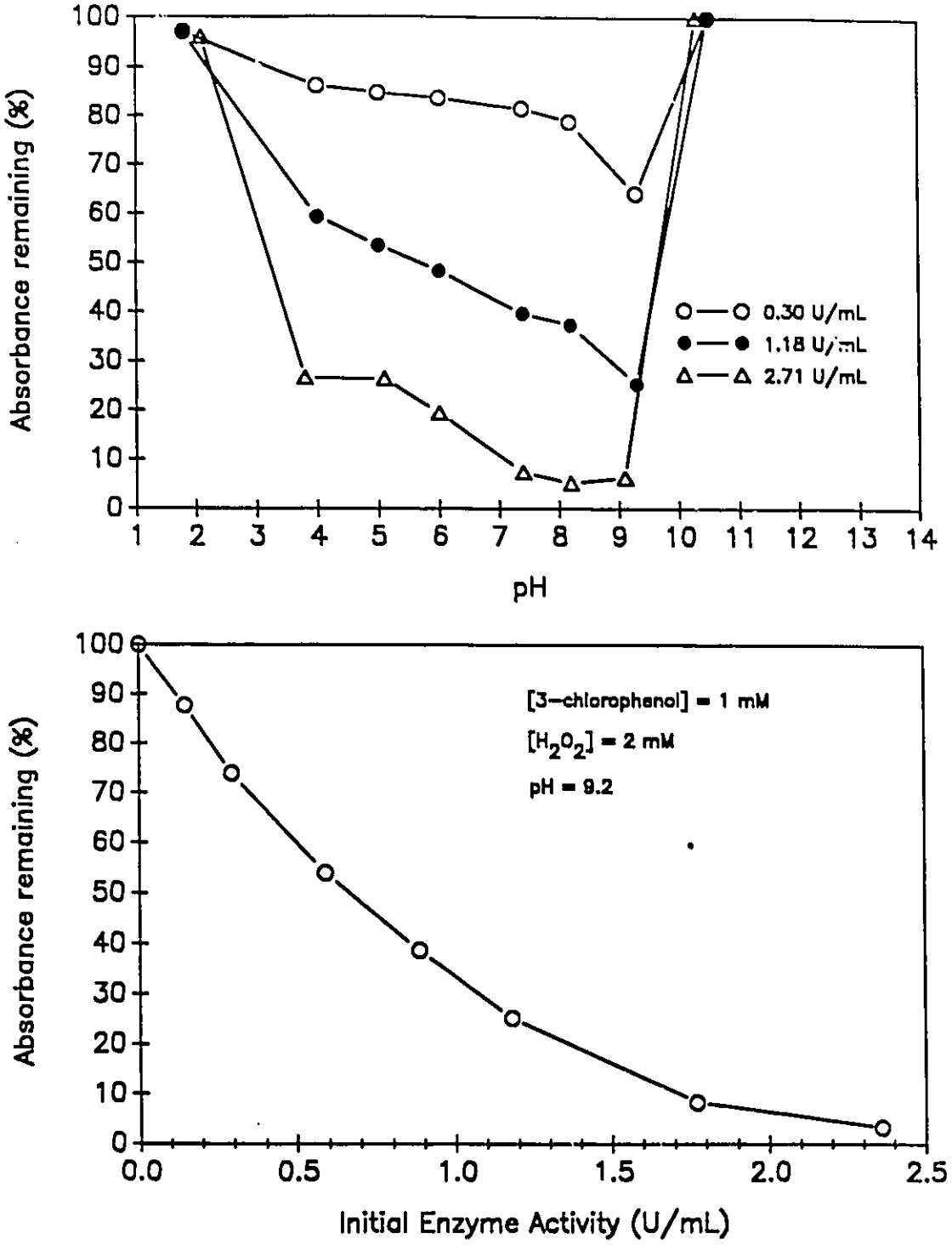

Figure D-11: Clearance of 3-Chlorophenol as a Function of (a) pH and (b) HRP Dose at the Optimal pH

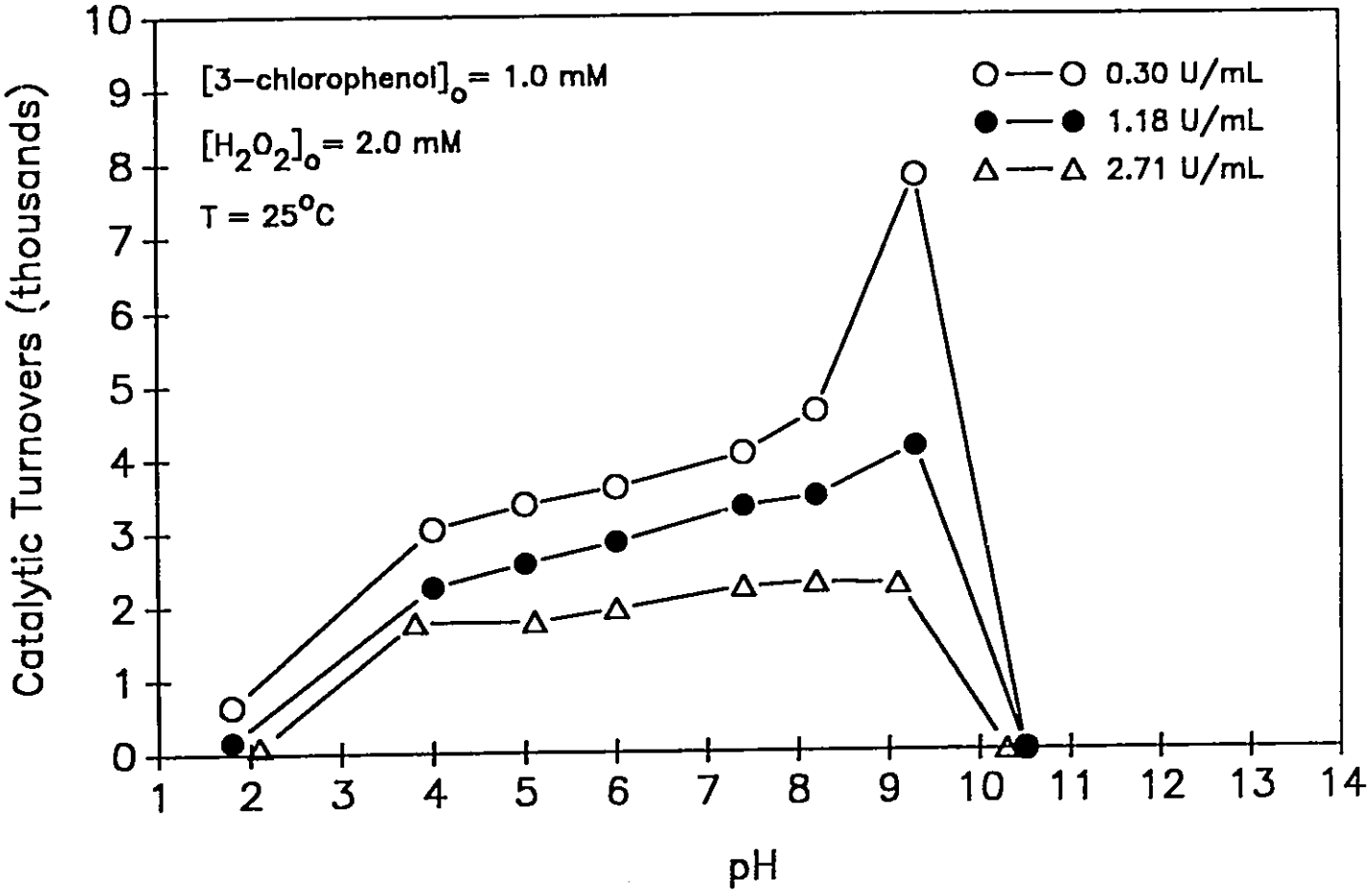

Figure D–12: Catalytic Turnovers of 3–Chlorophenol as a Function of pH and HRP Dose

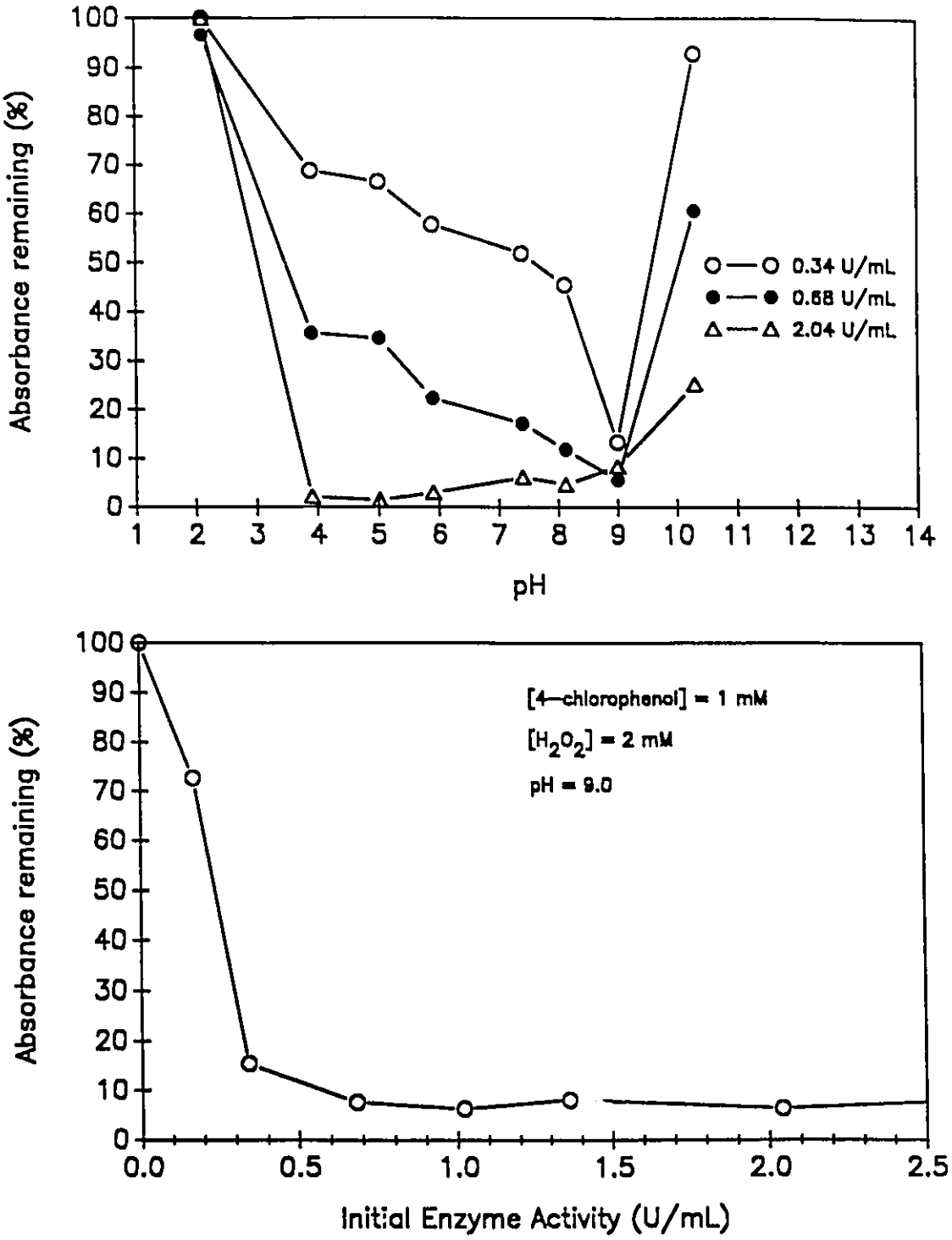

Figure D–13: Clearance of 4–Chlorophenol as a Function of (a) pH and (b) HRP Dose at the Optimal pH

