BIOSYNTHESIS OF AMINE OXIDES USING CALCIUM-ALGINATE IMMOBILIZED HOG LIVER MICROSOMES: AN EVALUATION OF PROCESS AND REACTOR DESIGN

by
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ABSTRACT

Title of Thesis: Biosynthesis of Amine Oxides Using Calcium-Alginate Immobilized Hog Liver Microsomes: An Evaluation of Process and Reactor Design.

Ioannis I. Valvis, Master of Science in Chemical Engineering, 1988.

Thesis directed by: Sam S. Sofer, Professor
Sponsored Chair in Biotechnology

An evaluation of the biocatalytic ability and the potential utilization of calcium-alginate immobilized hepatic microsomes (beads) for the biosynthesis of amine oxides has been performed. To accomplish this task, the following three areas were investigated: immobilization, catalytic biooxidation ability, and reactor configurations.

Immobilization was found to result in higher yields over free microsomes at the expense of higher cofactor requirements. Refrigerated drying of immobilized microsomes appears to be a better storage technique than wet storage in buffer. Smaller beads resulted in higher rates than larger beads, but size did not affect the overall yield.

Chlorpromazine (CPZ), diphenylamine (DPA) and 4-hydroxy-2,2,6,6-tetramethylpiperidine (TMP) were used as substrates to demonstrate the catalytic ability of

microsomal enzymes to biooxidize tertiary and hindered secondary amines. The biooxidation of all three compounds to their oxides, by hog liver microsomes, required the presence of NADPH and oxygen. CPZ was converted to its N-oxide while, DPA and TMP were converted to nitroxide free radicals.

Optimal reaction requirements for each substrate were established. NADPH is a reaction limiting factor for the oxidation of all three compounds. Biooxidation of the substrates increased, up to two-fold, by addition of n-octylamine, suggesting that mixed function amine oxidase (MFAO) is responsible for the N-oxidation of the amines.

The progress of the previously described reactions was followed by means of oxygen uptake and HPLC. The identification of CPZ N-oxide was done using NMR, HPLC and TLC. ESR spectroscopy was used to verify the formation of nitroxide free radicals.

A recirculation flow reactor is recommended for large scale production with immobilized whole microsomes. This configuration helps to maintain the physical integrity of the biocatalyst over longer reaction periods. The recirculation flow reactor also gives higher yields than a batch reactor.

PREFACE

This thesis presents a study that is done, for first time, under the supervision of a new sponsored chair in N.J.I.T. specialized in work related to the biotechnology field.

For the purpose of the researchers that are interested to enter this field, I would like to outline a brief definition of the term "Biotechnology" as:

" the application of scientific and engineering principles to the processing of organic and inorganic materials by biological agents to provide goods and services."

In this definition, "scientific and engineering principles", covers a range of disciplines, but rely heavily on biochemistry, microbiology, genetics, biochemical and chemical engineering. The concept of "biological materials" refers to a wide range of biological catalysts such as enzymes, microorganisms and animal and plant cells, while, "goods and services" covers products of industries concerned with food, pharmaceuticals, biochemicals, metal recovery and biological waste treatment.

The material presented in this work involves the utilization of hog liver microsomes in a bioprocess for

the synthesis of three amine oxides. Since this work was performed in three separate stages of this thesis research period, I feel it more appropriate to present the experimental methods and discussion of each stage in three individual chapters. In addition, it would be to the benefit of the reader to read the conclusions drawn from this work, first, and then read the material of chapters, II, III and IV.

AKNOWLEDGEMENTS

I wish to express my gratitude to my advisors, Dr. Sam S. Sofer and Dr. Dekang Shen for their guidance and moral support.

I would like to acknowledge Dr. Basil C. Baltzis for serving in my committee.

I would also like to thank Carl Camp for his help during the first steps of this work and Doug Lischick in recognition of his assistance on experimental work.

I wish to thank Dr. D.M. Ziegler (Clayton Foundation Biochemical Institute, University of Texas at Austin) for his generous gift of liver microsomes and for his advising on biochemical aspects related to microsomal oxidation.

Appreciation is also expressed to my parents Iakovos and Elephteria and my wife Maria for their encouragement and help which made this thesis possible.

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CHAPTER I

INRODUCTION

1.1 MICROSOMAL MFAO ACTIVITY AND BIOOXIDATION

Enzymes that catalyse the biotransformation of many foreign and endogenous compounds are located on the soft endoplasmic reticulum of the liver cells. When liver is removed and homogenized, the fragments of the broken tubular endoplasmic reticulum form microvesicles. These microvesicles are referred to as hepatic microsomes and can be isolated by centrifugal fractionation. The procedure is described by Ziegler and Pettit (1). Hog liver microsomes were used in this work.

Hog liver microsomes are enriched with cytochrome P-450 and mixed function amine oxidase (MFAO). These enzymes are capable not only of attacking the hydrocarbon skeleton but also of oxidizing the amine group of many organic molecules.

Ziegler and Mitchell (2) have reported that purified mixed function amine oxidase catalyzed the oxidation of tertiary aliphatic amines to form N-oxides. Sofer and Ziegler (3) have also reported that the N-oxides of five phenothiazine drugs were obtained in reactions catalyzed

by glass-immobilized purified microsomal mixed function amine oxidase.

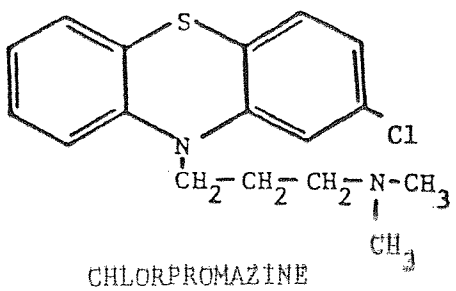
MFAO is a flavoprotein which has also been found responsible for the N-oxidation of many unhindered and hindered secondary amines (4,5). Specifically, the biooxidation of certain secondary amines results in the formation of stable nitroxides. Although nitroxide radicals have been postulated as unstable intermediates in the hepatic microsomal oxidation of primary and secondary amines (6), the biooxidation of certain secondary amines and their hydroxyl derivatives to stable nitroxide radicals has been reported.

Rauckman and Rosen (5,7) have reported that MFAO is responsible for the oxidation of 1-hydroxy-2,2,6,6-tetramethylpiperidine to 2,2,6,6-tetramethylpiperidine-1-oxyl while 2,2,6,6-tetramethylpiperidine-1-oxyl is reduced to 1-hydroxy-2,2,6,6-tetramethylpiperidine by cytochrome P-450. They also report that the biooxidation of 2,2,6,6-tetramethylpiperidine to 2,2,6,6-tetramethylpiperidine-1-oxyl by free liver microsomes was found to be a rate limiting step. Stier and Reitz (8,9) have similarly reported that 2,2,6,6-tetramethylpiperidine is oxidized by rabbit microsomes to 2,2,6,6-tetramethyl-piperidine-1-oxyl in the presence of oxygen and NADPH.

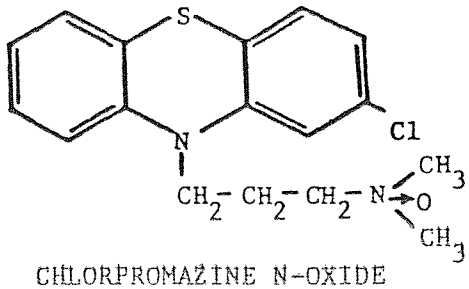
Stier and Reitz also reported that a stable equilibrium between the oxidation of 2,2,6,6-tetramethylpiperidine and the reduction of 2,2,6,6-tetramethylpiperidine-1-oxyl was observed by E.S.R. spectroscopy, but, they gave no experimental details.

In this study, an *in vitro* investigation of the biotransformation of CPZ, DPA, TMP, TMPOH and TMPO by free and immobilized hog liver microsomes is presented. Almost all the runs were made at air-saturation conditions, at 37^{o}C and pH 7.4. Some runs were performed at pure oxygen concentrations.

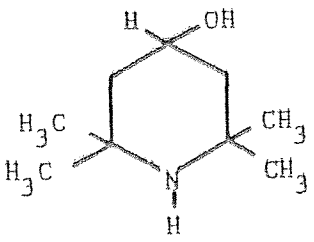
The biooxidation of all three compounds required the presence of the cofactor, reduced nicotinamide adenine dinucleotide phosphate (NADPH), and atmospheric oxygen. To reduce the cost of cofactor requirement, NADPH was regenerated from its oxidized form ($NADP^{+}$) in the presence of glucose-6-phosphate (G-6-P) and glucose-6-phosphate dehydrogenase coenzyme (G6PDH). The mechanism, function, and the effect of NADPH are presented in a later chapter. The structures of the compounds being investigated are shown Figure 1.1.



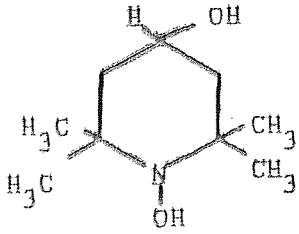
CHLORPROMAZINE



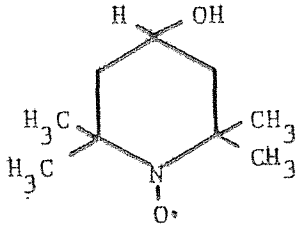
CHLORPROMAZINE N-OXIDE



TMP



TMPOH



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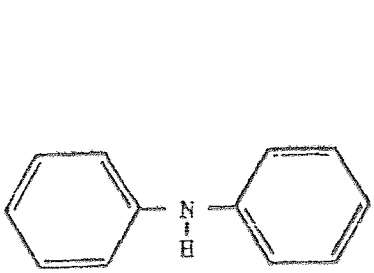
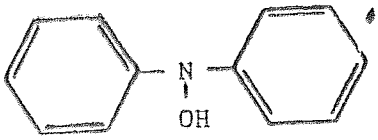
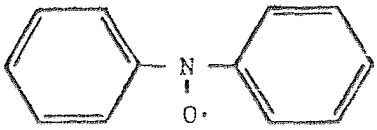


DPA



DPNOH



DPNO

Figure 1.1 The structures of the compounds investigated.

1.2 IMMOBILIZATION OF MICROSOMAL ENZYMES

Immobilization refers to confinement of a biological system (enzymes or microorganisms) into some type of non-toxic matrix by means of chemical or physical methods. In our case, entrapment of a membrane-bound enzyme system (hepatic microsomes) within a hydrophilic matrix (Ca^{++}-alginate gel) is used. Numerous methods have been developed for immobilization of enzymes (10,11).

There are many advantages with immobilization of enzymes. The most important is the enhancement of the resistance to environmental changes, which is dependent upon the physical integrity of the immobilized enzymes and the immobilizing matrix.

Microsomal enzymes are extremely thermally unstable and they decompose easily after a short term exposure to temperatures as low as room temperature. Current work has shown drastic increases in the microsomal enzymes' half-lives in comparison to the half-lives of free cell microsomes and pure enzymes. As a result, immobilization leads to higher reaction yields. Earlier work by Osinowo and Sofer (12) have reported the optimal operating conditions for drug oxidation by purified mixed function amine oxidase immobilized on alumina beads.

Higher activity has been found to be another important characteristic of immobilization. This has been

attributed to the filtration of natural inhibitors existing in crude microsomal preparations. Such inhibitors get washed out during the curing and the storage period of the immobilized enzymes (beads). Loss in the protein content of beads during the curing and storage periods suggests that the nature of the inhibitors is enzymic in nature. Camp (13) has reported similar observations.

In maintaining higher activities and obtaining higher yields at a lower enzyme cost, immobilization is a significantly better approach than the use of microsomes or the purified enzymes in their free form. This is especially true when optimum storage conditions and adequate bead design exist. Moreover, immobilized microsomes can be dried and stored for long periods. An extensive investigation on bead design using microorganisms entrapped in alginate gel has been performed by Lakhwala (14) of NJIT. In his work, higher rates were obtained using microorganisms in free form over immobilized microorganisms, but, higher yields were obtained in the later case.

Operational advantages due to the fixed geometry of the immobilized enzymes are also important aspects in the use of the immobilization technique. Many reactor design

studies related to mass transfer problems can be made by varying the reactor configuration and the size or the geometry of the immobilized enzymes. Fluidized bed configurations can be used with immobilized enzymes to study the effect of bead size and flow rate on reaction rate. Nan K. Chien (15) studied the effect of flow rate and bead size in a recirculation flow reactor. The use of recirculation flow reactor to produce amine oxides has been reported elsewhere (3).

Immobilized enzymes can also serve as models for studying natural *in vivo* membrane bound enzymes.