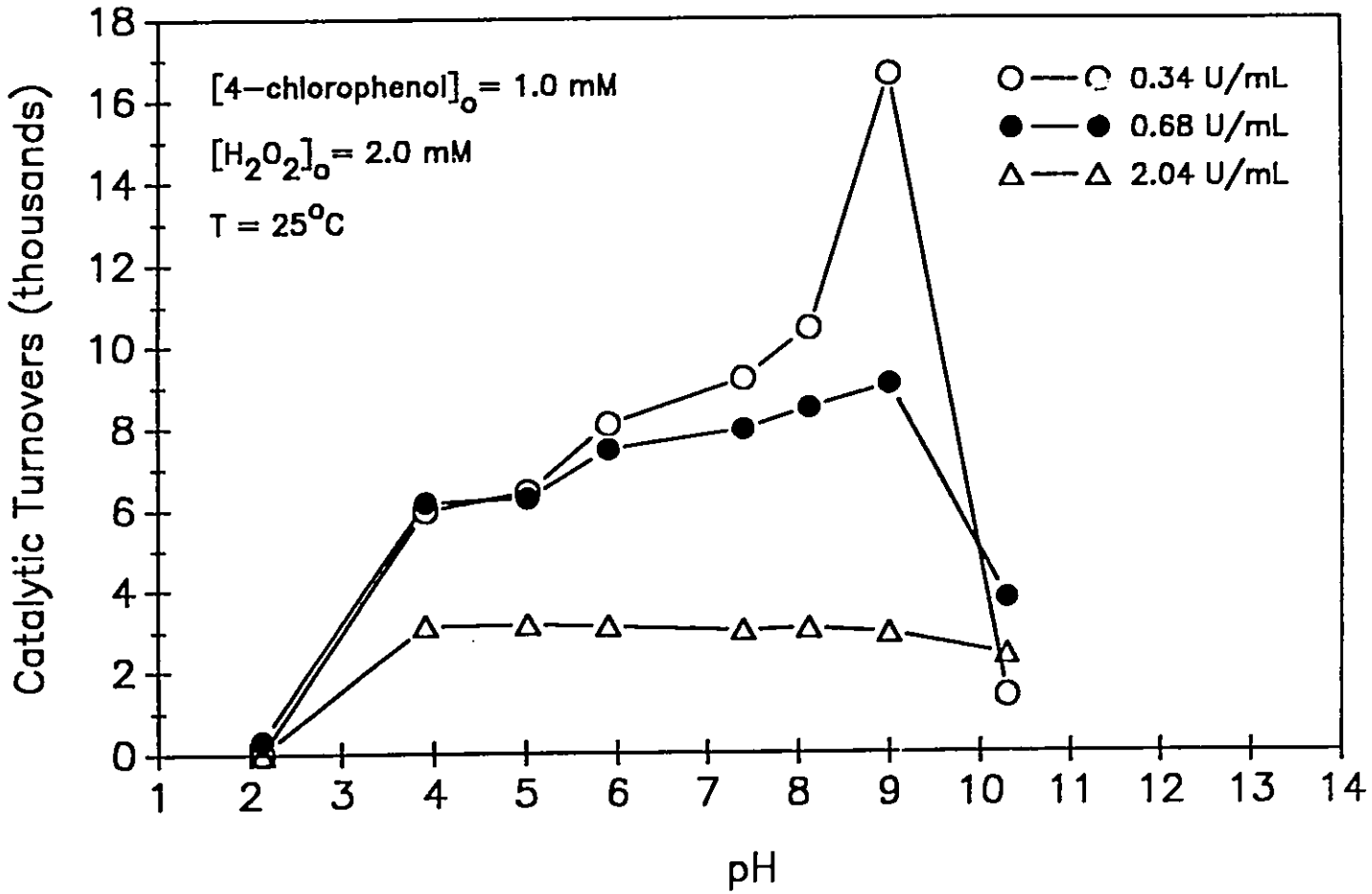

Figure D–14: Catalytic Turnovers of 4–Chlorophenol as a Function of pH and HRP Dose

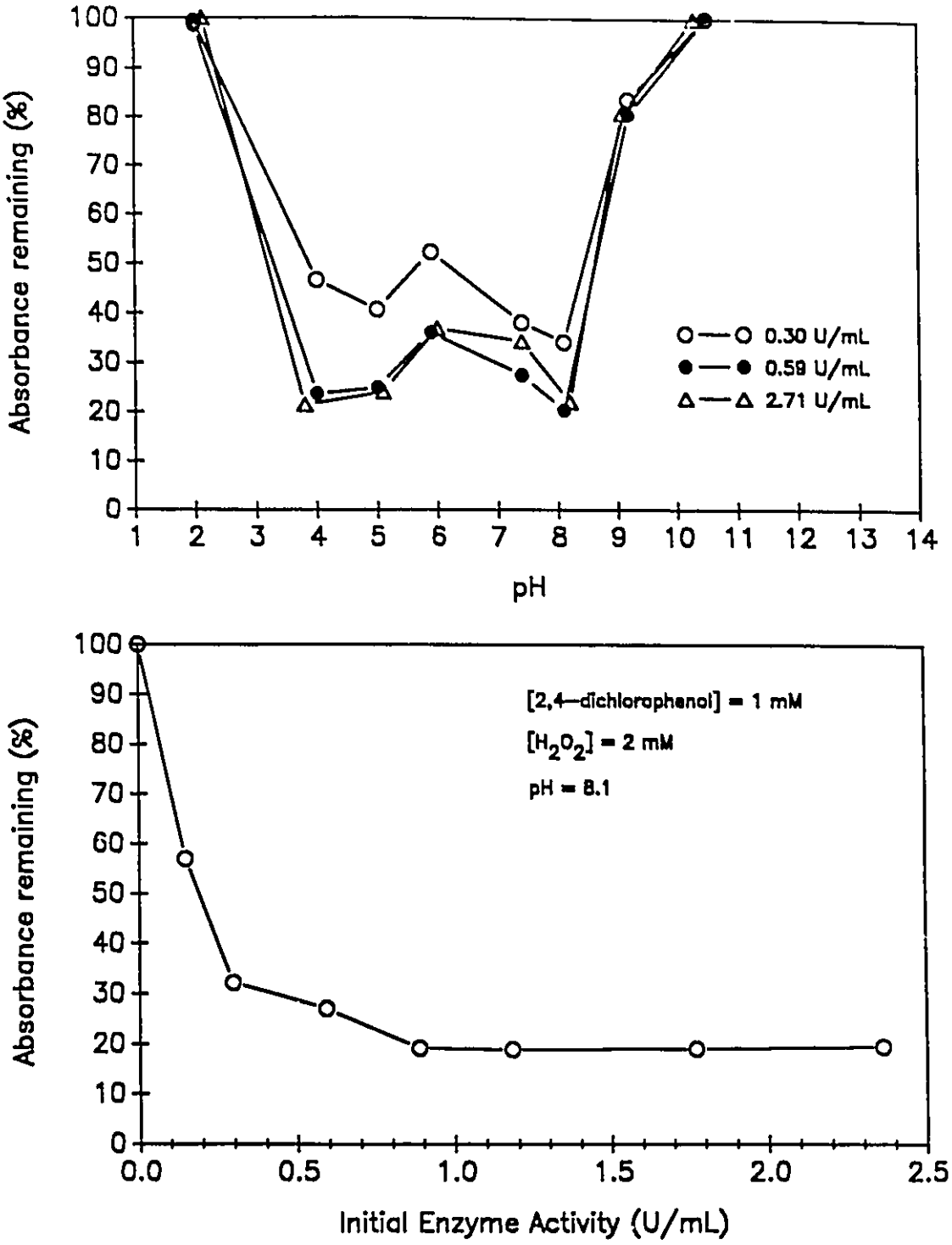

Figure D–15: Clearance of 2,4–Dichlorophenol as a Function of (a) pH and (b) HRP Dose at the Optimal pH

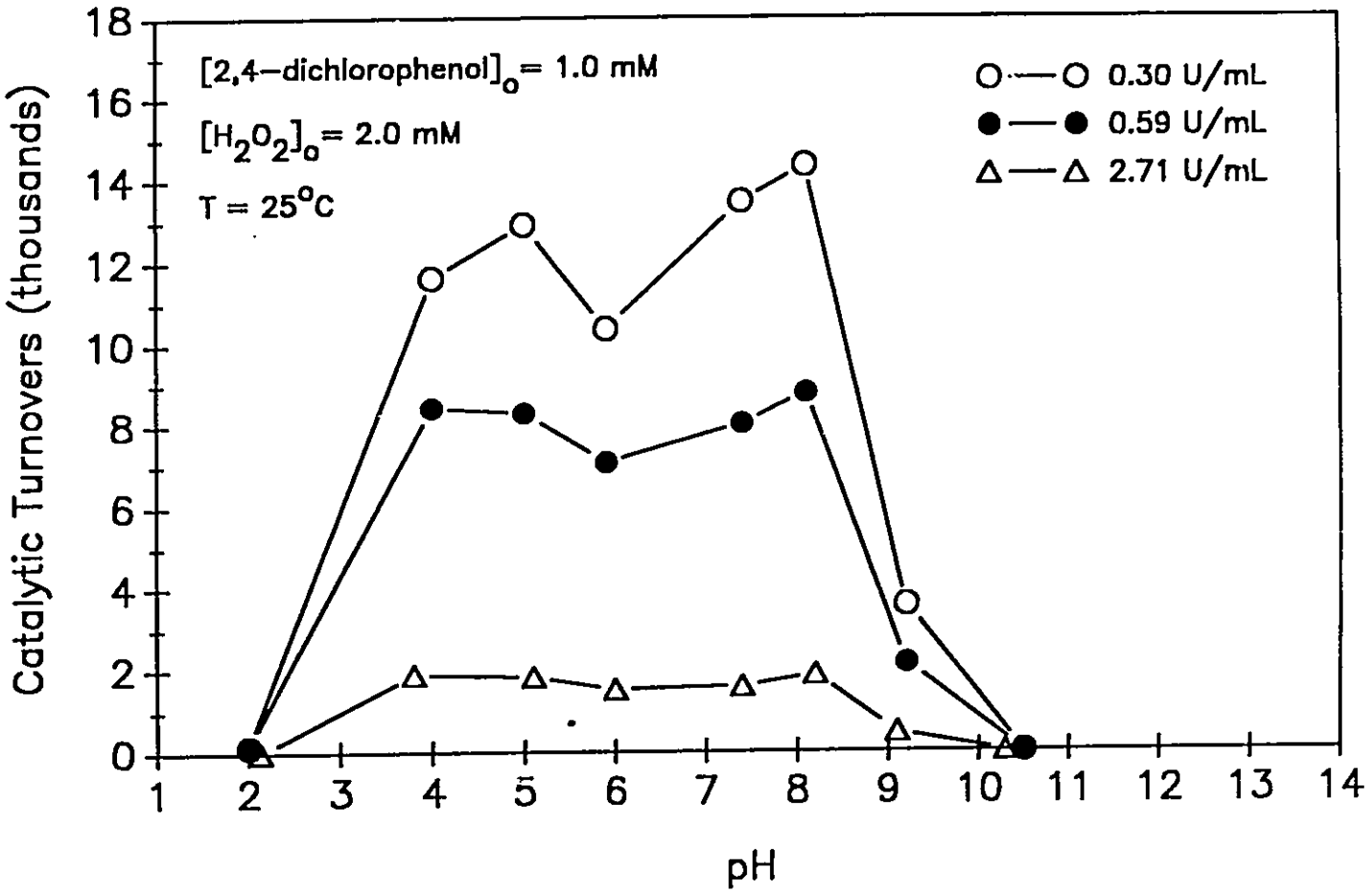

Figure D–16: Catalytic Turnovers of 2,4–Dichlorophenol as a Function of pH and HRP Dose

APPENDIX E

Clearance of Phenol and 4–Chlorophenol as a Function of Time in a Batch Reactor at Several Aromatic, Peroxide and Horseradish Peroxidase Concentrations at Neutral pH and 25°C

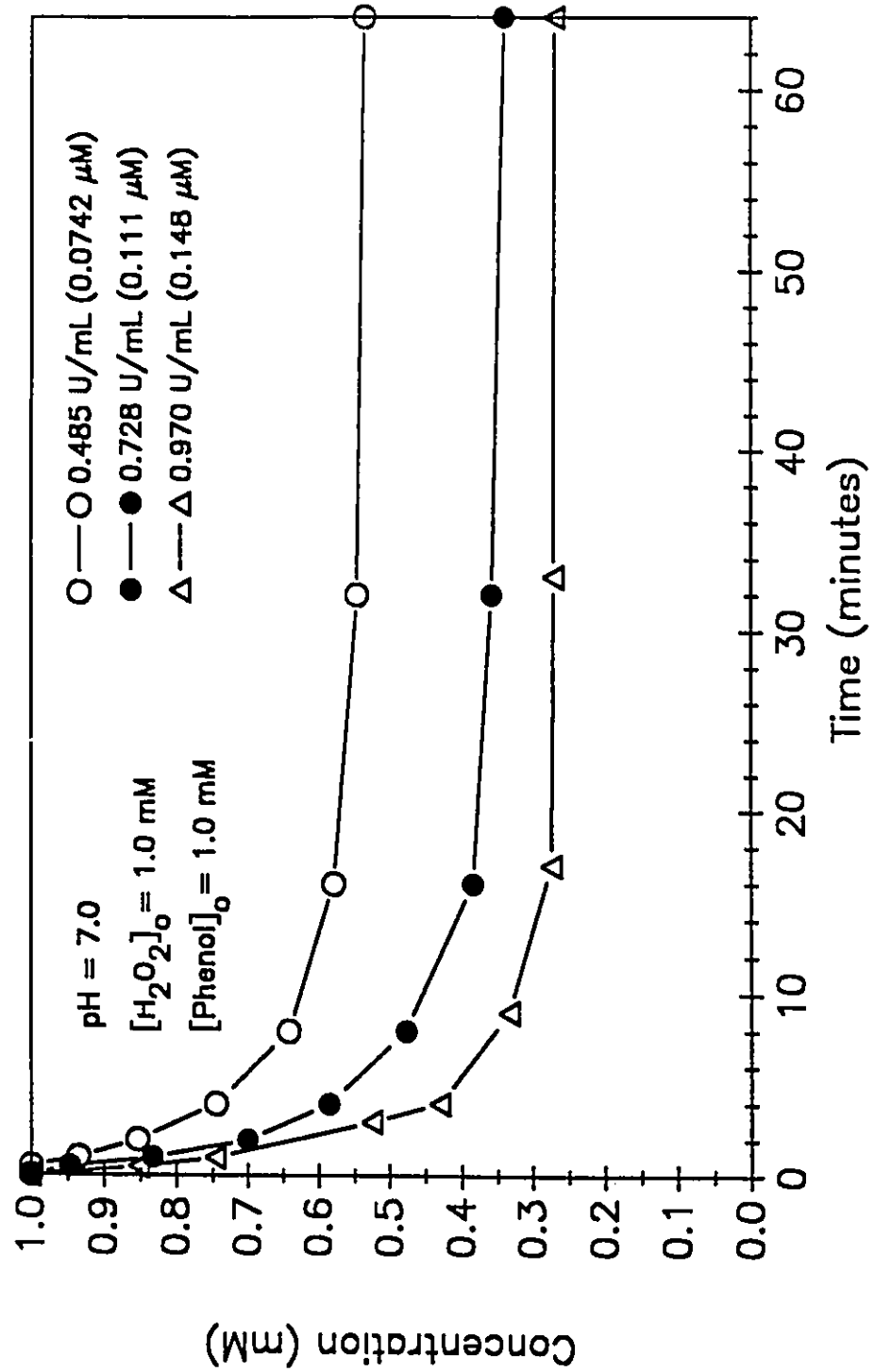

Figure E–1: Reaction of Phenol as a Function of Time in a Batch Reactor at Various Initial HRP Concentrations

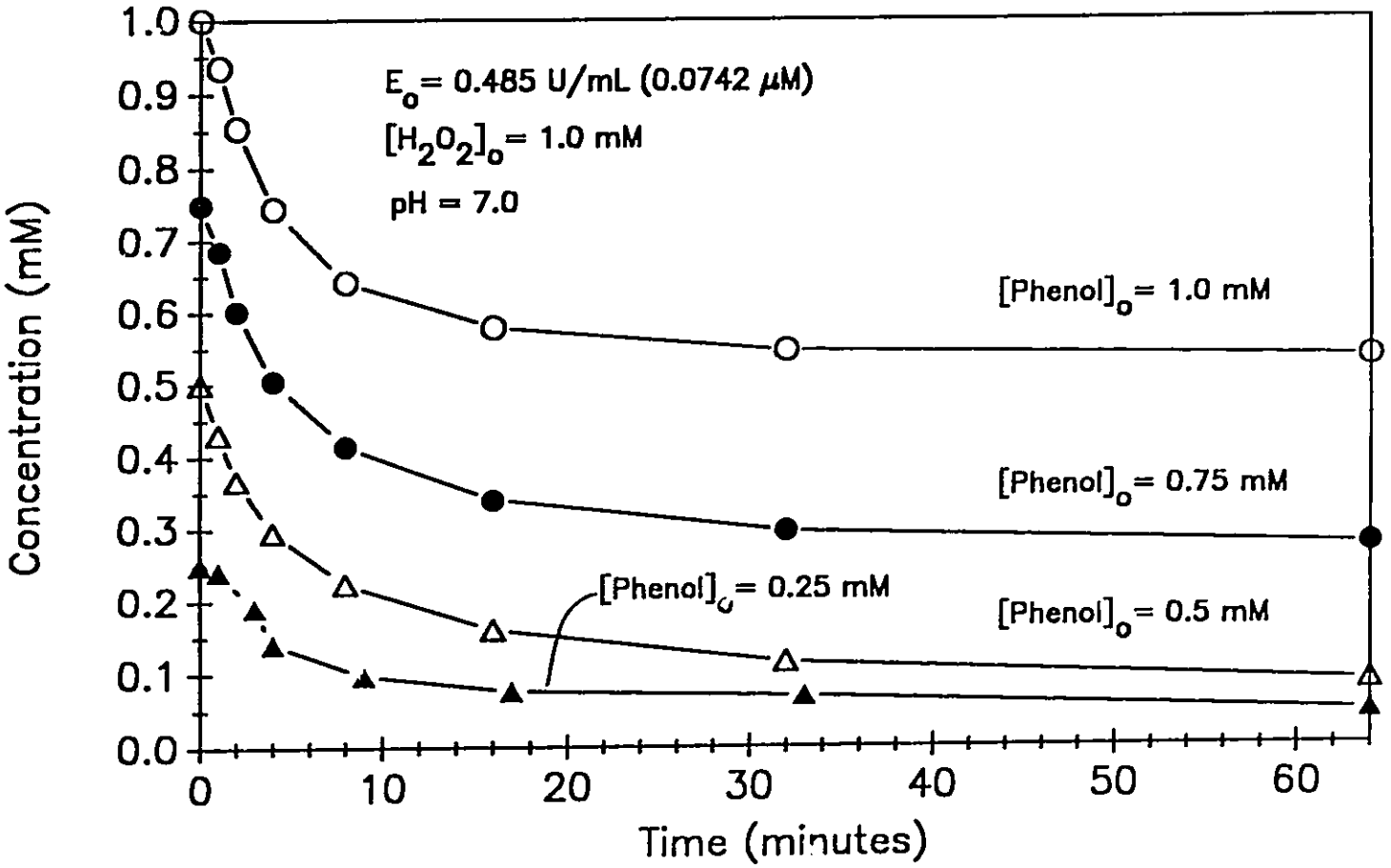

Figure E–2: Reaction of Phenol as a Function of Time in a Batch Reactor at Various Initial Concentrations of Phenol

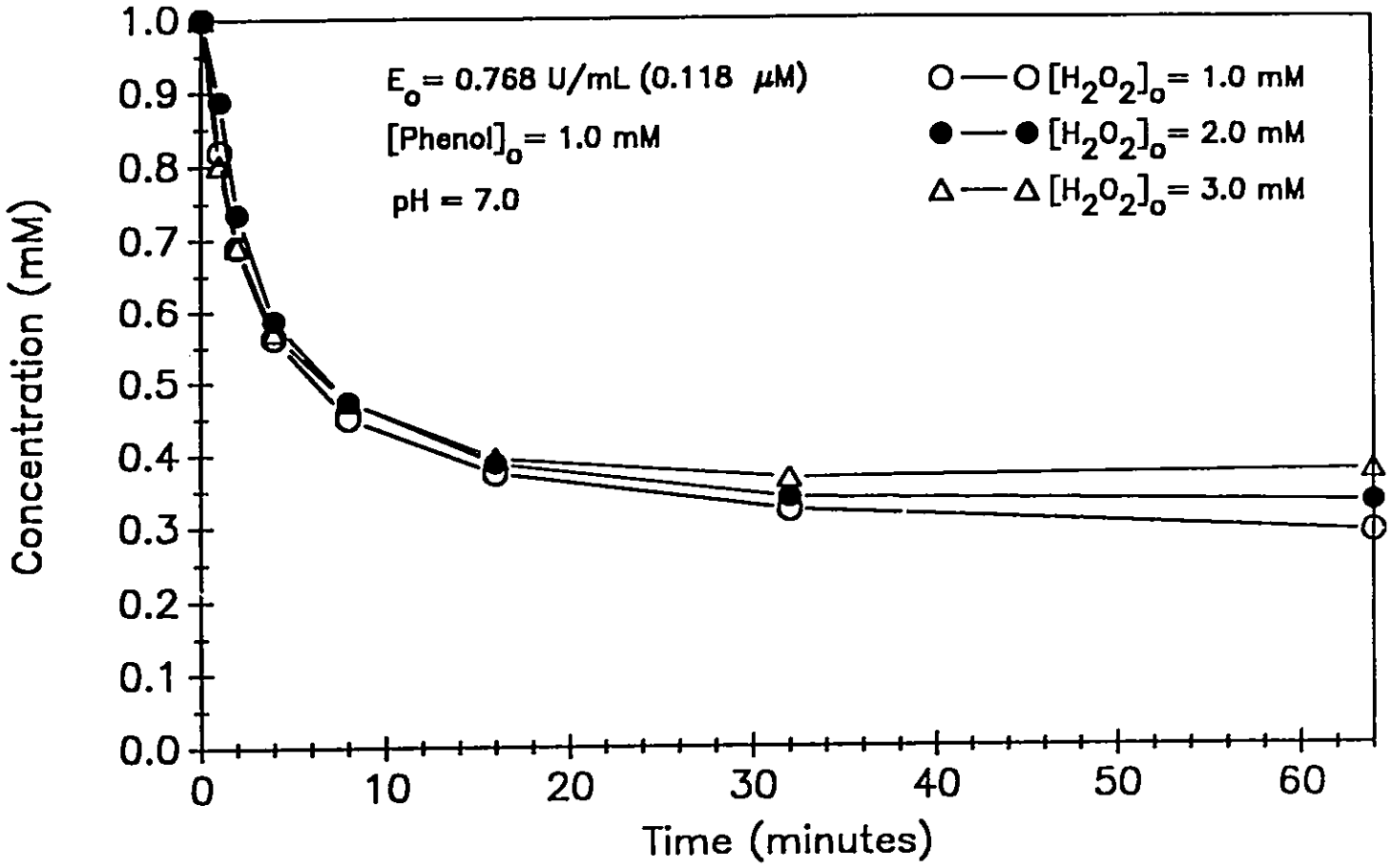

Figure E–3: Reaction of Phenol as a Function of Time in a Batch Reactor at Various Initial Concentrations of Hydrogen Peroxide

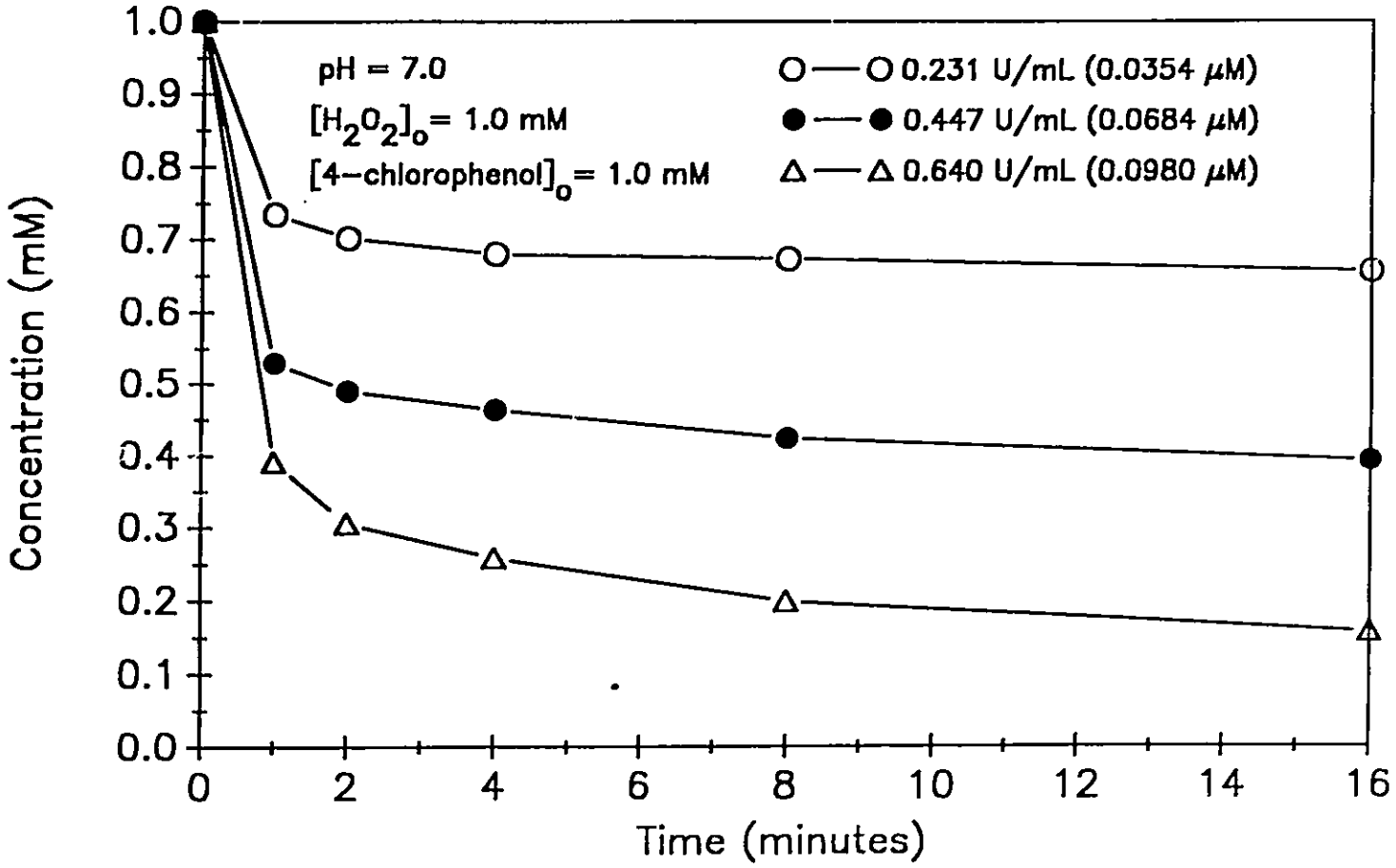

Figure E–4: Reaction of 4–Chlorophenol as a Function of Time in a Batch Reactor at Various Initial HRP Concentrations