1.3 APPLICATIONS OF MICROSOMES TO METABOLIC STUDIES AND THE SYNTHESIS OF MEDICINALLY VALUABLE CHEMICALS

The potential applications of hepatic microsomal enzymes are discussed in works reported by Sofer (11) and Camp (17).

1.3.1 METABOLIC STUDIES

Most of the drug oxidation reactions by which drugs and many foreign chemicals are metabolized have been shown to occur in liver microsomes. Some drugs are converted to metabolites having pharmacological properties that are further improved, or similar to, the parent compound.

Others evoke their action only through the formation of metabolites. Thus, utilization of microsomes for metabolic studies *in vitro* can supply us with valuable information on the *in vivo* activity of new drugs.

In addition to identification of metabolites, information on the metabolic pathways and on the requirements for metabolic activation has been obtained. For example, carbon monoxide was found to inhibit the nitroreductive ability of cytochrome P-450 activity while phenobarbital and n-octylamine were found to induce the action of cytochrome P-450 and activate mixed function amine oxidase oxidative action, respectively.

Studies on the mechanisms through which liver microsomes catalyze the oxidative reactions have revealed that atmospheric oxygen and not the oxygen of water is incorporated into substrates (18,19). This finding was of considerable importance because it suggested that NADPH reduces a component in microsomes which reacts with oxygen to form an "active oxygen intermediate". This intermediate is, then, transfered to the drug substrate. Later studies identified this intermediate as C(4a)-hydroperoxyflavin (20).

In this study diphenylamine was chosen, because there is no literature reported on the metabolic transformation of secondary arylamines.

1.3.2 SYNTHESIS OF N-OXIDES AND GLUCURONIDES

One of the applications of liver microsomes is the production of metabolites, such as N-oxides and glucuronides, for toxicological studies and potential use as new drugs.

N-Oxidation of the terminal piperazine nitrogen of a number of phenothiazines by mixed function amine oxidase-enriched liver microsomes has been reported (3).

Phenothiazine compounds are extensively used as antiphsychotic drugs. The N-oxides of these compounds can offer similar medicinal effects and can be easily excreted, as a result of their increased polarity over the parent compounds. Studies on the pharmaco-dynamics of polar compounds such as N-oxides and glucoronides have also shown reduction in their toxicity as well as in other undesired side effects. For example, imipramine N-oxide has been reported to exhibit therapeutic actions similar to its parent compound but it causes less drop in blood pressure and reduces toxicity (21).

Although N-oxidation of many coumpounds results in "detoxification", in other cases, N-oxidation of arylamines results in the formation of highly carcinogenic metabolites. Jenner (22) discussed the toxicological effects of <u>in vivo</u> formation of arylhydroxylamines. It

has also been stated in the literature that mixed function amine oxidase is presumably responsible for the N-oxidation of aromatic amines and arylnitro compounds (23). Thus, <u>in vitro</u> investigation of the microsomal biotransformation of compounds leading to toxic metabolites is of equal importance.

Glucuronidation is a known "detoxification" action of the liver. Glucuronidation (conjugation with UDP-glucuronic acid) is catalyzed by UDP-glucuronyl-transferase, an enzyme primarily present in liver microsomes. Various drugs as well as bilirubin and some steroid hormones are conjugated by this enzyme with UDP-glucuronic acid to form glucuronides. The production of various glucuronides by simultaneous immobilization of cytochrome P-450 and glucuronyltransferase has been reported (10). The glucuronides of 7-hydroxyphenazine and of 7-hydroxyprochlorperazine in a reaction catalyzed by glass immobilized microsomes has been reported elsewhere (16).

1.3.3 SYNTHESIS OF NITROXIDES

Due to their stability, a number of nitroxide free radicals have found applications as diagnostic drugs for nuclear magnetic resonance imaging (MRI), and as spin

labels for drug detection and study of biological membranes.

Contrast agents have been investigated in an effort to increase the differences between the proton relaxation times of normal and pathologic tissue, and thus enhance the detection of pathogenic regions. Synthesis of paramagnetic nitroxide analogues of cholesterol that can help us understand the distribution and movement rate of cholesterol itself is suggested by Maurin (24). This work suggests liver microsomes can be suitable catalysts for production of nitroxides from certain secondary amines.

With respect to the *in vivo* biotransformation of the secondary amines and their nitroxides investigated in this work, very little is known. As a result, valuable information on the metabolism of those amines and nitroxides in the liver can be obtained. Information on the metabolism of nitroxides is very important, since it can reveal the metabolic fate of nitroxides and can determine their potential utilization as spin labels or contrast agents.

CHAPTER II

OBJECTIVES

The primary objective of this study is to evaluate the biocatalytic ability and potential utilization of calcium-alginate immobilized liver microsomes in a process for the synthesis of des/amine oxides. The "biocatalytic ability" means the versatility of microsomal enzymes to catalyze a variety of medicinally important compounds. "Potential utilization" refers to the utilization of the immobilization technique in combination with an adequate reactor configuration to establish a process for a successful and economic production of amine oxides.

A secondary objective will be to determine the feasibilty of the entire process for generally studying the *in vivo* metabolism tertiary and hindered secondary amines.

Accomplishment of the primary objective involves the following steps:

1) Investigation of the microsomal biooxidation of more than one potential substrate of the oxidative or reductive enzymes present in the microsomes. In this work, chlorpromazine HCl salt, diphenylamine

and 4-hydroxy-2,2,6,6-tetramethylpiperidine were used.

2) Investigation of the mechanisms involved in the biotransformation of the amines. This will facilitate future selection of other potential substrates with similar structures for N-oxide synthesis. In addition information on the identity of the enzymic system involved in the biotransformation will be obtained. N-octylamine, a known activator of MFAO is to be used for enzyme identification purposes.

3) Obtain oxidation rate data and other valuable information related to reactor design, such as the effect of bead size, number of beads, diffusional characteristics, and enzyme and oxygen requirements.

4) Investigation of cofactor and substrate requirements, for optimal reaction conditions and the determination of their effects on immobilized microsomes over free microsomes.

5) Determine optimal storage conditions and preparatory protocol for bead making.

6) Compare batch versus recirculation flow as two potential reactor configurations for large scale production of amine oxides.

7) Identification of all amine oxides by adequate analytical methods.

CHAPTER III

N-OXIDATION OF CHLORPROMAZINE BY ALGINATE-IMMOBILIZED HOG LIVER MICROSOMES

3.1 MATERIALS AND METHODS

3.1.1 MATERIALS

Alginic acid (grade IV), $NADP^+$, G-6-P, G6PDH, and Tris-buffer were obtained from Sigma Chemical Co. CPZ was supplied by Smith Kline and French Labs. (Philadelphia, Pa.). NADPH was regenerated from its oxidized form, $NADP^+$, using G-6-P and G6PDH. Hog liver microsomes were the generous gift of Prof. D.M. Ziegler, University of Texas at Austin. The preparation of microsomes is described elsewere (1).

3.1.2 BIOCATALYST PREPARATION

A 2% sodium alginate gel was prepared by dissolving 2 g alginic acid and 2 g sodium chloride into a gently heated and well stirred 100 ml, 0.05 M Tris buffer solution of pH 7.4. Beads were prepared by mixing cold sodium alginate gel solution with an equal volume of liver microsomes. The mixture was homogenized and then extruded

through a syringe (21 G $1^1/_2$ "), drop by drop, into cold stirred 0.1 M $CaCl_2$ solution of pH 7.4 (see appendix A). For the preparation of smaller size beads, a 23 G $^3/_8$ " syringe was used. The protein content of each bead and free microsomes used was of the order of 5.0 mg/10 beads and 2.1 mg/5 drops of microsomes respectively. Dried beads where prepared by blowing cold air and applying vaccum in the chamber where the beads were being dried. A schematic of the installation is given in Appendix A. The "Lowry method" (28) was used to determine the protein content of the biocatalyst. The protein calibration curve is given in Appendix A.

3.1.3 ANALYSIS

The N-oxidation of CPZ and NADPH was monitored by oxygen uptake measurements. The installation of the 1.9 ml microassay reactor used for this work as well as the schematic of the recirculation flow reactor that was used for preliminary studies are shown in Appendix A. The procedure for oxygen measurements in the microassay reactor is described in the following chapter. The formation of CPZ N-oxide was verified by TLC, HPLC and proton NMR.

For HPLC analysis the following reversed columns were used: Altech, Spherisorb 5um 250x4.6 mm following an

SSI, Soft Seal Alkyl 18/5 um 150x4.6 mm; flow rate: 0.7 ml/min; UV detector set at 240 nm. Acetonitrile-Water 65%-35% was used as solvent.

A Clark-type D.O. probe [YSI 4004 Clark Oxygen Probe] was used to determine the content of oxygen in reaction medium.

Acetone-ammonia solution and MeOH-$CHCl_3$ solutions were used as solvents for TLC. The TLC plates were developed in an iodine chamber. EtoAc was used to extract CPZ N-oxide from the reaction medium.

3.1.4 INSTRUMENTATION (4004 Clark-type oxygen probe)

Oxygen uptake measurements were made by means of a YSI, 4004 Clark-type oxygen probe. This probe is a complete polarographic system consisting of platinum cathode, silver anode, and KCl solution held captive around the electrodes by a Teflon membrane fastened with an "O" ring. When polarizing voltage is applied across the probe, all oxygen in the probe is consumed (reduced) at the cathode and current flows in direct stoichiometric relation to the rate of oxygen consumption (reduction). Oxygen then diffuses through the membrane at a rate proportional to the oxygen concentration outside the probe, since the oxygen concentration in the probe is near

zero. When steady state conditions are reached, in about 30 seconds, current flows through the probe at a rate in proportion to the external oxygen pressure. The polarizing voltage in our probe is 0.8 volts and the oxygen depletion rate is 8E-11 grams oxygen /sec per microamperes.

At air saturation conditions the oxygen solubility in buffer solution of pH 7.4 at 37^{o}C is about 200 nmoles/ml. An amplifier/offset - box neccesary for the supplying of the external voltage and for the interfacing to a recorder device, was built in the laboratory.

3.2 RESULTS AND DISCUSSION

Chlorpromazine has been found to be oxidized to chlorpromazine N-oxide by hog liver microsomes in a reaction requiring NADPH and oxygen. The N-oxidation of chlorpromazine by hepatic microsomes has been the subject of many reported studies (3,13,17). This work uses chlorpromazine as model substrate of MFAO to answer questions that regard the design of a bioprocess system for the production of amine oxides at a larger scale.

Above all, this thesis work in combination with Camp's M.S. thesis work can give a more complete insight on the alginate-immobilization process, process design, product analysis, and reactor configuration evaluation.

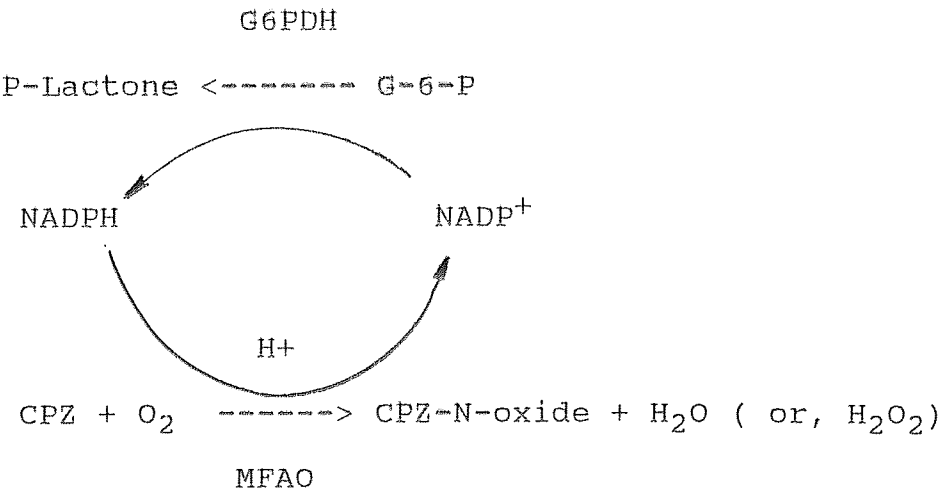
3.2.1 THE MECHANISM OF MICROSOMAL N-OXIDATION OF CPZ

Mixed function amine oxidase (MFAO) is an important oxidative enzyme present in hog liver microsomes. In terms of its structural identity, MFAO has been known to be a favoprotein that can readily catalyze the oxidation of many tertiary amines with highly electrophilic nitrogens. In our study the N-oxidation of chlorpromazine (CPZ) was found to require the presence of reduced nicotamide adenine dinucleotide phosphate (NADPH) and atmospheric

oxygen. The N-oxidation of CPZ can be described by the following scheme:

$$\text{CPZ} + \text{O2} + \text{NADPH} \xrightarrow[\text{MFAO}]{\text{H}^+} \text{CPZ-N-oxide} + \text{NADP}^+ + \text{H2O}$$

A more specific description of the mechanism has been given by Poulsen and Ziegler (25). Since $NADP^+$ was used to regenerate NADPH in the presence of G-6-P and coenzyme G6PDH, the following scheme describes the biooxidation of CPZ:



In the absence of substrate (CPZ) the reaction proceeds towards the formation of hydrogen peroxide (H_2O_2).

3.2.2 ENZYME REQUIREMENT AND MAXIMUM OXIDIZING CAPACITY

In determining the amount of immobilized microsomal enzyme required for the oxidation of 1 mg of CPZ, the progress of CPZ bioxidation by 20 beads was followed by means of oxygen uptake measurements. The beads contained a total of 1 mg of microsomal protein and were used until their complete inactivation. With exception of G-6-P /G6PDH, the concentration of $NADP^+$ and CPZ were maintained at a level such that neither of them becomes a limiting factor during the progress of the reaction. The initial concentration of G-6-P and G6PDH in the reactor were 0.88 mM and 3 unit/ml respectively. The results are shown in Table 3.1.

According to the above table an average of 440 nmoles/hr can be consumed by 1 mg of alginate immobilized microsomal enzyme for a time span of two hours, at 37°C and pH 7.4 . The physical integrity of the catalyst was maintained in good condition during the entire period.

During the performance of these runs three addional observations were made regarding the stoichiometry and the

mechanism of CPZ oxidation: first, after the initial injection of 400 nmoles CPZ, the oxidation rate (as seen by the slope of the oxygen uptake) began to increase at a point at which 400 nmoles of oxygen had been consumed since the begining of the run; second, upon addition of additional 400 nmoles of CPZ the slope of oxygen uptake increased drastically; third, $NADP^+$ was found to be a limmiting factor in the biooxidation of CPZ.

Table 3.1. Oxygen consumption by immobilized microsomes.

Run #	Storage $CaCl_2$ (hrs)	Time Buffer (hrs)	CPZ Initial. Oxid. Rate (nmoles/min/ mg protein)	Oxygen Consumed (nmoles)	Time Required (hrs)
1	20	3	1.54	1080	2.0
2	50	3	1.72	512	1.1
3	50	22	2.03	785	2.0
4	75	3	1.72	800	2.0
5	75	48	2.06	810	2.0

According to the above observations it was conluded that there is a 1:1 ratio between the CPZ N-oxidation by immobilized hog liver microsomes and the amount of atmospheric oxygen depleted in the bioreactor. The same result was qualitatively observed by following the progress of CPZ biooxidation with TLC.

Another conclusion drawn from the first and second observation was that the mechanism of CPZ oxidation appears to switch to the mechanism of hydrogen peroxide formation until more substrate is introduced. Further investigation to verify this conlusion is recommended.

In determing whether G-6-P and G6PDH were limiting the biooxidation of CPZ, the reaction previously described was repeated maitaining CPZ and all cofactors and coenzymes in excess. This time 1650 nmoles of oxygen were consumed in a period of 3.7 hours. After 4 hours some of the beads began to break down while their color indicated the washing out of enzymes.

Based on the above data, it was calculated that 1.71 mg of microsomal protein is required for the conversion of 1 mg of CPZ in 3.7 hours.

In Figure 3.1, the dashed curve shows the biooxidation of CPZ maintaining CPZ concentration at 0.33 mM while all cofactors and coenzymes are in excess. In the

same figure, the biooxidation of CPZ (0.33 mM) by 20 beads in the presence of 0.60 mM $NADP^+$ (0.60 mM), 0.88 mM G-6-P and 2 units/ml G6PDH is shown. From this figure the immobilized enzyme's half-life at 37°C was calculated to

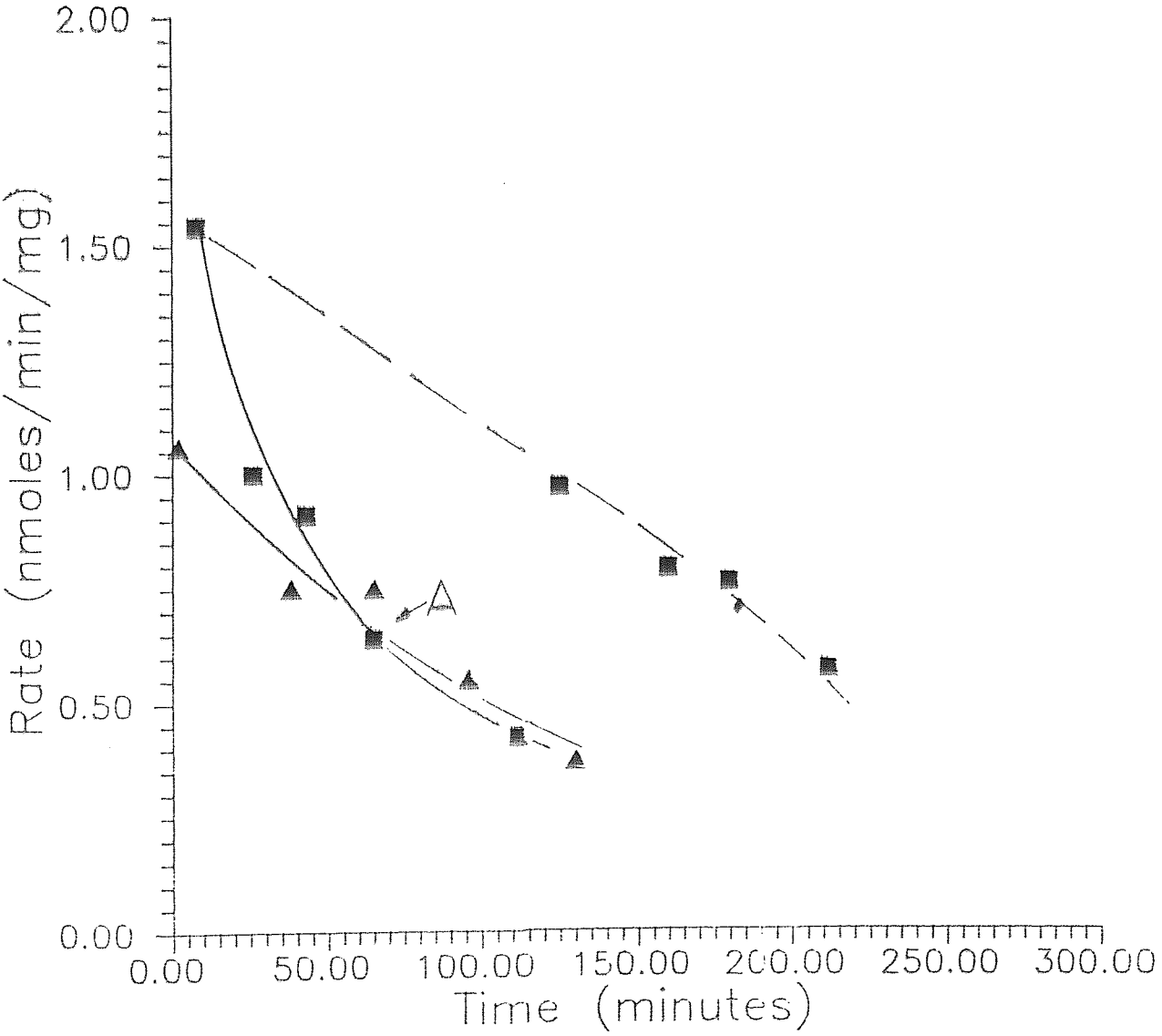


Figure 3.1 Biooxidation of CPZ. Dashed: CPZ conc. was maintained constant at 0.33 mM and all cofactors were in excess. (-■-■-): CPZ (0.33 mM) is biooxidized completely until point A; then, the reaction mechanism switches to the biooxidation of NADPH ($NADP^+$ 0.60 mM). (-▲-▲-):biooxidation of NADPH ($NADP^+$ 0.60 mM)

be 2.9 hrs. The half-life of free microsomes at 37°C was found to be in the range of 12 to 15 minutes.

3.2.3 THE $NADP^+$ REQUIREMENT

$NADP^+$ (used to produce NADPH by regeneration) was found to be a limiting factor in the biooxidation of CPZ by alginate immobilized microsomes. Figure 3.2 shows that the minimum requirement for maximal rate of NADPH oxidation occurs at $NADP^+$ concentration of 1.5 mM. In determining the minimum requirement of $NADP^+$, G-6-P/G6PDH was kept in excess, that is 3.0 mM G-6-P and 4 units/ml G6PDH. Based on the literature 2 units per ml of G6PDH is considered sufficient for a 1 to 2 hour run.

3.2.4 THE EFFECT OF BEAD SIZE ON CPZ BIOOXIDATION

Figure 3.3 shows the effect of bead size on the CPZ oxidation rate, based on the same protein content. Thus, 40 beads were compared to 20 larger beads. The ratio of the a large bead over the volume of a smaller bead was 2.5. The two types of beads were prepared using a 23 G $^3/_8$" syringe and a 21 G $1^1/_2$" syringe, respectively. The total surface area of the 40 small beads was calculated to

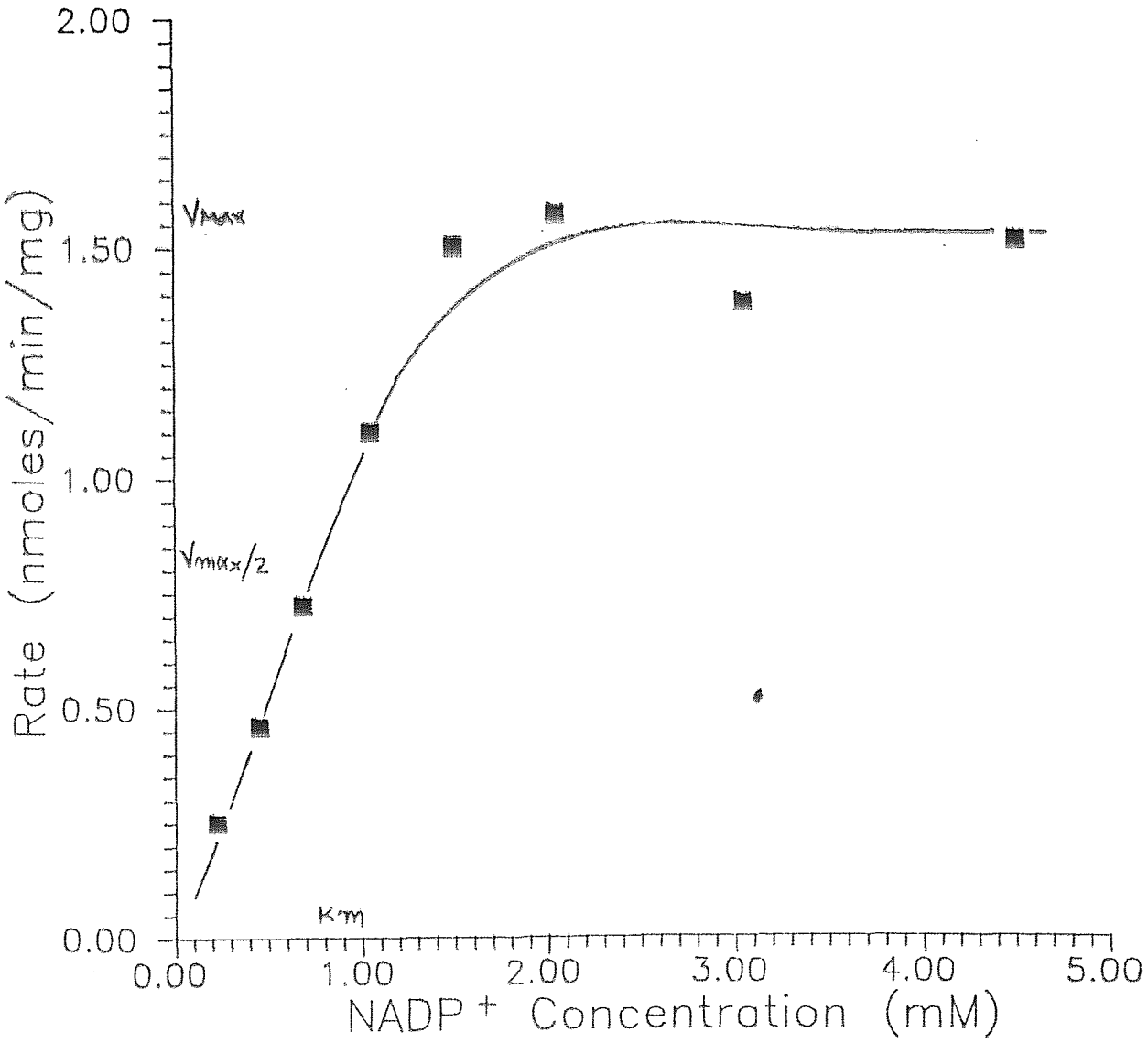


Figure 3.2 The $NADP^+$ requirement for microsomal biooxidation.