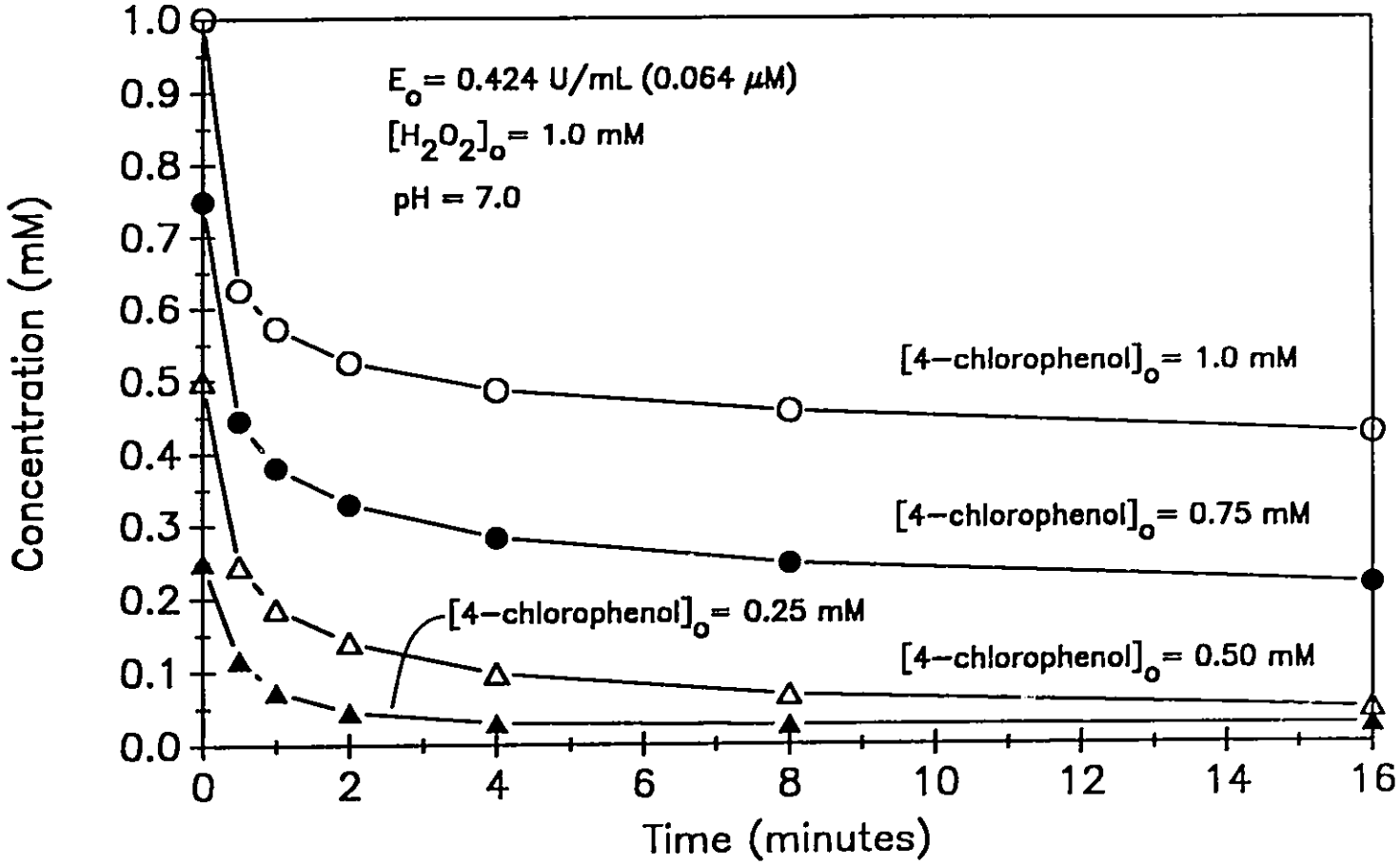

Figure E–5: Reaction of 4–Chlorophenol as a Function of Time in a Batch Reactor at Various Initial Concentrations of 4–Chlorophenol

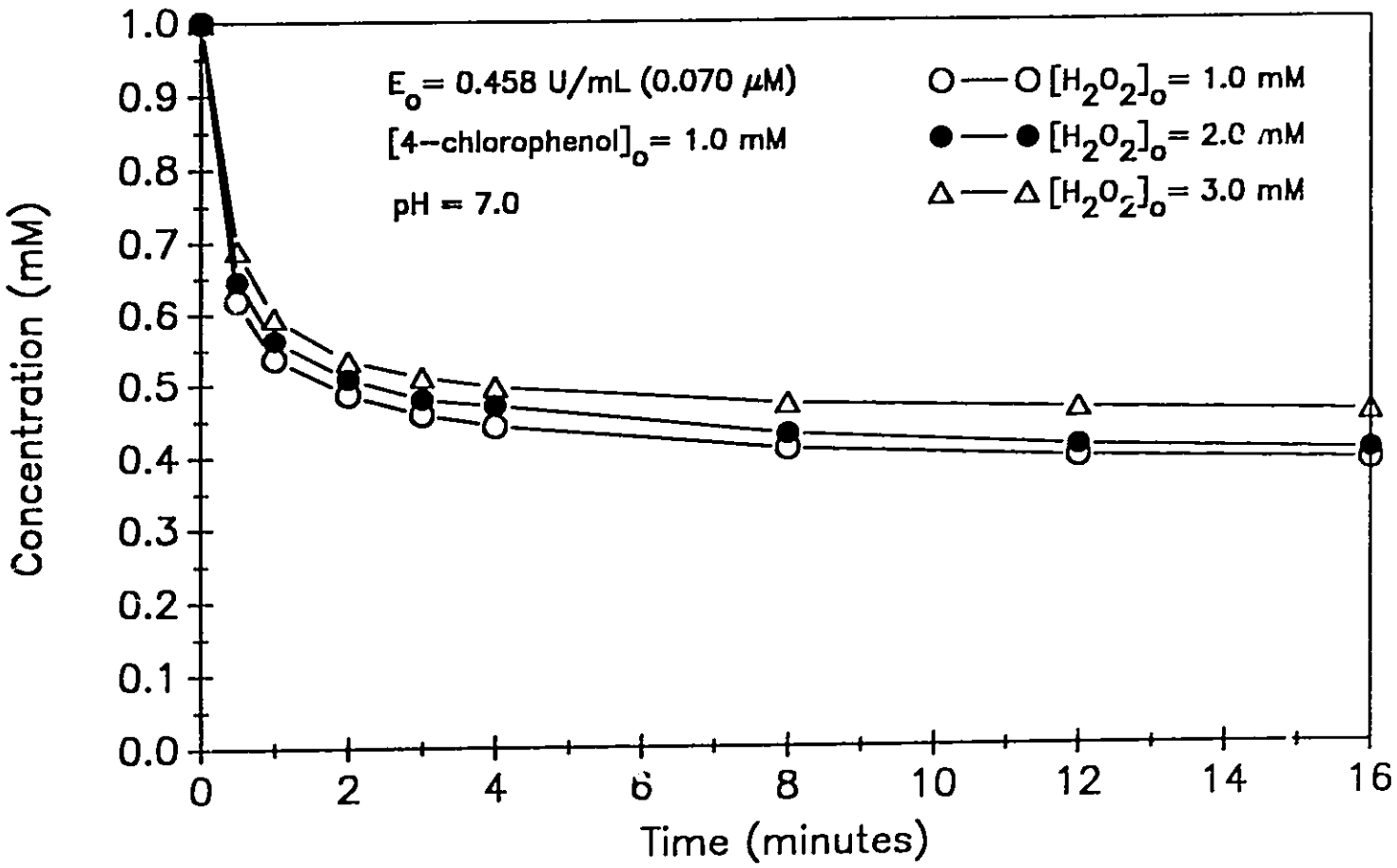

Figure E–6: Reaction of 4–Chlorophenol as a Function of Time in a Batch Reactor at Various Initial Concentrations of Hydrogen Peroxide

APPENDIX F

Sensitivity Analysis of the Pseudo–Steady State Kinetic Model

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Sensitivity Analysis of the Pseudo–Steady State Kinetic Model

The pseudo–steady state model developed in this study is based on six constants (k_1, k_7, k_8, k_9, k_{app} and K_s). The number of catalytic turnovers (K_s) was measured in this laboratory. The remaining five kinetic rate constants were measured by other authors under conditions which were not completely identical to those used here and by each other. Despite these differences, it has been demonstrated that the model fits experimental data for 4–chlorophenol satisfactorily. A sensitivity analysis is necessary to determine the relative effect that variations in the measurement of these constants will have on model output.

A sensitivity analysis was performed by fixing the input conditions in the kinetic model and varying the values of the model constants one at a time. The input conditions that were chosen were 1 mM 4–chlorophenol, 1 mM H_2O_2 and 0.5 U/mL horseradish peroxidase. The model constants for this system are:

$$k_1 = 2.0 \times 10^7 \ M^{-1} s^{-1}$$

$$k_{app} = 31 \ M^{-1} s^{-1}$$

$$k_7 = 4.2 \times 10^{-3} \ s^{-1}$$

$$k_8 = 1.13 \times 10^7 \ M^{-1} s^{-1}$$

$$k_9 = 1.1 \times 10^6 \ M^{-1} s^{-1}$$

$$K_s = 9\ 000 \text{ turnovers}$$

Figure F–1 shows that the model is extremely insensitive to changes in the value of k_1. Increasing its value has no effect on model results since this rate constant does not limit the rate of catalytic turnovers; this rate is limited by the smallest rate constant in the catalytic cycle, k_9. Decreasing the value of k_1 shows some effect, however, changes on the order of one magnitude are needed before the effect becomes significant.

The rate constant k_{app} describes the rate of formation of compound III from compound II. Since most of the active enzyme at any one instant is present in the compound II state (due to the limiting value of k_9 in the catalytic cycle), the value of k_{app} has significant effect on the model predictions. For example, Figure F–2 shows that with increasing values of k_{app} the initial period of rapid reaction rate is shortened and the period of slow reaction rate is lengthened dramatically due to overprediction of compound III formation. The opposite is experienced with decreased values of k_{app}. Therefore, this rate constant is responsible for describing the time at which the reaction rate changes from extremely rapid to extremely slow. In addition, the degree of compound III formation and its effects on the overall reaction time are extremely sensitive to changes in k_{app}.

Figure F–3 shows that the model is sensitive to changes in the value of k_7. This rate constant describes the rate of release of compound III back to the active native form of the enzyme. When increased significantly, this constant minimizes