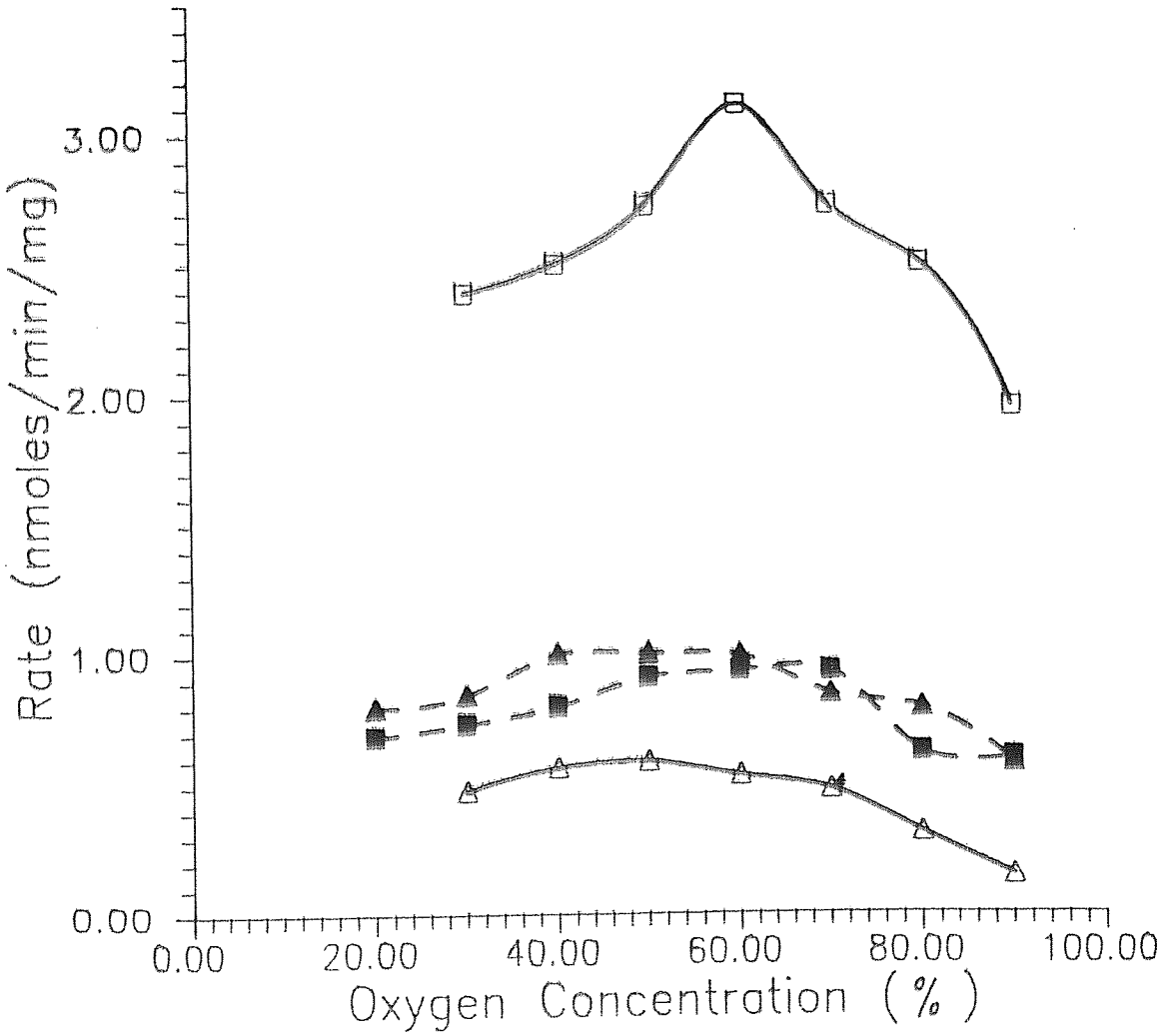


Figure 3.3 The effect of bead size on the biooxidation of CPZ. Dashed, (▪—▪): 40 drops (23 G $^{3}/_{8}$ ") of gel-microsome mixture prior extrussion process; (▲—▲): 20 drops (21 G $1^{1}/_{2}$ ") of gel-microsome mixture prior extruson process. Solid, (□—□): 40 beads (23 G $^{3}/_{8}$ "). Solid, (△—△): 20 beads (21 G $1^{1}/_{2}$ "). A 5.6-fold increase over the larger beads was obtained using the smaller beads.

be 3.7 times greater than the total surface area of the 20 larger beads.

It is clear from the figure that immobilization can lead to higher rates providing an optimum bead size. By increasing the exterior surface of the catalyst the accessible surface for adsorption increases. In our case, although the total volume of the larger beads (i.e biocatalyst) is 25% greater, the smaller beads provide 3.7 times greater exterior surface. As a result a surface phenomenon resistance exists, in addition to film resistance.

Based on the amount of oxygen consumed by the two sizes of beads, it was found that in both runs the same yield was obtained, but the time required was approximately 5-fold more for the larger size beads.

The preparation procedure was followed for both types of beads; therefore, the structure of the porous matrix for each type should be the same. Since the oxidation rate of CPZ obtained by the smaller beads was found to be 5.6 times greater than the one obtained by the larger beads, one can conclude that internal diffusion is a controlling factor for the reaction condidions examined (0.5 mM CPZ, 0.25 mM $NADP^+$). Lastly, immobilization appears to result in higher rates than free cell microsomes, provided an optimum bead size is used.

3.2.5 THE EFFECT OF CPZ CONCENTRATION ON BIOOXIDATION RATE

Thirty beads were used to study the effect of initial CPZ concentration on biooxidation rate. In this study, the cases of two different reaction conditions were examined. In the first case, the initial concentration of $NADP^+$ was 0.5 mM with excess of G-6-P/G6PDH solution. Here, the beads were cured for 20 hours in $CaCl_2$ 0.1M and then washed in buffer for 3 hrs. A comparison to free microsomes containing the same amount of protein was, also, performed. This study is represented by the solid curves of Figure 3.4.

In the second case, 30 drops (i.e. 30 beads) of alginate gel-microsome mixture prior extrusion and 30 beads cured for 20 hour in $CaCl_2$ and stored for 25 hrs in buffer solution pH 7.4 solution, were used. This time the initial concentrations of $NADP^+$ G-6-P and G6PDH were 1.5 mM, 3.0 mM and 2 units/ml, respectively. This study is represented by the the dashed curves of Figure 3.4.

The results indicate that:

1) The 3.5-fold increase in the oxidation rate rates occured, partially due to extended storage in buffer. It has been observed by many researchers

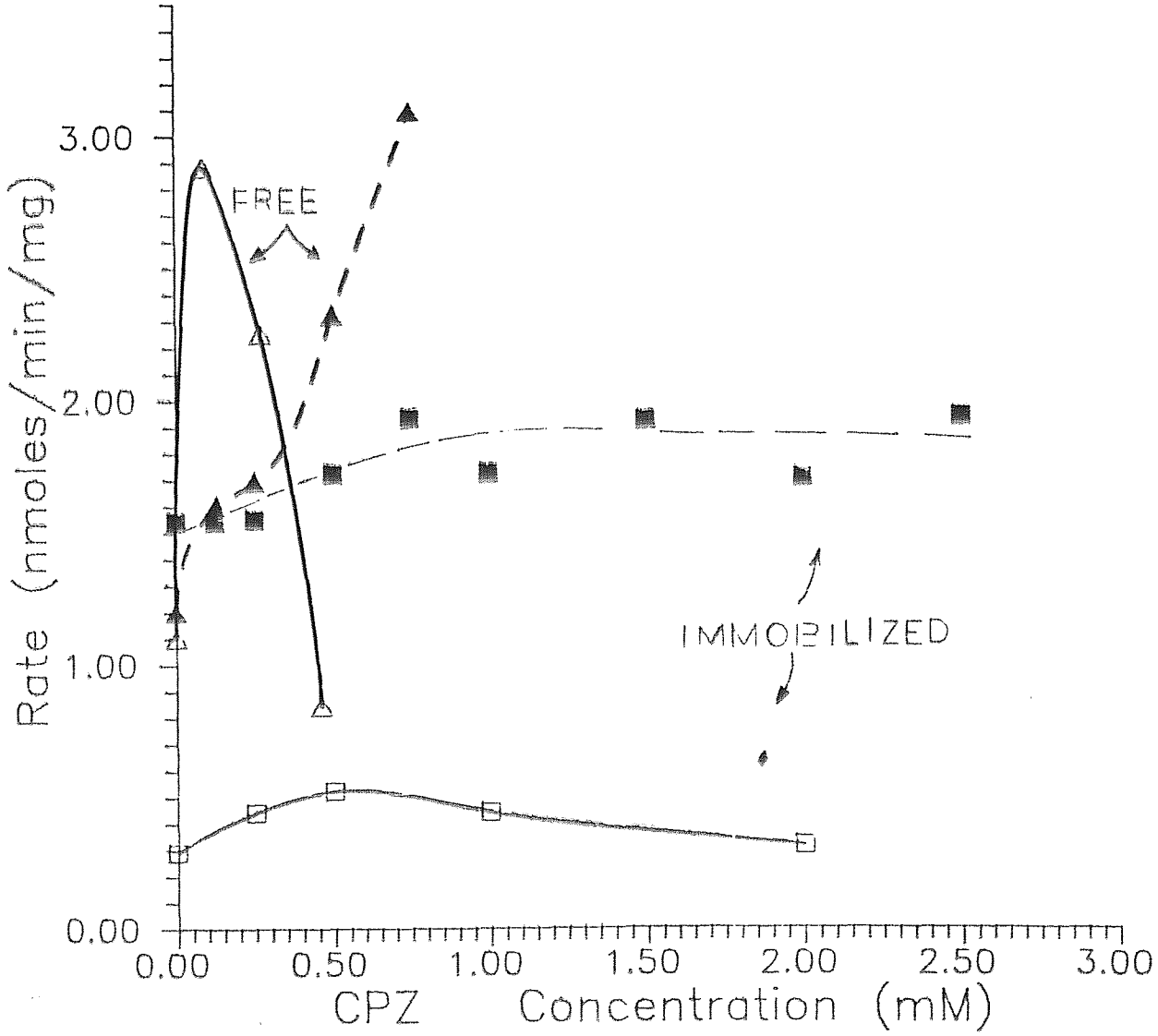


Figure 3.4 The effect of CPZ concentration on biooxidation by immobilized and free microsomes. Dashed: $NADP^+$, G-6-P and G6PDH were in excess. Solid, (-△-△): free microsomes suspended in the the reaction medium. Dashed, (▲ ▲): free microsomes and alginate gel in the same amounts as in beads. Interaction between the alginate gel and CPZ increases the concentration requirement of CPZ for saturation with free microsomes.

(13,26) that extended storage periods in buffer facilitate the wash out of natural inhibitors and cause an increase of the activity. Cavagnaro (26) suggests that this phenomenon is attributed to contaminating proteins. He also showed that up to 2.3-fold and 2.7-fold increase in specific activity occured after a single and a double washing procedure of rat microsomes, respectively.

At this point, it should be noted that during the entire period of this thesis work, protein content measurements indicated 3-4% loss of protein takes place during the curing process, while a lower percentage (1-2%) of protein loss takes place during storage in buffer.

2) The CPZ requirement for saturation increases in the second case. This can be partially explained by the freeing of active sites and pores in the catalyst after the washout of the contaminating enzymes.

3) As it has been shown in the previous section, increase in the initial $NADP^+$ concentration from 0.5 mM to 1.5 mM can cause increase on the biooxidation rate of NADPH and thus accelerate the biooxidation of CPZ proportionaly. As a result, the higher rates and the shifting of the CPZ saturation requirement could be more likely due to NADPH limitations.

4) CPZ inhibition is less pronounced in immobilized cell microsomal reactions. This could be explained by the

higher density of enzymes in a unit volume of immobilized matrix and the high CPZ concentration requirement for saturating the beads. In addition, a more favorable rearrangement of the enzymes in the immobilizing matrix could possibly attribute to a less pronounced inhibition with immobilized microsomes. Inhibition occuring at higher concentration levels of substrate or product has been found to be one of the advantages of immobilization.

5) In the presence of alginate gel, free microsomes require higher CPZ concentration to reach maximal velocity and saturation. This suggests that CPZ gets absorbed into alginate gel, and it can also explain the higher CPZ concentration requirements for saturation.

Applying the results of the second case to construct a Lineweaver-Burke plot, a V_{max} value of 1.91 nmoles/min/mg protein and a K_m value of 0.37 mM were obtained. In the presence of alginate gel, the V_{max} value of free microsomes was calculated to be 3.42 nmoles/min/mg protein. Upon addition of n-octylamine to 2 mM, a maximum activation of 1.2-fold for immobilized microsomes and 1.5 fold for free microsomes were observed. This findings indicated that the N-oxidation of CPZ is primarily due to the microsomal activity of MFAO enzyme.

3.2.6 THE EFFECT OF $CaCl_2$ CONCENTRATION USED DURING THE CURING PROCESS ON THE ACTIVITY OF IMMOBILIZED MICROSOMES

The effect of $CaCl_2$ concentration that is used for curing the alginate microsomal beads was investigated. To do this the activity of 20 beads was obtained by measuring the oxygen uptake during the biooxidation of 0.7 mM NADPH. The immobilized microsomes were allowed to cure for 9.5 hours in 0.05 M, 0.1 M $CaCl_2$; then, were stored in 0.05 M Tris-buffer pH 7.4 for 17 hours. The results are shown in Figure 3.5.

The results suggest that the lower the $CaCl_2$ concentration, the higher the beads' activity. However, it is was observed that the loss of activity at higher $CaCl_2$ concentrations was not due to the inactivation of the microsomes but it was due to the poor porosity of the beads. During a number of general studies with immobilized microsomes it was found out that curing of 300 beads in 100 ml of 0.1 M $CaCl_2$ is an adequate preparatory procedure for curing. In any case, the washing of microsomes with freshly made buffer several times enhances microsomal activity.

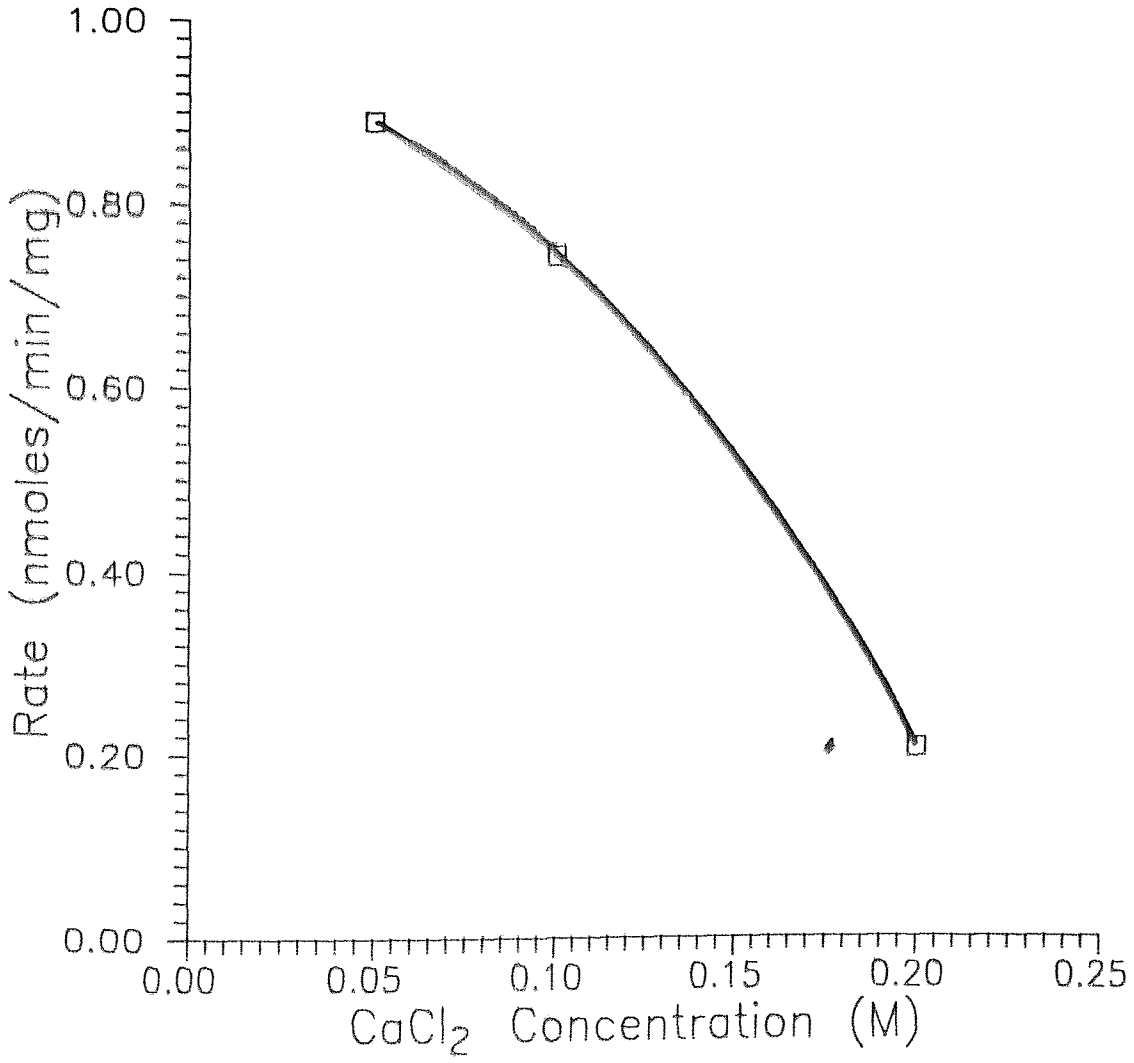


Figure 3.5 The effect of $CaCl_2$ concentration on the activity of immobilized microsomes. Beads were cured in $CaCl_2$ for 9.5 hours and stored in 0.05 M Tris-buffer, pH 7.4, for 17 hours.

3.2.7 POTENTIAL STORAGE CONDITIONS FOR IMMOBILIZED MICROSOMES

The storage of immobilized microsomes at 7-9^{o}C, in 0.1 M $CaCl_2$, 0.05 M Tris-buffer pH 7.4 and in dry refrigerated conditions, were examined.

Figure 3.6 shows the rate profile as a function of oxygen concentration. This figure shows the biooxidation of 0.5 mM CPZ by 30 beads in the presence of 0.5 mM $NADP^+$ and G-6-P/G6PDH excess. The beads were cured in 0.1 M $CaCl_2$, stored in buffer for 13 hours and then stored in dry refrigerated conditions. It appears that the beads were able to retain their activity for a period of 5 days. On a one-day basis, beads stored in buffer showed a 27 % activity increase over the ones remained stored in dry refrigerated conditions. The 11 % deviation between the dried beads stored for 1 day and those stored for 5 days is possible due to a descrepancy in protein content.

Figure 3.7 shows the activity of 30 beads stored under various conditions. The activity here was measured in the same way as in Figure 3.6. It is clear that $CaCl_2$ and refrigerated dry storage result in the same activity. Measurements of activity after storage in buffer suggest an increase in activity. The reasons for this have been explained earlier. However, beads stored in

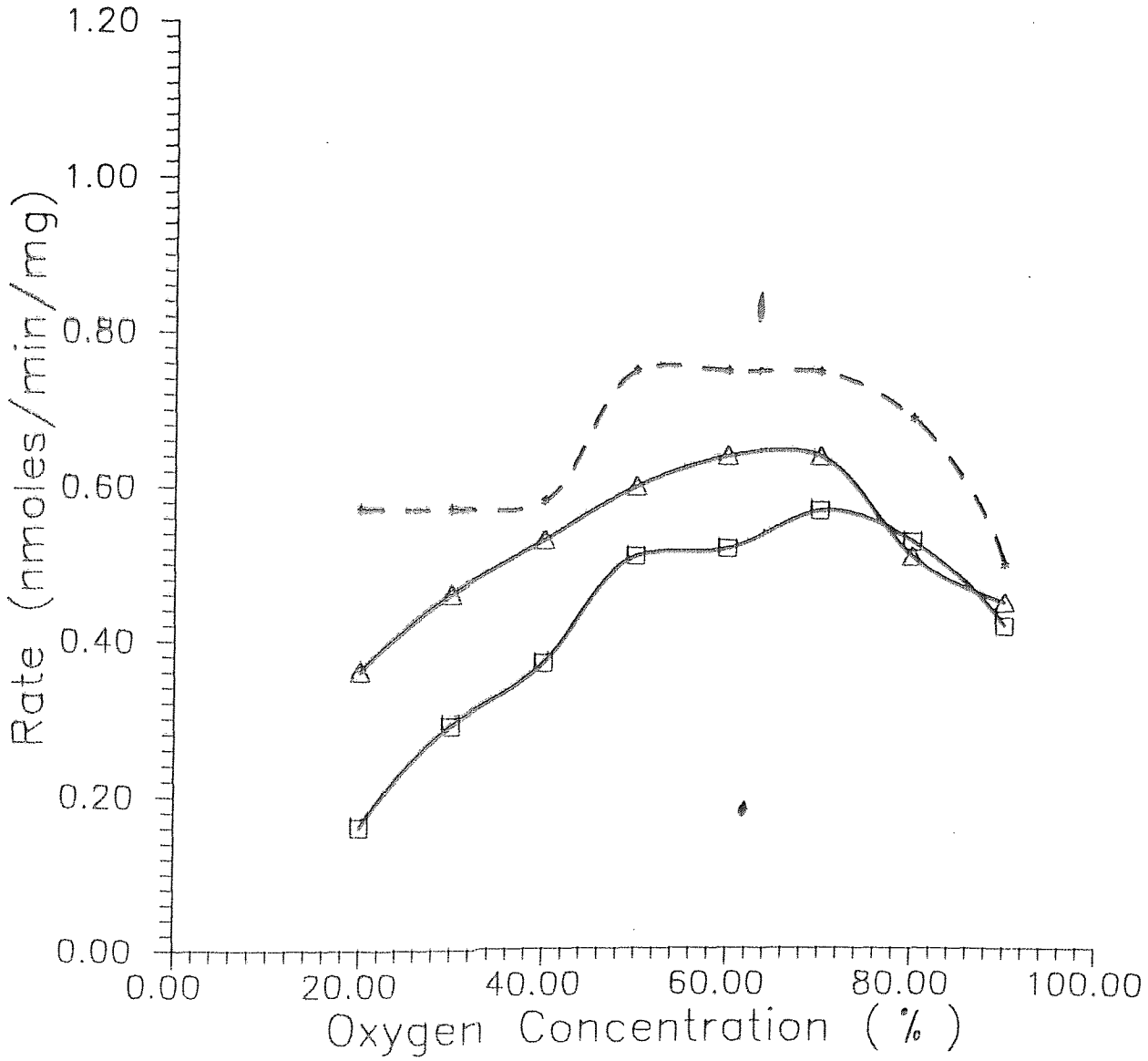


Figure 3.6 CPZ biooxidation by refrigerated dry immobilized microsomes. Dashed: stored in Tris-buffer for 1 day after 20 hours of curing in 0.1 M $CaCl_2$. Solid, (△—△), 1 day under dry storage; (□—□), 5 days under dry storage. The dry beads were soaked in 0.05 M Tris-buffer for 4-5 hours, before they were used.