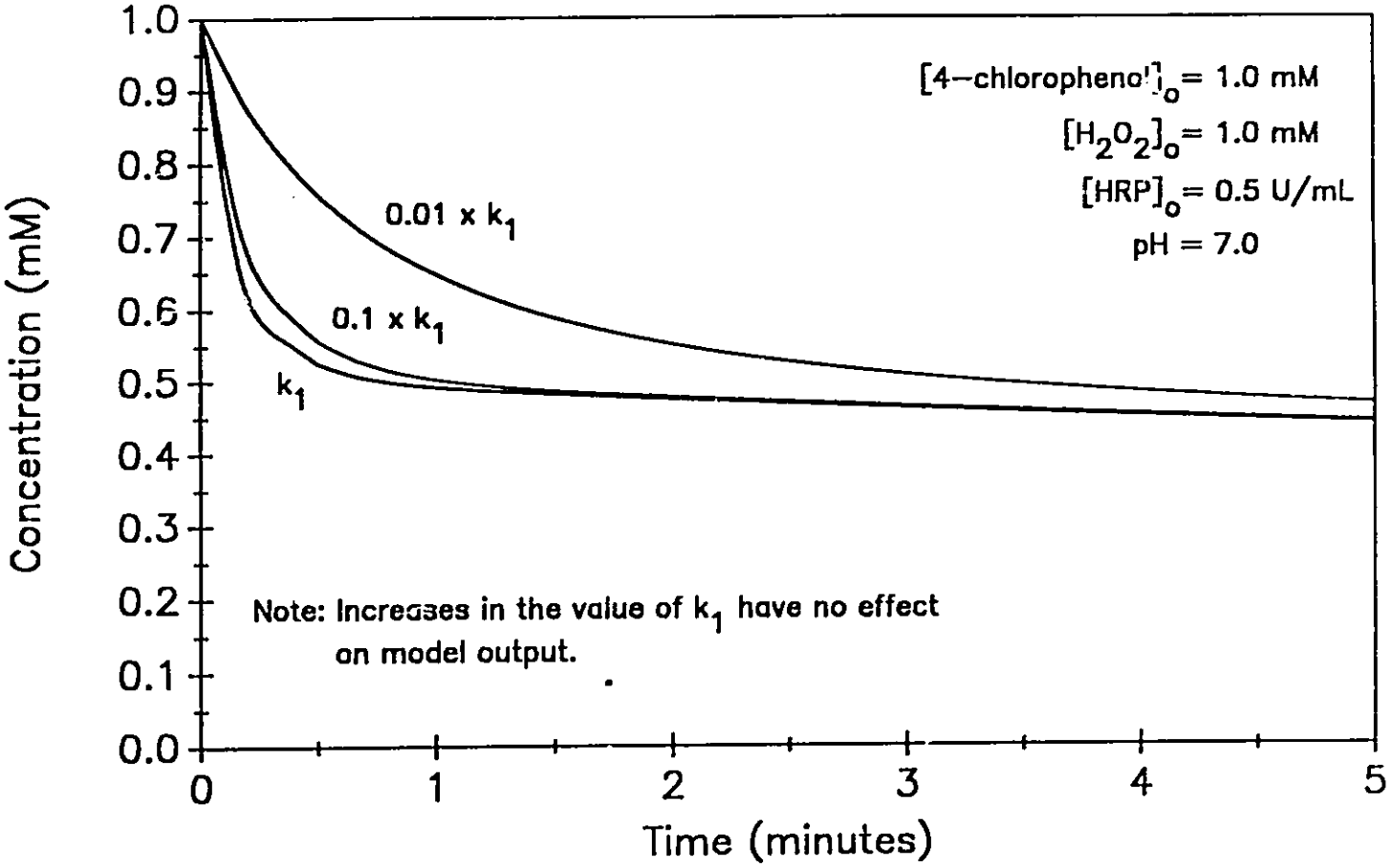

Figure F–1: Effect of Changes in k_1 on Model Output

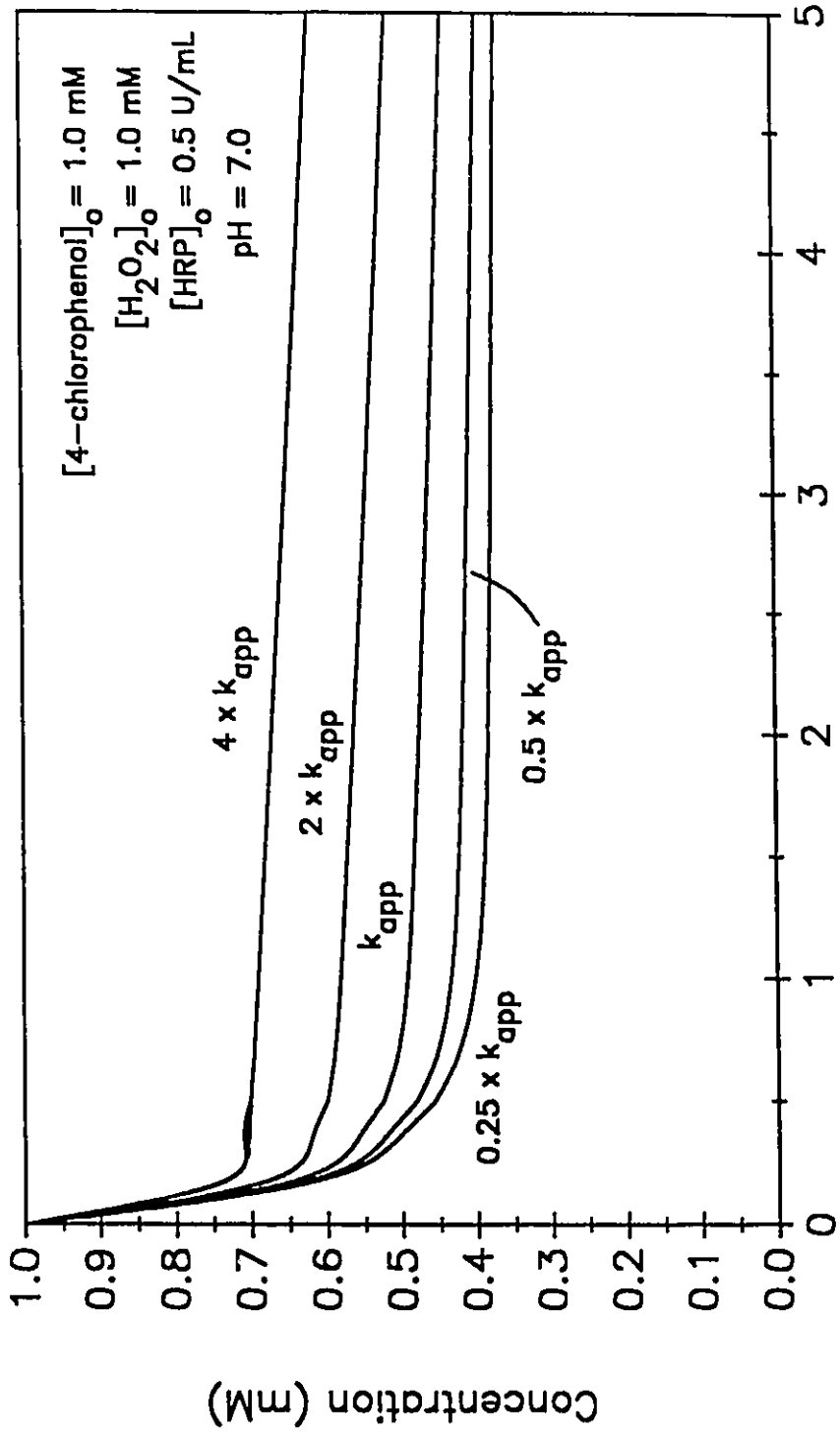

Figure F–2: Effect of Changes in k_{app} on Model Output

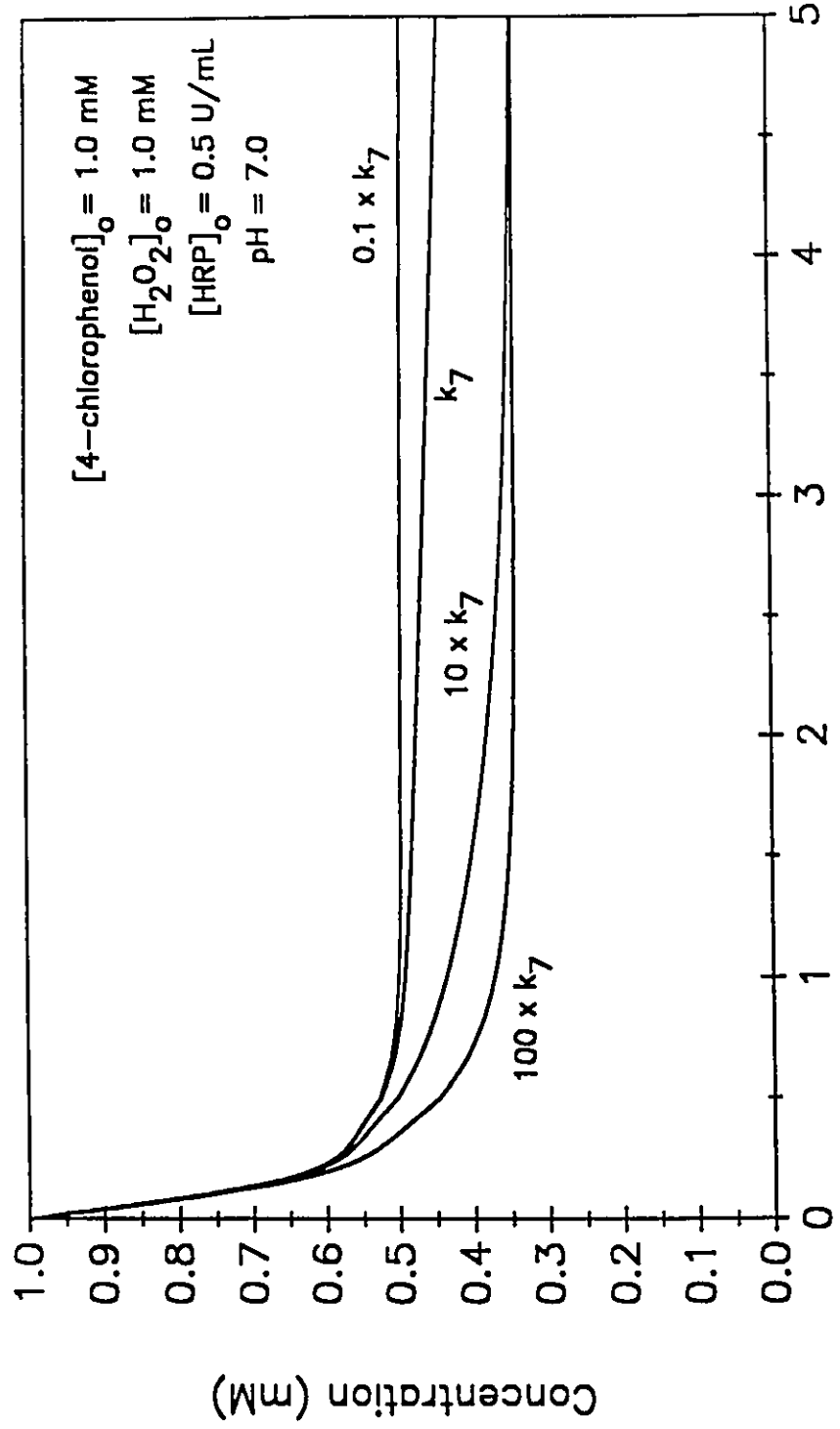

Figure F-3: Effect of Changes in k_7 on Model Output

the effect of compound III formation resulting in a premature prediction of the time of complete reaction. When decreased, the effects of compound III formation are overpredicted, with very slow return of the enzyme back to the native state.

Figure F–4 demonstrates that the pseudo–steady state model is fairly insensitive to major changes in the rate constant k_8. As in the case for k_1, k_8 is not the limiting rate constant in the catalytic cycle; therefore, increases in its value have minimal effect on model output. However, when k_8 is decreased to values below k_9 ($k_8 \simeq 10 \times k_9$), it becomes the limiting rate constant and begins to demonstrate more significant effects on the model. Therefore, overestimation of k_8 has little effect on model results but underestimation can result in significant changes in modelling capability.

The constant k_9 is the limiting rate constant in the catalytic cycle and hence was expected to cause the greatest sensitivity in the model. This was verified as shown in Figure F–5 where variations in k_9 result in major changes in model output. This suggests that accurate measurements of k_9 are critical to model performance.

The degree of removal predicted by the model under any conditions is totally dependent on the number of turnovers that the enzyme can achieve. Therefore, any change in the value of K_s will result in a change in the degree of removal achieved by the enzyme before or at the end point of the reaction. This is demonstrated in Figure F–6.

These results have indicated that the model is insensitive to changes in k_1 and k_8 because they are not the limiting rate constants in the catalytic cycle.. The degree of compound III formation and its effect on the time at which the reaction goes to completion is very sensitive to changes in k_7 and k_{app}. The value of k_9 is critical since it is the rate limiting step in the catalytic cycle. It defines the overall rate of reaction and the amount of compound II available for conversion to compound III. The model is extremely sensitive to changes in K_s because it defines the degree of removal that can be achieved by a given dose of enzyme.