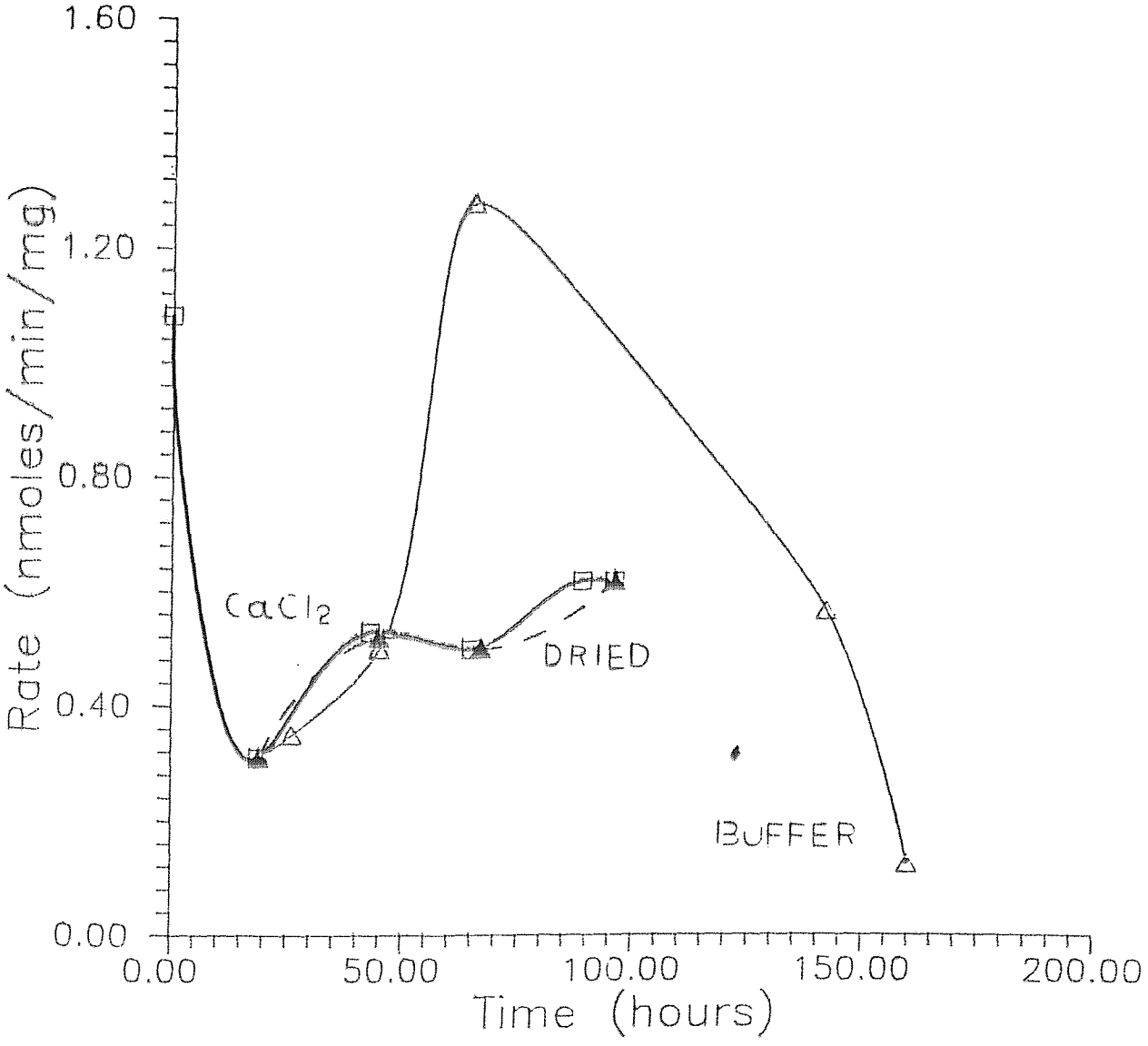


Figure 3.7 The effect of various storage conditions on the activity of immobilized microsomes. Dark solid: storage in $CaCl_2$. Thin solid: storage in Tris-buffer, after 20 hours of storage in $CaCl_2$. Dashed: storage under refrigerated dry conditions, after 20 hours of storage in $CaCl_2$.

buffer begin to become contaminated by bacterial sources. Such contamination was found to begin its progress after 4-5 days of storage in buffer. The drastic increase in activity after the third day is probably the result of an earlier bacterial contamination. In the presence of bacterial sources, increase in the oxygen consumption could be due to bacterial respiration.

In general, the above study suggests that refrigerated drying can be a promising way to store immobilized microsomes for long period of time. For a shorter than 5 day period, storage in 0.1 M $CaCl_2$ followed by a number of washings with Tris-buffer over a period of 5-10 hours can be used.

3.2.8 BATCH VS RECIRCULATION FLOW REACTOR CONFIGURATION

A small number of recirculation runs was performed using alginate immobilized liver microsmes. Here the objective was to deside whether the recirculation flow configuration is a potential configuration for larger scale production of amine oxides, when immobilized microsomes are used.

By comparison to the batch 1.9 ml microassay reactor, recirculation offers a number of advantages. Recirculation flow runs could operate at a wide range of

flow rates and for as long as 20 hours without destruction of the beads. With the batch configuration the beads begin to break down after a 4-hour run. The reason for this is the continous pounding of the beads by the stirring mechanism (in this case is the spin bar).

The presence of diffusional gradients, either external or internal to the biocatalyst (immobilized enzyme matrix particles or beads), and axial or radial nonuniformities of flow, temperature and concentration in columns of biocatalyst, often result in significant distortion of reaction kinetic values such as V_{max}, K_m and % of active site in biocatalyst. Using the recirculation flow reactor system, the negative effects of gradients and nonuniformities can be be minimized. Consequently, the recirculation configuration can become a powerful research tool that can provide nearly gradient-free kinetic data in studies of heterogenous catalysis. A number of reaction kinetics equations that describe a recirculation reactor are given in Appendix B.

The result of both of the above mentioned advantages can provide higher reaction yields that are very important when dealing with costly materials such enzymes, and cofactors.

Another operational advantage of the recirculation system built is that the progress of reaction can be

monitored by the two attached D.O. impingement probes while the entire recirculation system operates as an open to the atmosphere system. The probe at the inlet can measure the oxygen concentration of the reservoir (that must be significantly larger than the reactor itself), while the probe at the outlet can provide us with the rate of the biooxidation taking place in the reactor. Such measurements, however, can be successfully made as long the oxygen gradient between the oxygen in the reactor bed and the atmosphere is kept at levels that do not act as the controlling mechanism of oxygen depletion.

The functions of the reservoir are: first, to act as a de-bubbler, allowing the gas (O_2) caught in the system to be purged within one pass through the loop, second, to act as a mixing tank, providing rapid despersion of any concentration gradients, and third, to supply the reactor with reactant fluid that is well saturated with oxygen and is at constant temperature.

With the recirculation flow configuration shown in Appendix A, the use of pure oxygen saturation conditions not only led to lower rates but their measurement by the probes was not feasible. It was found out that above 30 % pure oxygen the rate of oxygen diffusion was much higher than the oxidation rates. Specificaly, between 30% and 65

% pure oxygen concentration the oxygen diffuses out in a rate of 5 nmoles/min/% oxygen concentration that the reactor operates. Thus operating at 50 % oxygen saturation the oxidation must exceed 250 nmoles/min. Providing that the beads used in this study can result in maximal rates of 1.91 nmoles/min/mg, at least, 231 mgs of immobilized microsomes (i.e. 262 beads) to reach the diffusion rate of 250 nmoles/min would be needed. In addition, such a run would be very expensive as a result of its cofactor requirements.

Therefore, it can be concluded that oxidation rate measurements using the recirculation flow reactor system can be made only under air saturation conditions. At higher oxygen saturation conditions the higher enzyme requirement as well as the cofactor and coenzyme requirements will result in much higher cost of operation. If recirculation runs were to be made at higher oxygen concentrations, the use of immobilized purified enzyme would make oxidation rate mesurements easier.

3.2.9 IDENTIFICATION OF CPZ N-OXIDE

Identification of CPZ N-oxide was carried out using TLC, HPLC and proton NMR.

Analysis by TLC indicated that both, 9:1:0.3 acetone-H_2O-ammonia(6 M) solvent system and 2.5:7.5 MeOH-

$CHCl_3$ solvent system were found to be appropriate for TLC identification of the N-oxide. For the first system, the R_f values for CPZ and CPZ N-oxide were found to be 0.86 and 0.20. For the second system, the corresponding R_f values were 0.46 and 0.21 respectively. The results of the TLC development in the iodine chamber are shown in Figure 3.8.

The results from the analysis with HPLC are shown in Figure 3.9. The peaks of both CPZ and its N-oxide were well resolved. The retention time of CPZ and CPZ N-oxide were 200 secs and 320 secs, respectively. When additional CPZ was added to the bioproduct mixture (Figure 3.9.2A), the CPZ peak of the bioproduct mixture was identified (Figure 3.9.3A) and, consequently, the peak of CPZ N-oxide (Figure 3.9.3B). By comparison to the peak of the pure stating material (Figure 3.9.1A), it appears that in the presence of its N-oxide the retention time of CPZ shifts to shorter time.

The NMR spectra of pure CPZ and CPZ N-oxide (in the presence of a small amount of unreacted CPZ) are shown in Figure 3.10. The spectra in Figures 3.10.1 and 3.10.2 are of pure CPZ but the second has been obtained with lower concentration of CPZ. This was done for identifying the chemical shift of the protons in the NMR spectrum of

CPZ N-oxide (Figures 3.10.3) by comparison to pure CPZ of almost equal concentration.

The NMR spectra of chlorpromazine and its oxidized metabolite in Figure 3.10 show no differences in the patterns of aromatic protons associated with the phenothiazine ring (δ 7.35-6.50) and only a small change (δ, 4.1-4.05) of the methylene triplet α to the aromatic nitrogen atom. These observations demonstrate that neither the sulfur nor nitrogen atoms of the phenothiazine ring were oxidized. However, the upfield shift (δ, 1.72-1.80) of the pentet of the methylene protons β to the aromatic nitrogen atom, the slight downfield shift of the N-methyl couplet (δ, 3.05-2.98) α to the terminal nitrogen indicate that the oxidation occurred on the terminal (N-methyl) perazine nitrogen atom in the metabolite. The methylene singlet at δ, 2.65 of the two terminal methyl groups did not change. The spectrum of the metabolite is consistent with the NMR spectra of three other phenothiazines reported elsewhere (3). The results obtained by NMR suggest that the microsomal amine oxidase specifically catalyzes N-oxidation of only the terminal nitrogen atom of chlorpromazine.

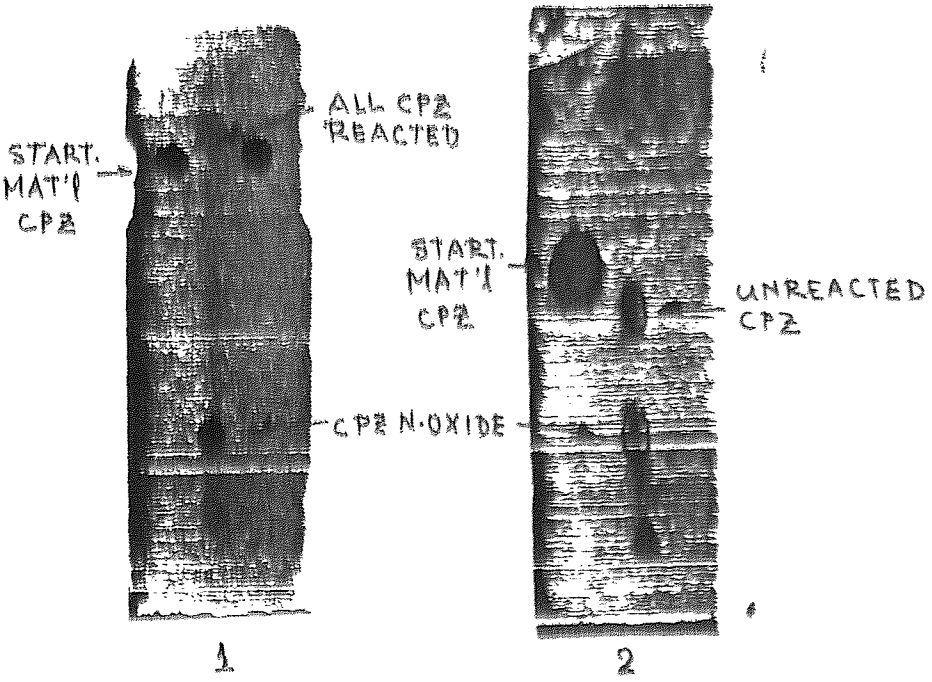


Figure 3.8 Identification of CPZ N-oxide by TLC, using, (1), acetone-ammonia 9:1 and (2), $CHCl_3$-MetOH 75%-25% .

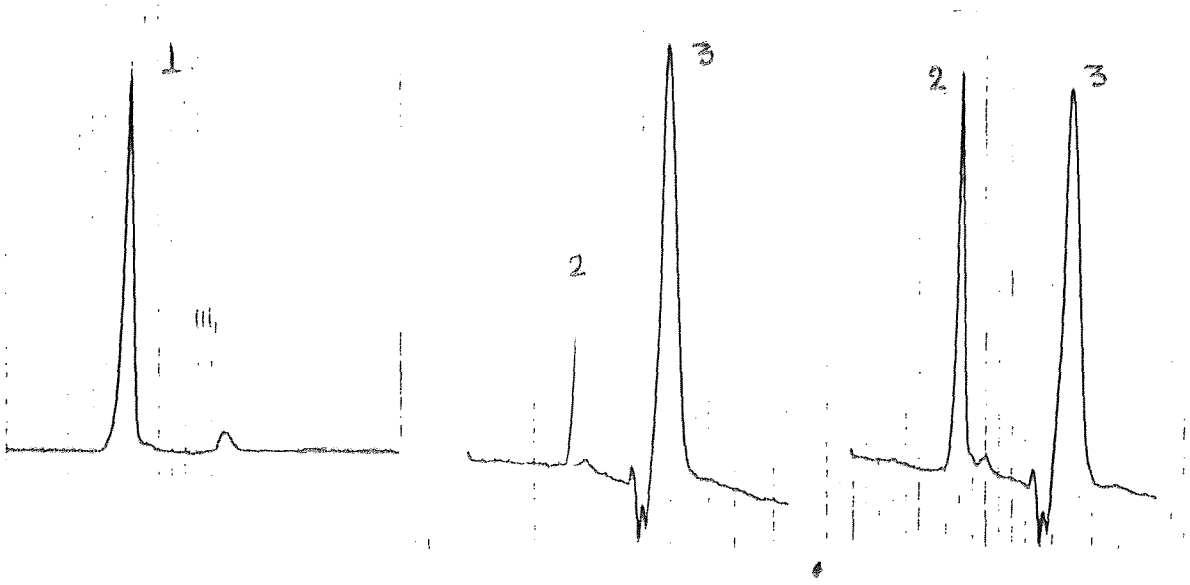


Figure 3.9 Identification of CPZ N-oxide by HPLC. (1), CPZ starting material; (2),(3), mixture of unreacted CPZ and bioproduct CPZ N-oxide (3), after introducing

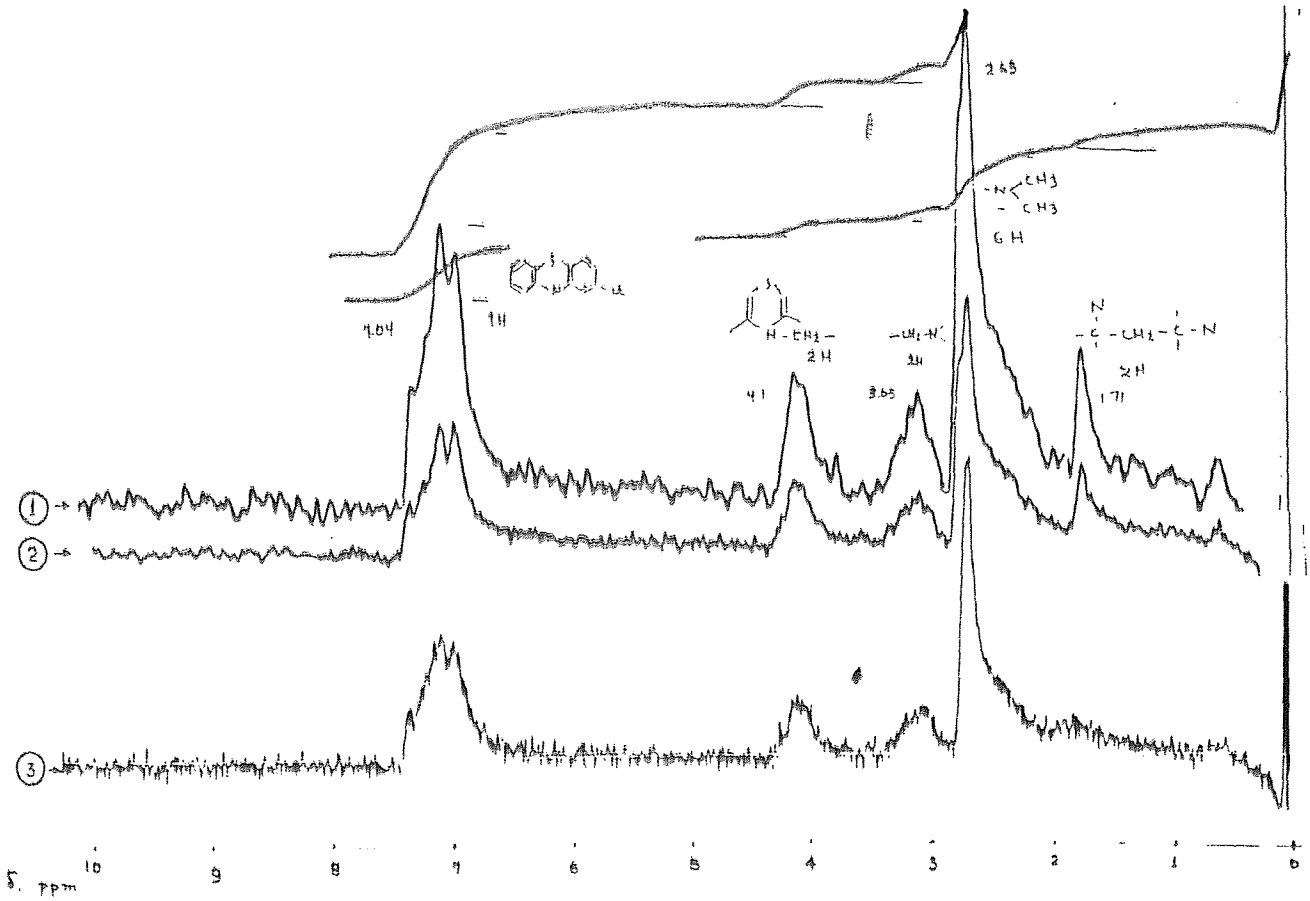


Figure 3.10 Identification of CPZ N-oxide by proton NMR. (1),(2), CPZ starting material; (3), mixture of bioproduct CPZ N-oxide and unreacted CPZ (97%-3%).

CHAPTER IV

STUDY OF THE METABOLIC PATHWAYS IN THE BIOOXIDATION OF 4-HYDROXY-2,2,6,6-TETRAMETHYLPIPERIDINE TO NITROXIDE FREE RADICAL

4.1 MATERIALS AND METHODS

4.1.1 MATERIALS

Alginic acid (grade IV), $NADP^+$, NADPH, G-6-P, G6PDH, TMPO and Tris buffer were obtained from Sigma Chemical Co. With the exception of the HPLC run NADPH was regenerated from its oxidized form, $NADP^+$, using G-6-P and G6PDH. TMP was obtained from FluKa Chemical Co. and TMPO was obtained from Sigma Co. TMPOH was obtained by reducing TMPO with ascorbic acid according to the literature (27). Hog liver microsomes were the generous gift of Prof. D.M. Ziegler, University of Texas at Austin. The preparation of microsomes is described elsewere (1).

4.1.2 BIOCATALYST PREPARATION

A 2% sodium alginate gel was prepared by dissolving 2 g alginic acid and 2 g sodium chloride into a gently heated and well stirred 100 ml, 0.05 M Tris buffer

solution. Beads were prepared by mixing cold sodium alginate gel solution with an equal volume of liver microsomes. The mixture was homogenized and then extruded through a syringe (21G $1^1/_2$ "), drop by drop, into cold stirred 0.2 M $CaCl_2$ solution of pH 7.4. The protein content of each bead and free microsomes used was of the order of 4.2 mg per 10 beads and 2.1 mg per 5 drops of microsomes respectively. The "Lowry method" (28) was used to determine the protein content of the biocatalyst.

4.1.3 INSTRUMENTATION (E.S.R.)

A brief discussion of the principles involved in the function of the main instrument used for identification of nitroxide free radicals is given below.

Electron paramagnetic resonance spectroscopy (EPR or ESR) is based upon the absorption of microwave radiation by an unpaired electron when it is exposed to strong magnetic field. The E-line system used for the analysis and identification of free radicals, biradicals, and triplet states. It is also used for studying: chemical kinetics, electron exchange rates, molecular structures, unpaired electron wave functions, and electrostatic fields of transition element ions.

The E-line system contains an operator console, a microwave bridge, a support table, and a magnet power

supply. The system is completely solid state in design with the exception of the tube of the osciloscope. The functional relationship of the system is illustrated in Appendix A.

The principles of EPR are similar to those of NMR spectroscopy. The unpaired electron, like the proton, has a spin quantum number of 1/2 and thus has two energy levels that differ slightly in energy under the influence of a strong magnetic field. In contrast to the proton the lower energy corresponds to m = -1/2 and the higher to m = +1/2; this difference results from the negative charge of the electron.

The energy required to give us the peak to peak spectrum is represented by the following equation:

$$E = h*v = u*b*H0 / I = g*b*H0$$

where g is the splitting factor and b is the Bohr magneton, which has value of 9.27E-21 erg Gauss. The value of g for unpaired electron is approximately 2.0000 depending upon the electron's environment. H0 is the field size of the spectrum (usually 3240 G). The resonance frequency for an unpaired electron is about 9.5E+9 Hz.

4.1.4 ANALYSIS

The biotransformation of TMP, TMPOH and TMPO was monitored by oxygen uptake measurements. The formation of TMPO during the progress of the reaction was followed with HPLC. Since neither TMP nor TMPOH absorb in the UV region, while TMPO does, HPLC served well as a qualitative and quantitative instrument.

For HPLC analysis the following reversed phase columns were used: i) Altech, Spherisorb 5um 250x4.6 mm following an SSI, Soft Seal Alkyl 18/5 um 150x4.6 mm; flow rate: 1 ml/min; UV detector set at 235.5 nm and ii) Column Resolution Inc., CRI C8 150x4.6 mm; flow rate 1 ml/min; UV set at 240 nm, were used. Acetonitrile-Water 65%-35% was used as solvent.

A Varian E-12 EPR spectrometer was used for the identification of the TMPO nitroxide radical.

A Clark-type D.O. probe [YSI 4004 Clark Oxygen Probe] was used to determine the content of oxygen in reaction medium.

The presence of TMPOH and TMPO in the reaction medium at the end of reaction was also verified by TLC. EtoAc was used as solvent. TMPO and TMPOH were detected by UV light after being developed in an iodine chamber.

4.1.5 OXYGEN UPTAKE AND HPLC MEASUREMENTS

Oxygen uptake measurements were made in a microassay bioreactor consisting of a 1.9 ml water jacketed reactor vessel equipped with a 0.1 ml magnetic bar. The oxygen content in the reactants was monitored using a Clark-type oxygen probe. The installation of this reactor is shown in Appendix A.

The reaction assay began by adding 1.6 ml 0.05 M Tris buffer (pH 7.4) followed by addition free microsomes or beads and saturating with air. Further additions to the reaction medium consisted of 0.5 ml G-6-P/G6PDH solution containing 5.5 umoles/ml G-6-P and 20 units/ml G6PDH followed by 50 ul of 44 umoles/ml $NADP^+$. Two minutes after the addition of $NADP^+$, the substrate to be tested was injected into the bioreactor. The effect of n-octylamine was invetigated by adding various amounts of a water saturated solution 4 minutes after injecting the substrate.

Since immobilization helps to keep the reaction medium free of microsomal particles, 10 ul samples of the reaction medium could be taken for HPLC analysis during the progress of the reaction.

In addition to the 1.9 ml microassay runs, a duplicated 9.5 ml batch run was performed to follow the

progress of TMP biooxidation at the same conditions used during the microassay runs. This time the progress of the reaction was followed by measuring the formation of TMPO using HPLC. In this run 50 beads were used in a reaction allowed to progress for 3 hours. At the end of each run, the mixtures were raised to pH 10, the unreacted substrate and its oxidative products were extracted with chloroform and, then, TLC analysis followed.

4.2 RESULTS AND DISCUSSION

4-Hydroxy-2,2,6,6-tetramethylpiperidine was found to be oxidized to 4-hydroxy-2,2,6,6-tetramethylpiperidine-1-oxyl (a nitroxide radical) in a reaction requiring oxygen and NADPH. As it is shown in chapter V, diphenylamine can also be oxidized to its nitroxide radical derivative by hog liver microsomes in the presence of NADPH and oxygen. Following the findings of the works of Rauckman-Rosen and Stier-Reitz, the biooxidation of TMP is investigated in this chapter.