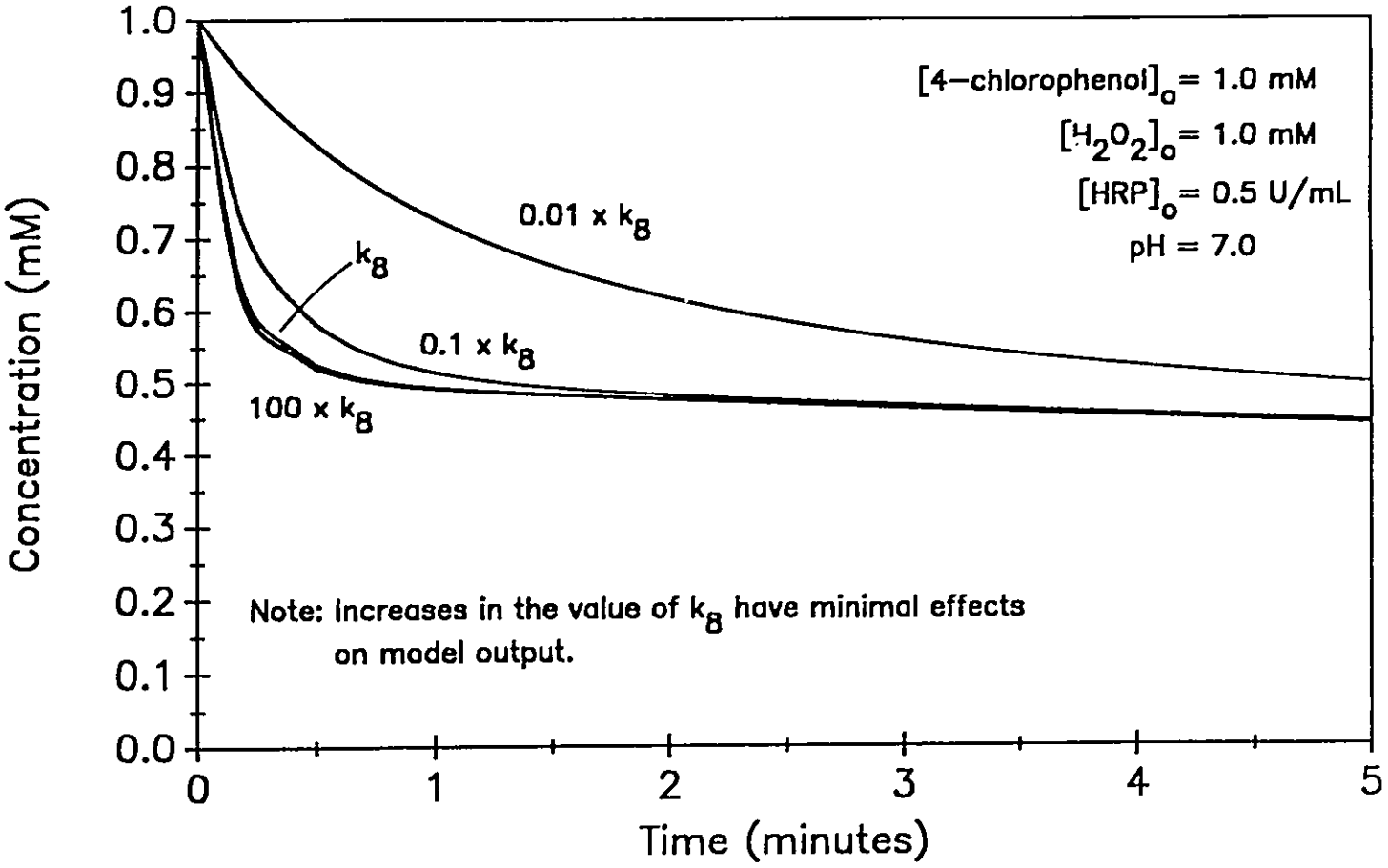

Figure F–4: Effect of Changes in k_8 on Model Output

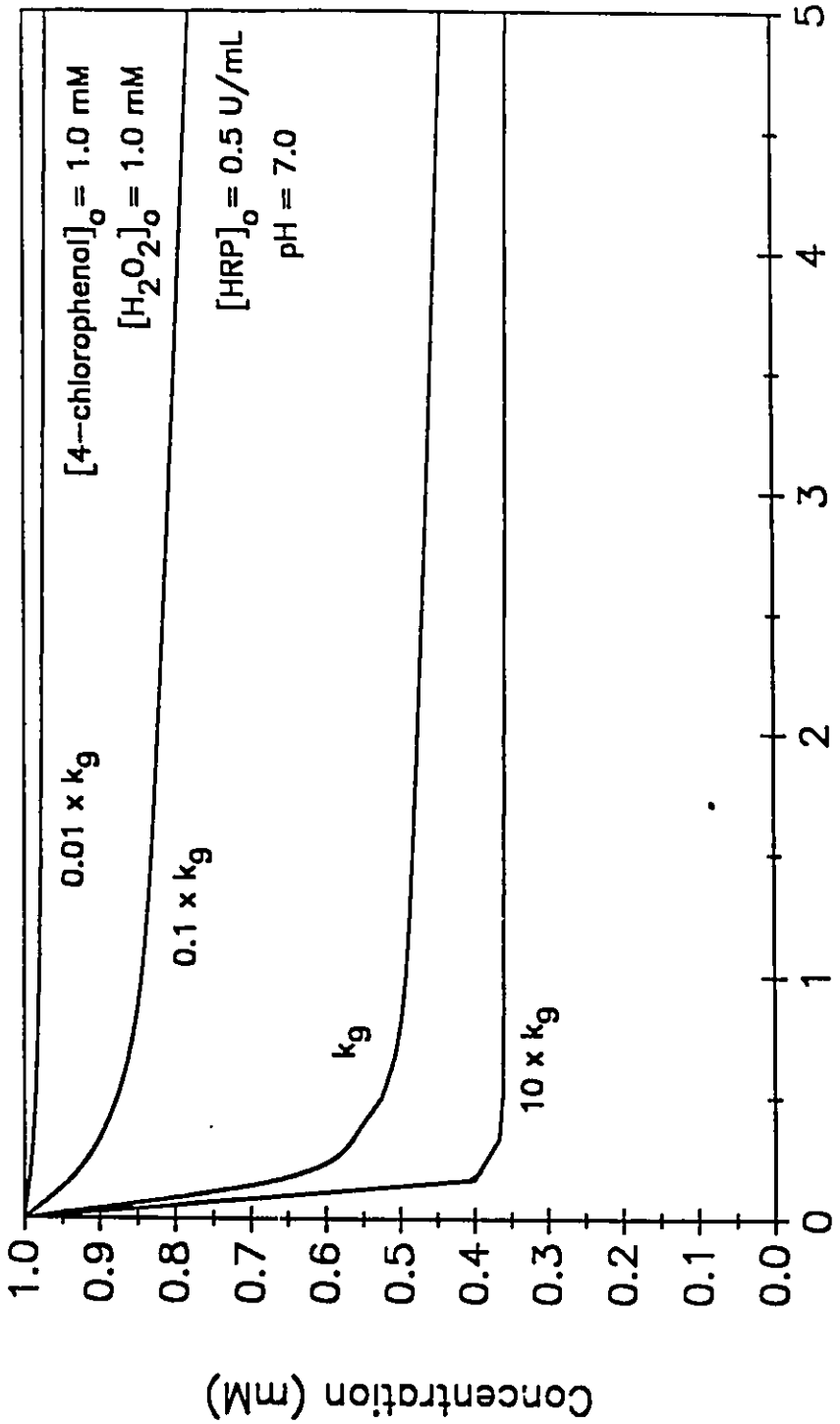

Figure F–5: Effect of Changes in k_g on Model Output

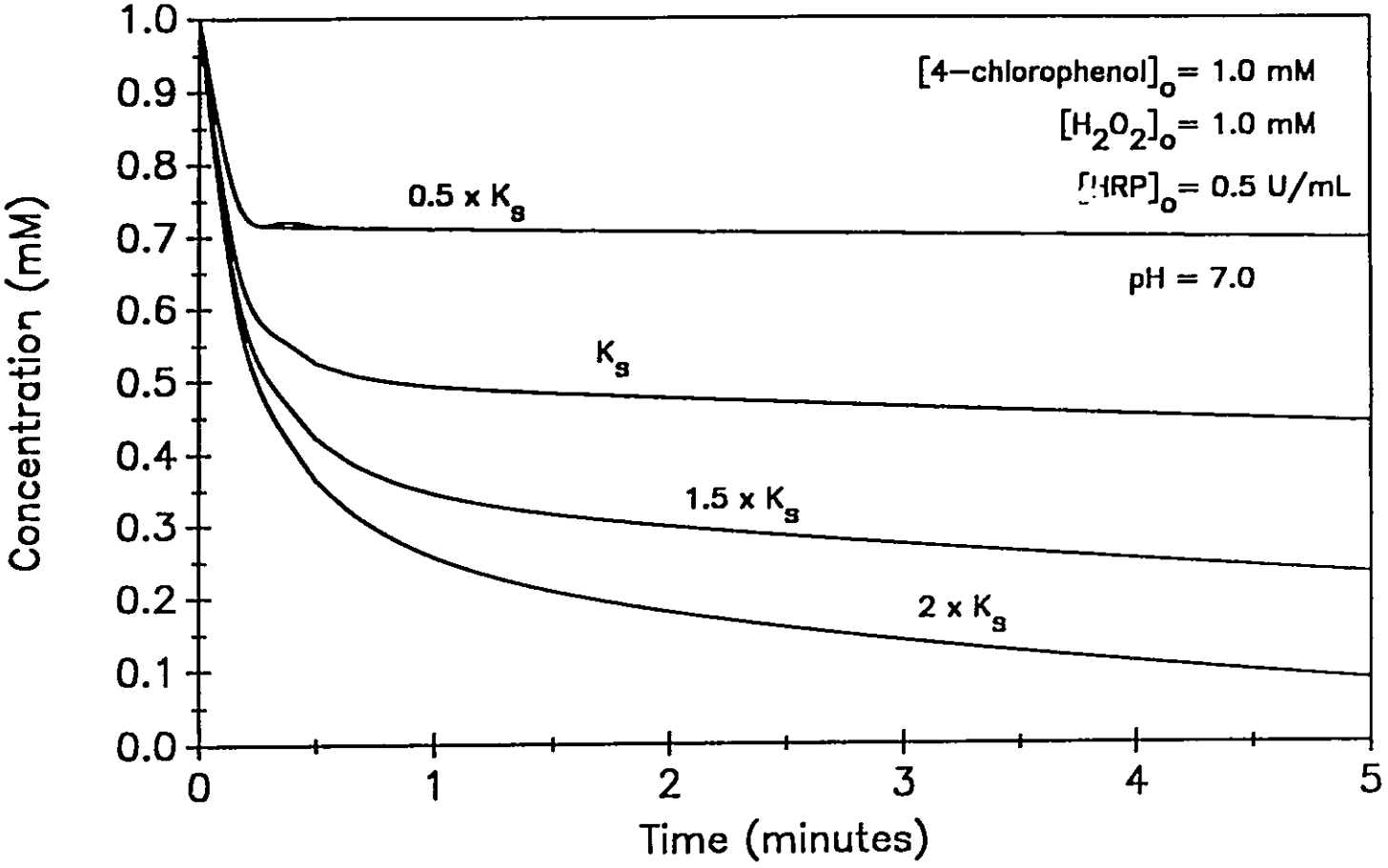

Figure F–6: Effect of Changes in K_s on Model Output

APPENDIX G

Removal of Phenol and 4–Chlorophenol in Single and Staged CSTR's With Fixed Inlet Aromatic and Peroxide Concentrations, Enzyme Dose, and Total Retention Time (pH 7.0 and 25°C)

APPENDIX G

Removal of Phenol and 4–Chlorophenol in Single and Staged CSTR's With Fixed Inlet and Aromatic Concentrations, Enzyme Dose, and Total Retention Time (pH 7.0 and 25°C)

All single and multiple CSTR experiments described in this appendix were conducted under the following conditions:

- a constant flowrate (2.0 mL/min) of influent consisting of 1 mM aromatic and 1 mM peroxide fed to the first in the series of reactors.
- a constant total flowrate (0.010 mL/min) of enzyme supplied to the reactor system. Enzyme flowrate was minimal with respect to influent flowrate (< 1%) and was divided equally among the reactors in the series.
- influent was buffered by phosphate buffer at pH 7.0 and 25°C
- a constant total system volume (120 mL) resulting in a fixed hydraulic retention time (1 hour):
    - single reactor – 120 mL
    - two reactors in series – 60 mL each
    - three reactors in series – 40 mL each
    - four reactors in series – 30 mL each
- the reactor system was allowed to come to equilibrium over a period of three hydraulic retention times (3 hours) before samples were taken. At the end of this period, six samples of effluent were taken at 10 minute intervals. A volume of 9.5 mL of effluent was collected and mixed continuously in a sample tube containing 0.5 mL of 0.4 mg/mL catalase to halt the reaction.
- samples were centrifuged at 3000 G for 40 minutes prior to analysis.

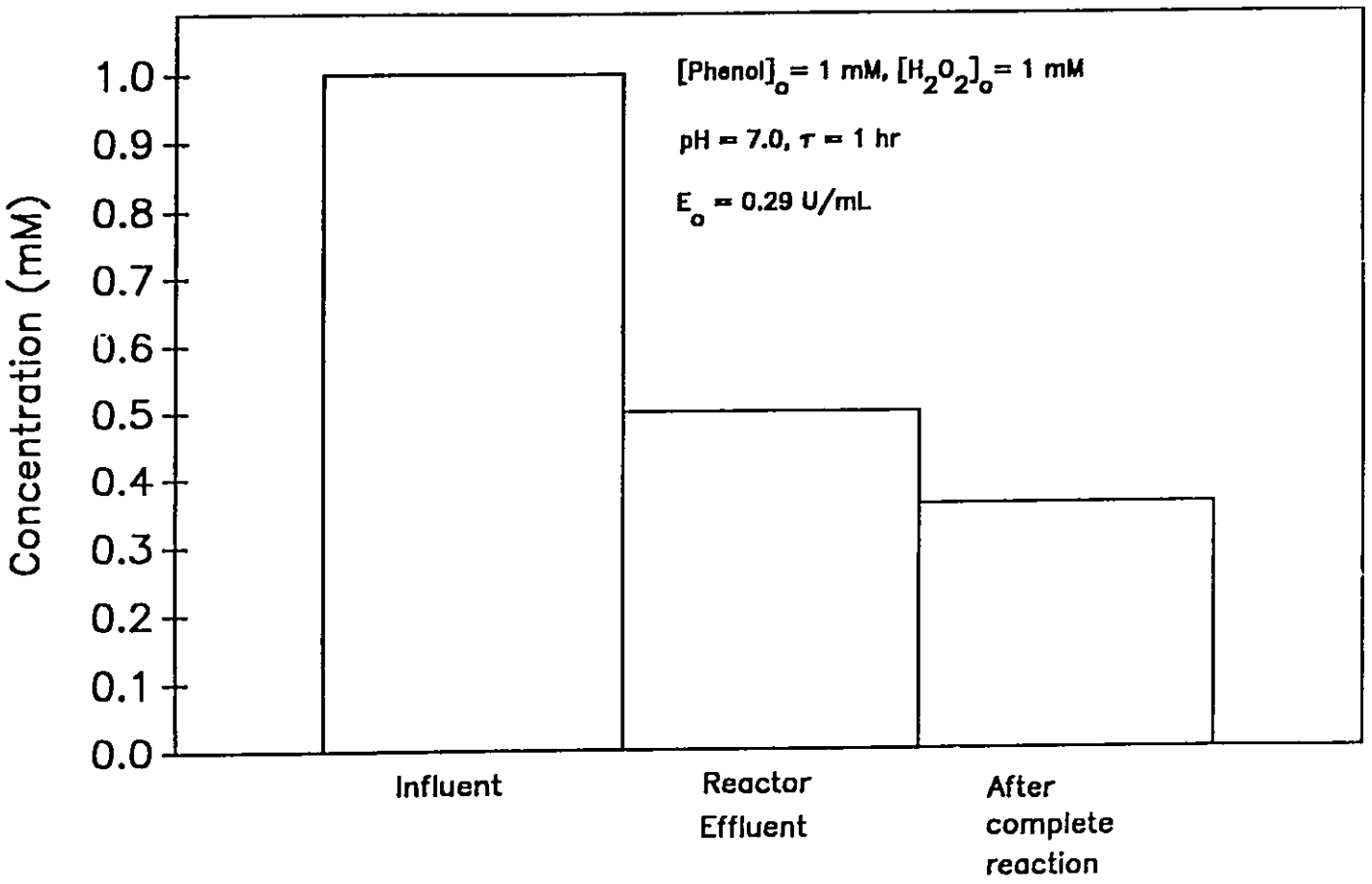

Figure G–1: Removal of Phenol in a Single CSTR at Neutral pH and 25°C

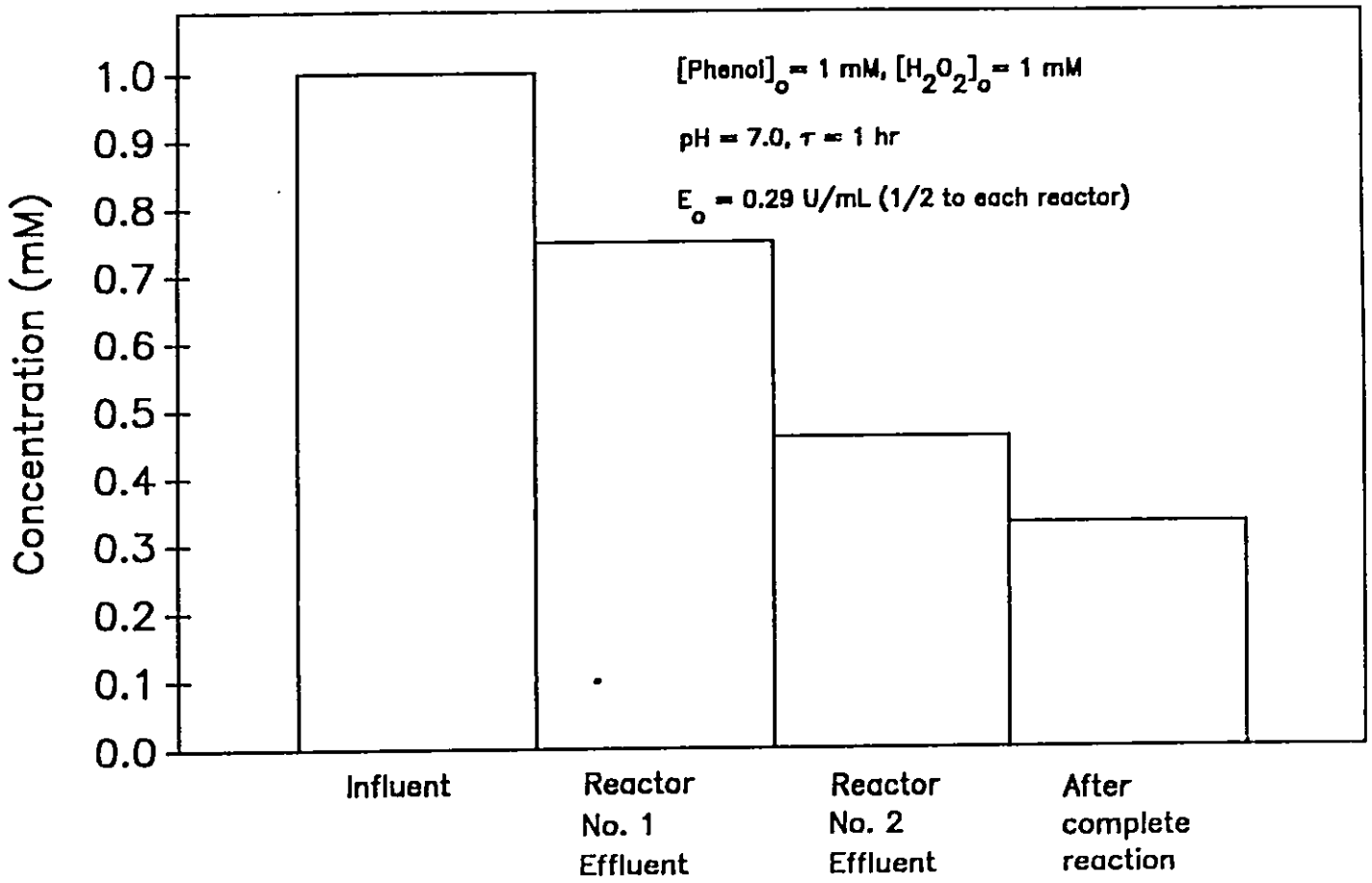

Figure G–2: Removal of Phenol in Two CSTR's in Series at Neutral pH and 25°C

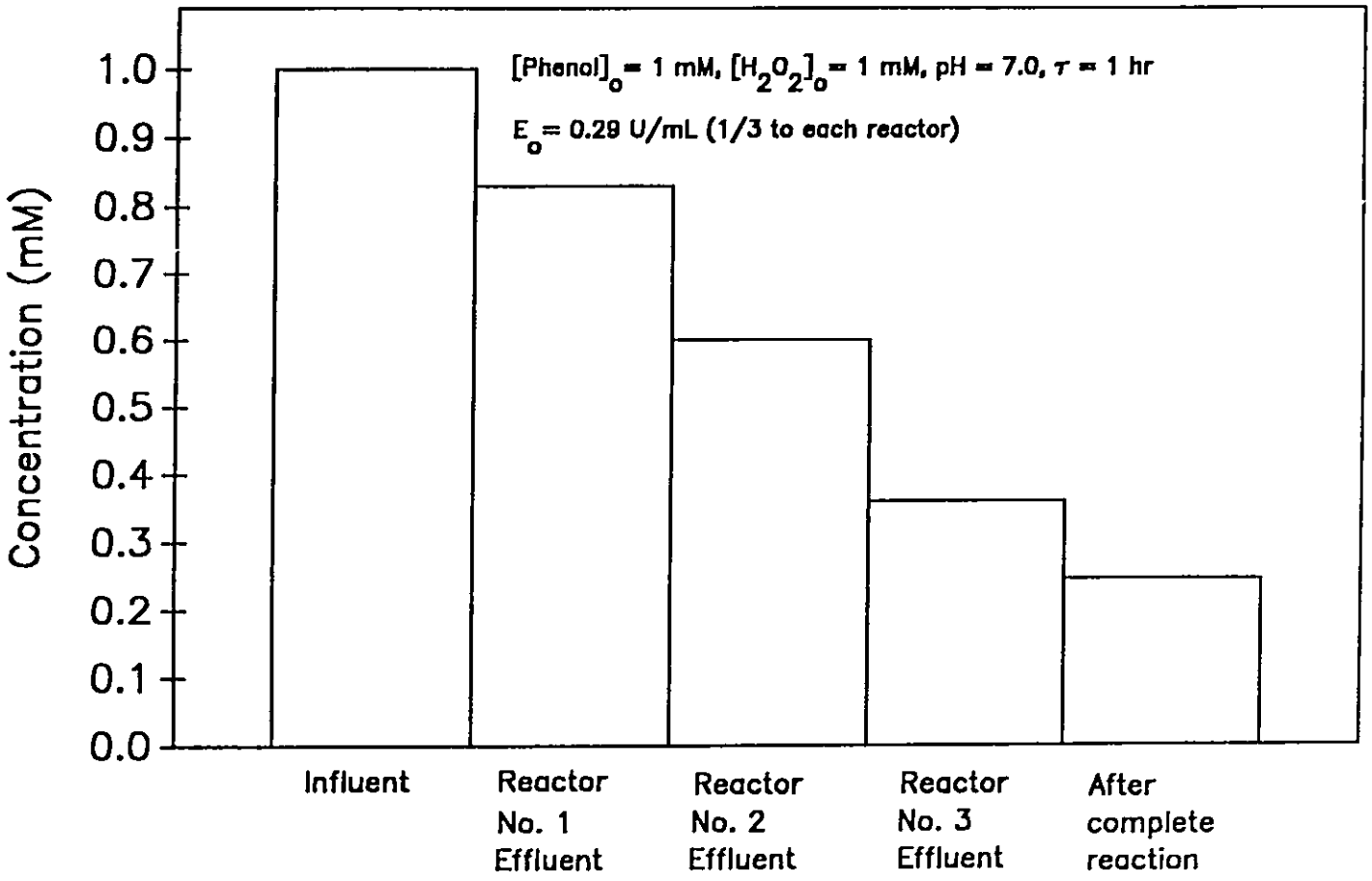

Figure G–3: Removal of Phenol in Three CSTR's in Series at Neutral pH and 25°C

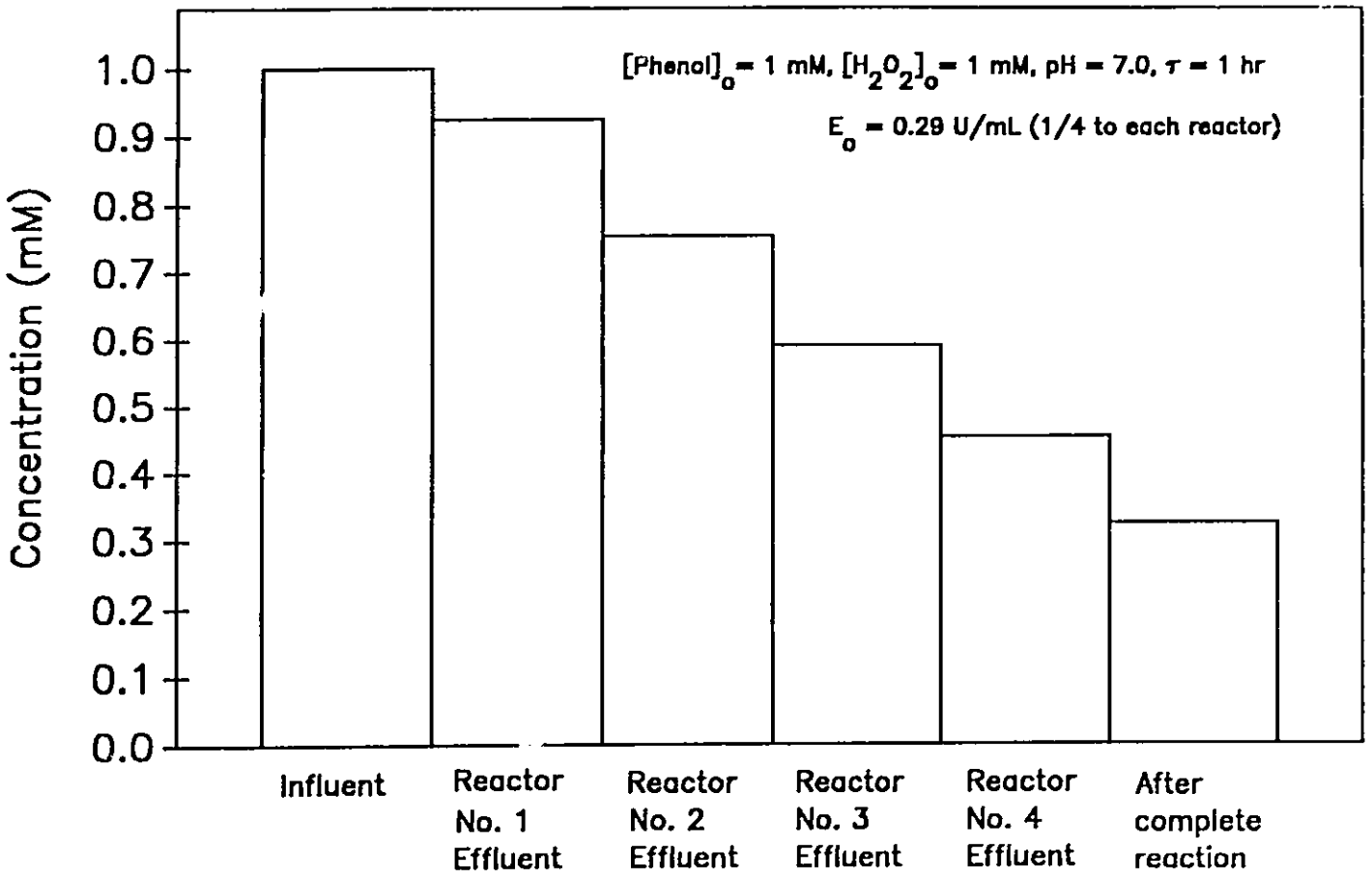

Figure G–4: Removal of Phenol in Four CSTR's in Series at Neutral pH and 25°C

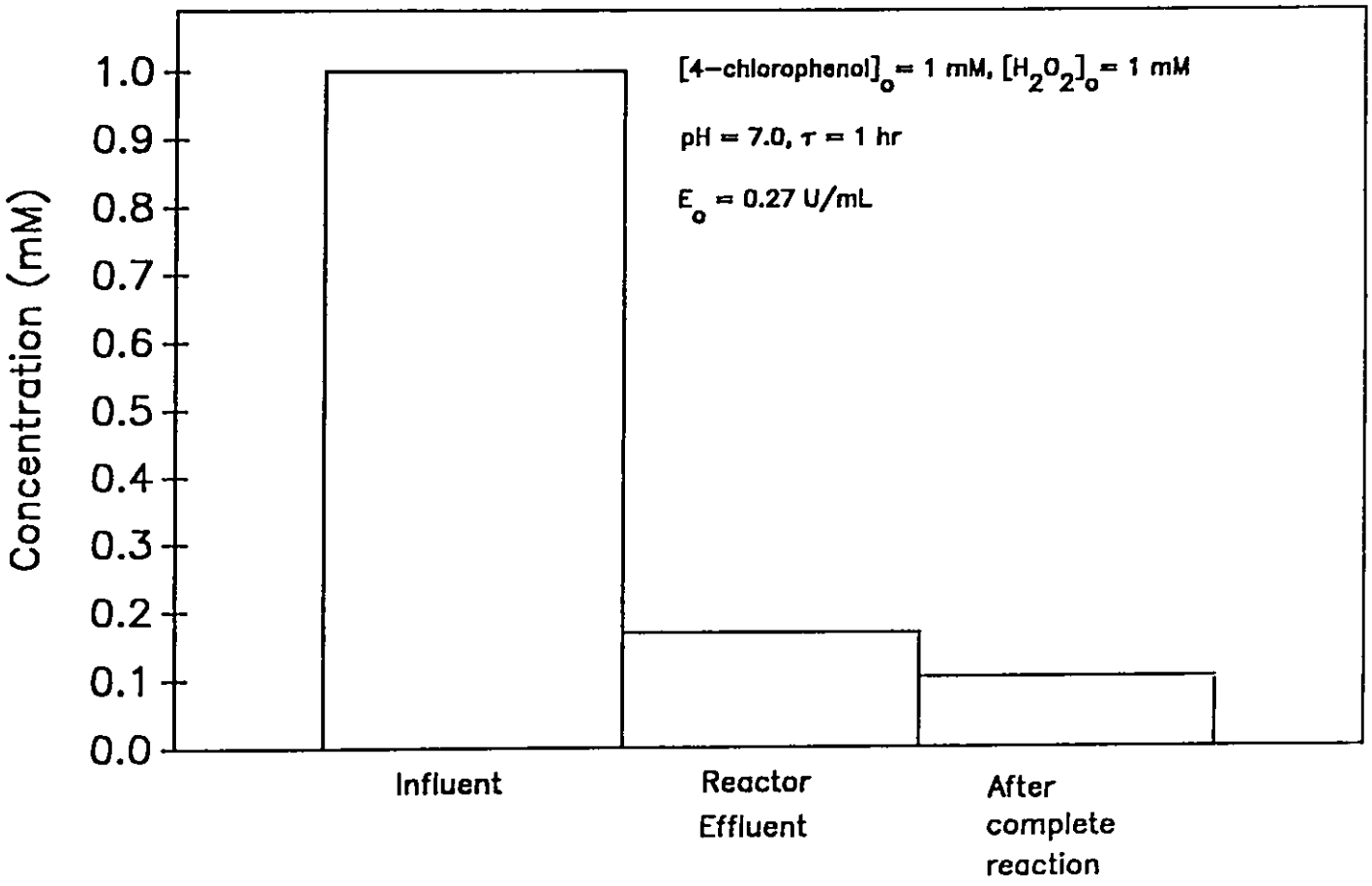

Figure G–5: Removal of 4–Chlorophenol in a Single CSTR at Neutral pH and 25°C

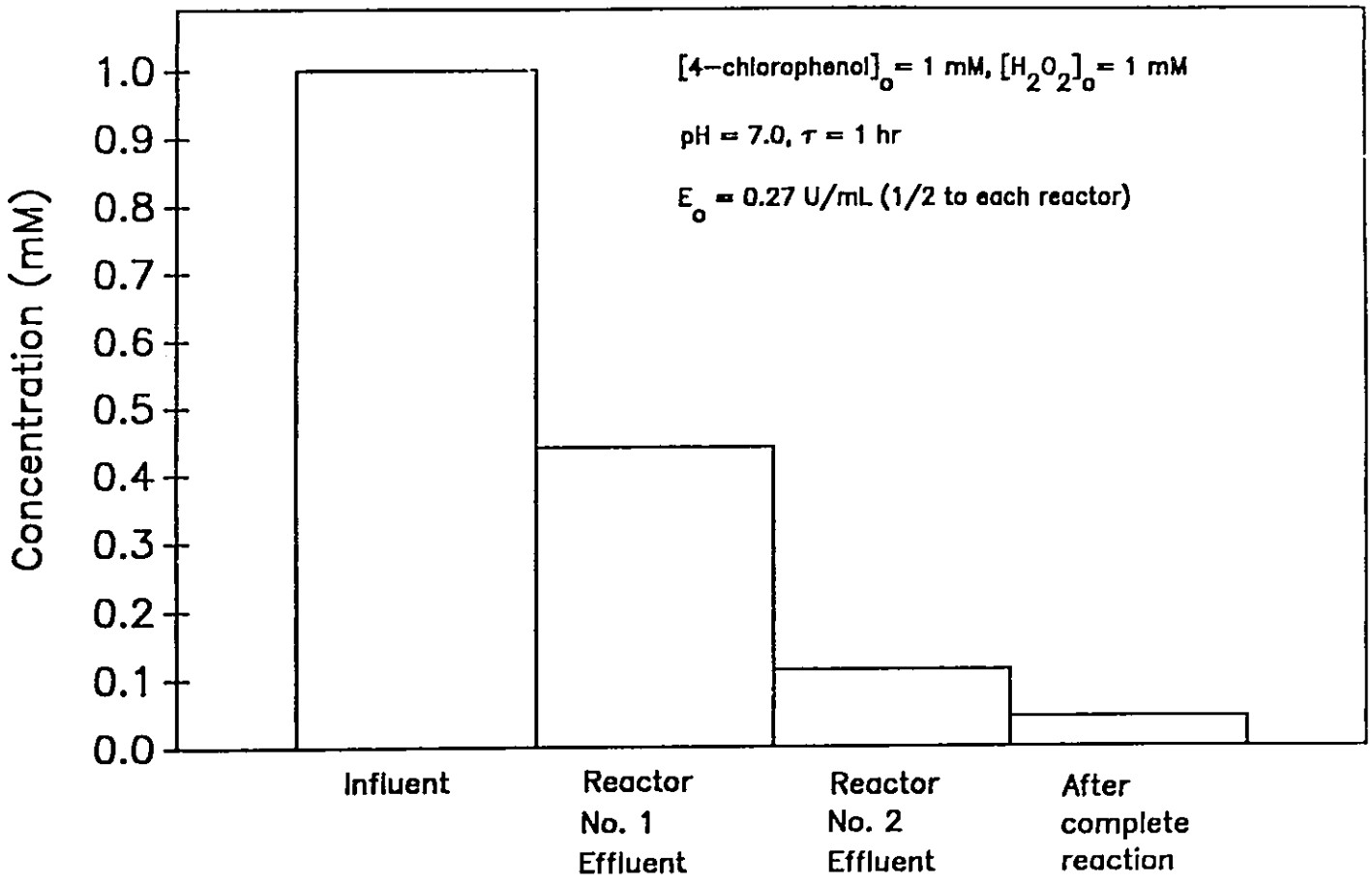

Figure G–6: Removal of 4–Chlorophenol in Two CSTR's in Series at Neutral pH and 25°C

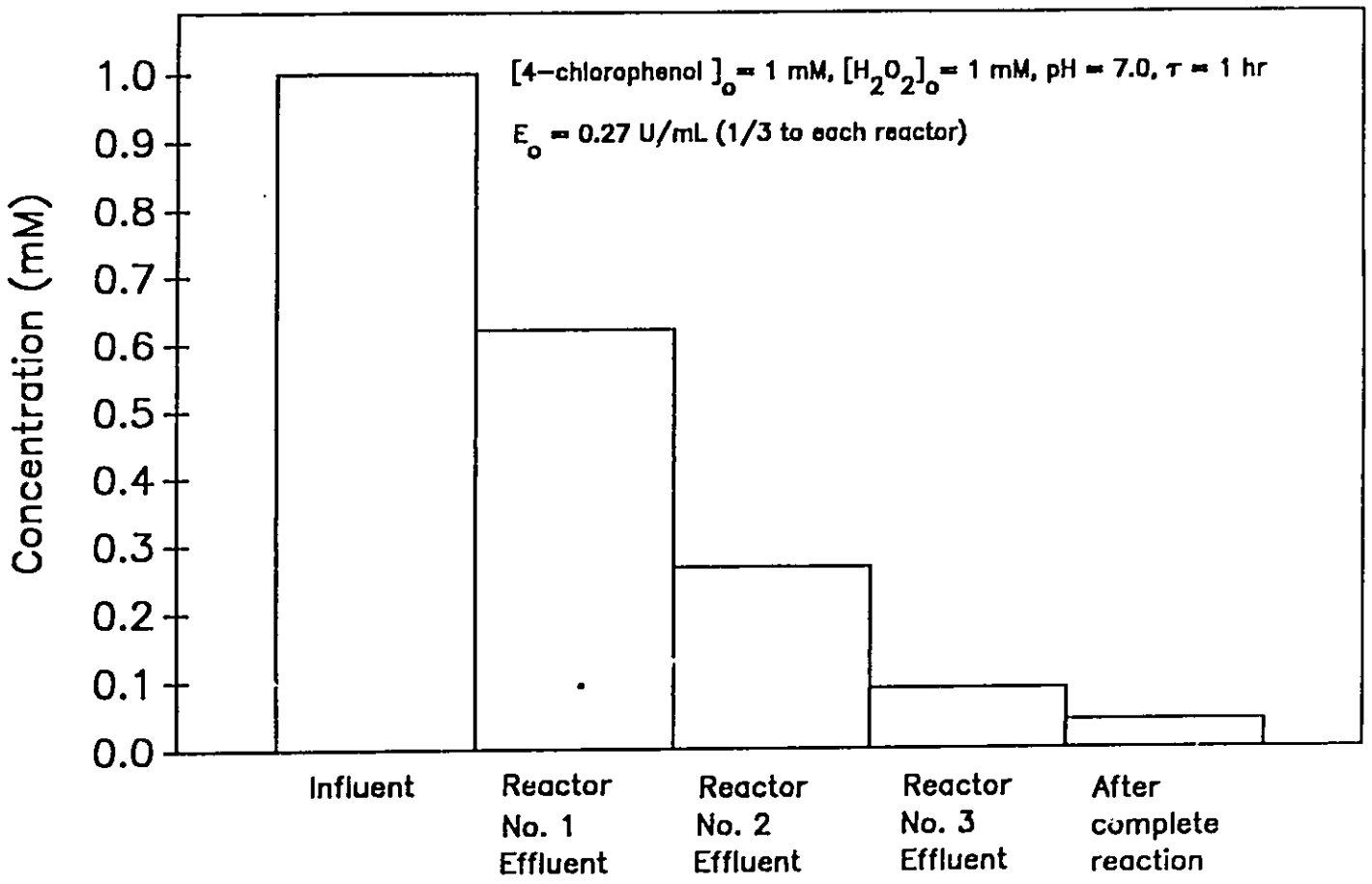

Figure G–7: Removal of 4–Chlorophenol in Three CSTR's in Series at Neutral pH and 25°C

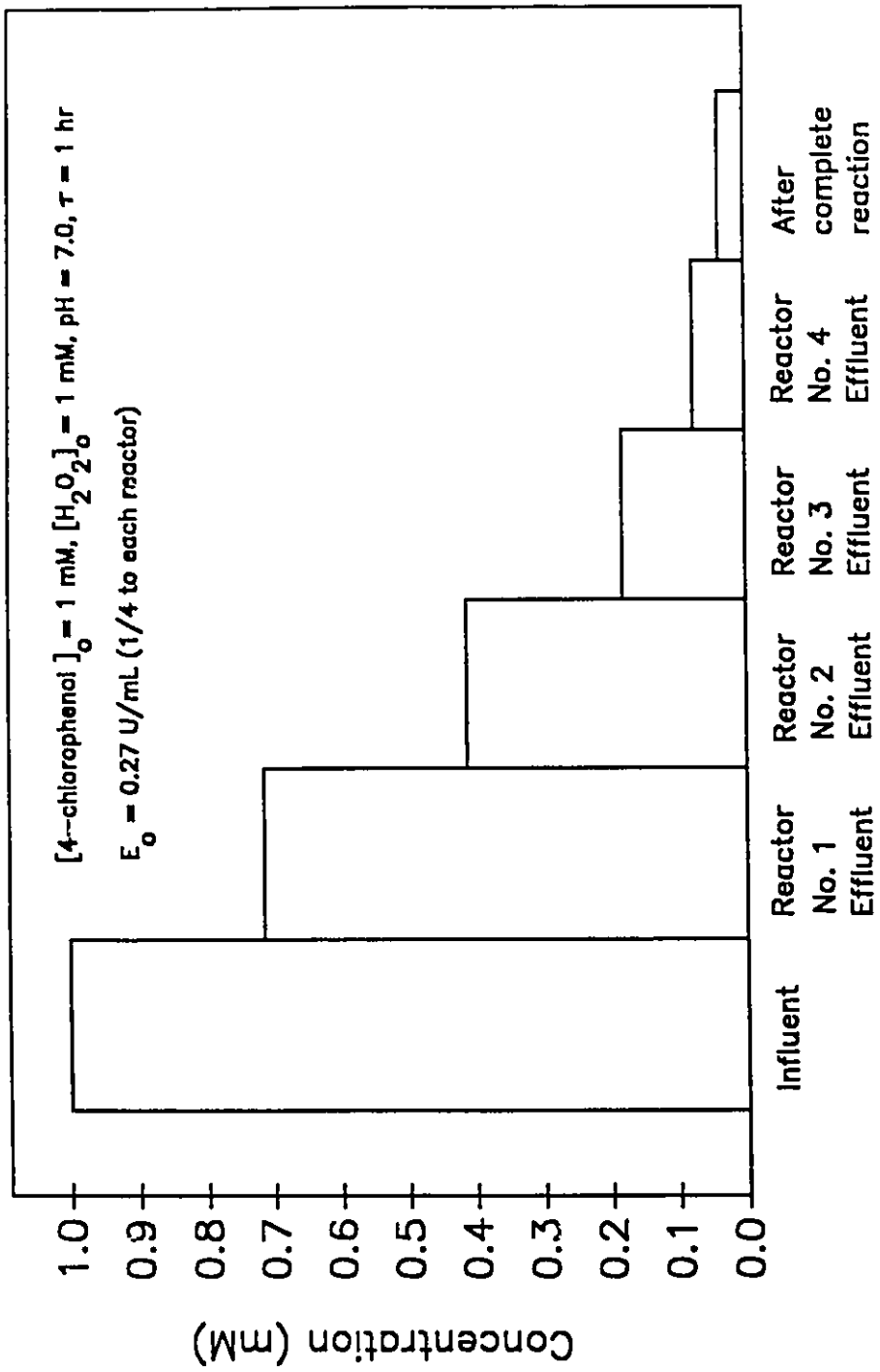

Figure G–8: Removal of 4–Chlorophenol in Four CSTR's in Series at Neutral pH and 25°C

VITA AUCTORIS

1963	James "Jim" Anthony Nicell was born in Montreal, Quebec, Canada on July 28 to parents Patrick and Barbara.
1980	Awarded High School Diploma from Pierrefonds Comprehensive High School, Montreal, Quebec.
1981	Awarded Secondary School Diploma from Vincent Massey Secondary School, Windsor, Ontario.
1985	Awarded the Degree of Bachelor of Applied Science in Chemical Engineering at the University of Windsor, Windsor, Ontario.
1986	Awarded the Degree of Master of Applied Science in Chemical Engineering at the University of Windsor, Windsor, Ontario.
1986–88	Lectured in the Department of Chemical Engineering at the University of Windsor, Windsor, Ontario.
1988–91	Candidate for the Degree of Doctor of Philosophy in Environmental Engineering at the University of Windsor, Windsor, Ontario.