4.2.1 TMP BIOOXIDATION

A number of runs was performed in a 1.9 ml microassay reactor in an attempt to investigate the biooxidation of TMP by measuring the oxygen consumption in the reactants. Using 10 beads (0.11 ml; 4.2 mg protein) and a G-6-P/ G6PDH/NADPH regeneration system, the NADPH oxidation rate was found to be 1.16 nmoles/min/mg protein. In the range of TMP concentration between 0.45 and 2.14 mM, the substrate dependent oxidation rate of NADPH did not change significantly and showed that no relation between TMP concentration and oxidation rate exists. TMP saturates the reaction medium at 0.45 mM and

no substrate inhibition occurs in the range of concentrations tested. Based on oxygen uptake measurements, an average TMP oxidation rate over 5 runs was calculated to be also 1.18 nmoles/min/mg protein. The same results were obtained using 10 beads consisting of 0.35 mg protein each. This implies that above 2 mg/ml of immobilized microsomal enzymes, the TMP-dependent oxidation rate of NADPH is not a linear function of microsomal enzyme concentration. Deviations from linearity at protein concentrations above 1.5 mg/ml, for mouse microsomes is reported elsewhere (29).

Using free microsomes (0.055 ml; 2.1 mg protein) and with basis of an NADPH oxidation rate of 4.57 nmoles/min/mg protein, the TMP depended oxidation rate of NADPH decreased from 4.71 to 2.83 nmoles/min/mg protein as TMP concentration increased from 0.45 mM to 1.64 mM respectively. The effect of TMP concentration on the NADPH oxidation rate, for immobilized and free microsomes, is shown in Figure 4.1. According to the data of Figure 4.1, substrate inhibition for free microsomes begins past 0.45 mM TMP while inhibition for immobilized microsomes is not observed for TMP concentrations up to 2.22 mM. At 0.45 mM TMP concentration and for free microsomes, the oxidation rate of TMP was maximum and nearly the same as

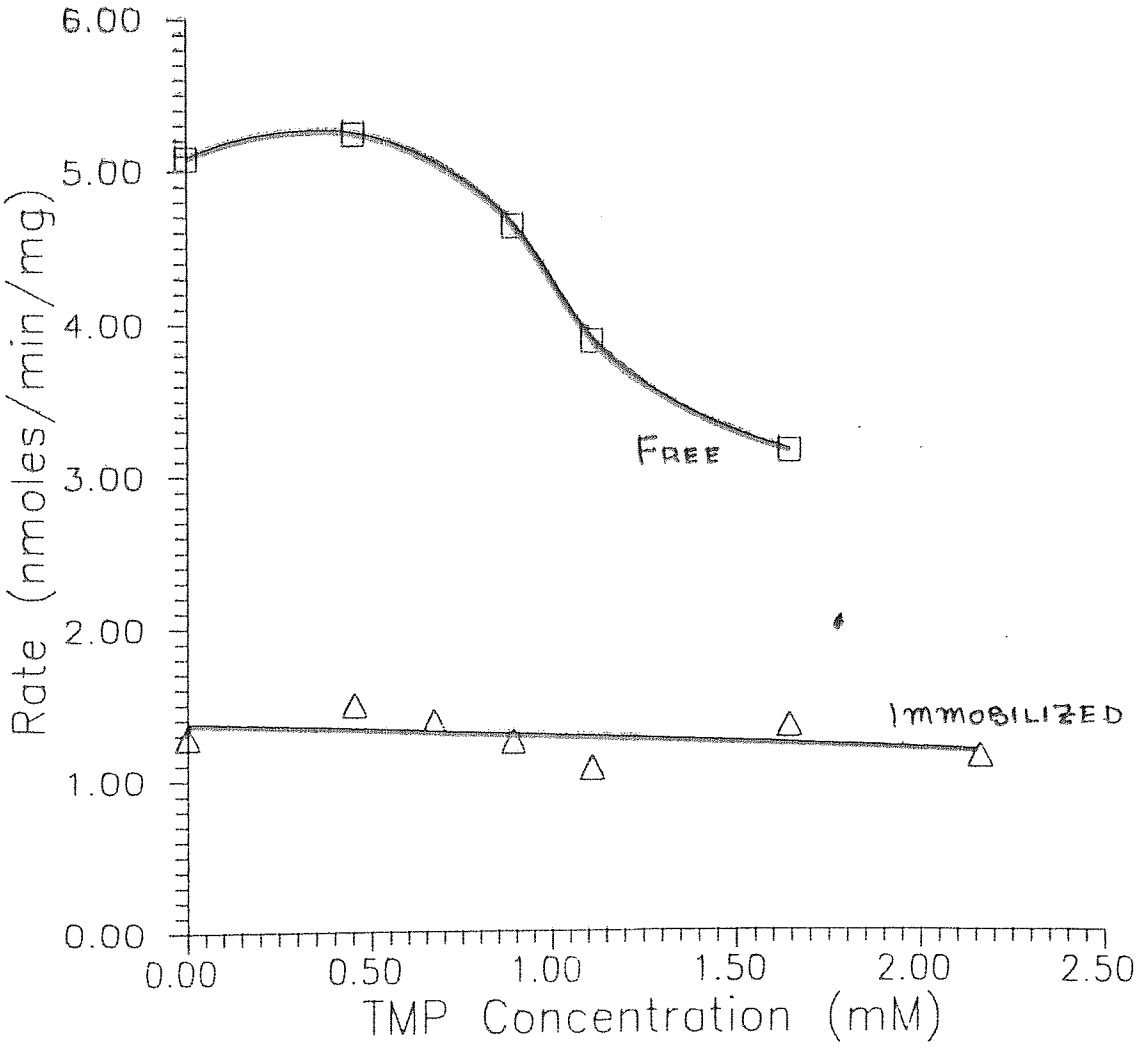


Figure 4.1 The effect of TMP concentration on biooxidation by immobilized and free microsomes.

the NADPH oxidation rate, 4.71 nmoles/min/mg protein.

In determining the extent of MFAO participation in the biooxidation of TMP, n-octylamine, a known activator for MFAO was used. In the presence of 0.45 mM TMP, the biooxidation of TMP increased by 2-fold and 1.5-fold after the addition of 2.24 and 1.25 mM n-octylamine, respectively. In the presence of 0.82 mM TMP, the oxidation rate increased by 1.3-fold after the addition of 0.78 mM of the activator.

It has been reported elsewere (30) that n-octylamine activates MFAO up to 2-fold. The major effect of n-octylamine on enzyme catalysis is that n-octylamine binding accelerates the breakdown of the C(4a)-hydro-peroxyflavin complex that is a reactive intermediate in the oxidation of nitrogen and sulfur bearing compounds. In a detailed analysis presented by N. Beaty and D. Ballou (20), it is reported that the breakdown of the C(4a)-hydroperoxy-flavin is the rate limiting step in the biooxidation of N- and S-bearing substrates. Rosen and Rauckman have also suggested that the biooxidation of TMP could be the rate-limiting step in the conversion of the amine to the nitroxide (5). A suggested scheme of the biooxidation cycle of secondary amines is shown in Figure 4.2. A similar scheme is given by Poulsen and Ziegler (25).

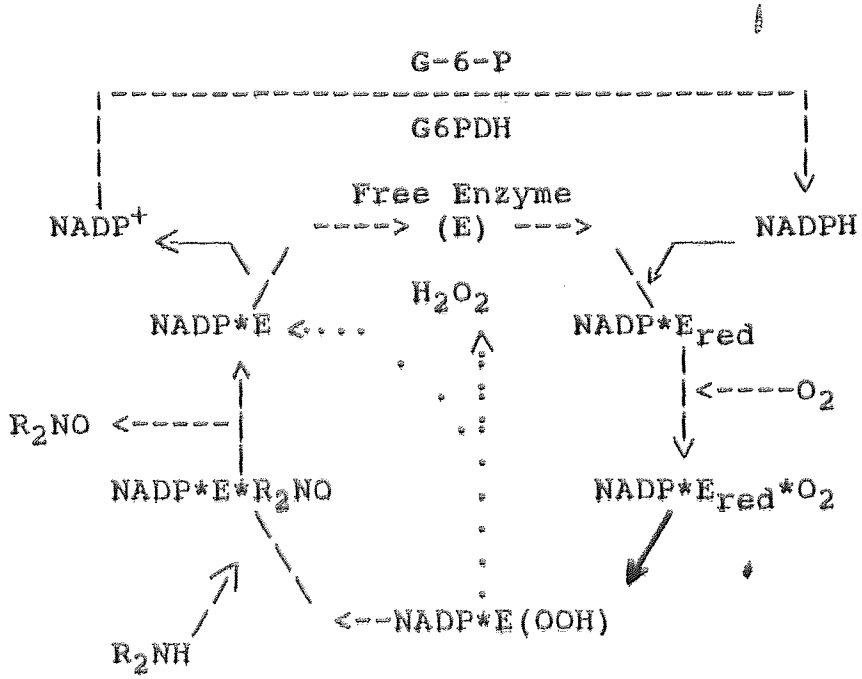


Figure 4.2 Mechanism scheme for the biooxidation of hindered secondary amines by MFAO. Abbreviations are: E and E_{red}, oxidized and reduced forms of enzyme; R_2NO, substrate and oxygenated substrate; E(OOH), C(4a)-hydroperoxyflavin intermediate. Heavy arrow indicates an irreversible step. Dotted line indicates an alternate route that that takes place in the absence of substrate and leads to the formation of hydrogen peroxide (H_2O_2).

In examining further the nitroxide formation from the amine, 50 beads were used to oxidize 0.45 mM TMP in a reaction consisting of 2.6 mM NADPH in 9.5 ml of 0.05 M Tris buffer pH 7.4. This run was performed at 37 ^{o}C and its progress was followed by HPLC for 165 minutes. The peak of TMPO standard identified by comparison of the retention time of TMPO purchased by Sigma Chemical Co.

The formation of TMPO was found to be a linear function of time with a rate of 0.48 uM/min or 0.22 nmoles/min/mg microsomal protein. Combining the NADPH and TMP oxidation rate obtained by oxygen uptake measurements and TMPO formation measured by HPLC we found a nearly 0.18:1:1 ratio between TMPO formation, oxygen consumption and TMP dependent NADPH oxidation. During the last stage of the reaction H_2O_2 addition did not result in increase of the TMPO peak. This indicates that hydrogen peroxide does not act as an oxidizing agent enhancing the formation of the nitroxide.

4.2.2 TMPOH BIOOXIDATION

TMPOH (0.45 mM) was allowed to be oxidized by 10 beads in the microassay reactor. The oxidation rate of TMPOH was found to be nearly the same as the NADPH oxidation rate. Upon addition of n-octylamine to 2.24 mM,

only 1.2-fold increase on the oxidation rate of TMPOH over the oxidation rate obtained without the activator (5.1 nmoles/min or 1.21 nmoles/min/mg protein).

The Haber-Weiss reaction (7) known to produce hydroxyl radicals during hydroxylamine oxidation should enhance the formation of nitroxides. Since this did not occur even with the addition of n-octylamine the Haber-Weiss reaction is less likely to occur. Instead Rauckman's and Rosen's theory of superoxide mediated oxidation of hydroxylamines is suggested to occur.

Using free microsomes we found that addition of 1.11 and 1.64 mM TMPOH decreased the TMPOH-dependent oxidation rate of NADPH to 2.32 and 1.42 nmoles/min/mg protein, respectively. This could be the result of a superoxide inhibitor present in the free microsomes but absent in immobilized microsomes due to the filtering/cleaning characteristic of immobilization (13). If superoxide dismutase, a superoxide inhibitor, is assumed to be present in microsomes, this could be supporting evidence for the superoxide mediated oxidation of hydroxylamines.

4.2.3 TMPO BIOREDUCTION

The effect of TMPO upon the oxidation rate of NADPH was also investigated by means of oxygen uptake measurements.

Using 10 beads, the TMPO-dependent oxidation rate of NADPH was found to increase as a function of the TMPO concentration. The same was observed for the free microsomes. By fitting the initial rate of TMPO dependent NADPH oxidation as a function of the TMPO concentration, the apparent half-saturation coefficients $(Km)_{app}$ are 0.0745 mM for immobilized microsomes and 1.63 mM for free microsomes; the maximum reaction velocities are 1.05 umoles/min/mg protein and 5.74 umoles/min/mg protein, respectively. In discussing the factors affecting the values of Km_{app} and Vmax one should consider that nitroxides carry an oxygen with an unpaired electron that can easily bind with reduced forms of an enzyme. In this case, due to the high density of enzymes in the immobilized matrix, the TMPO requirement for saturation could be less in immobilized microsomes. Such a study, however, is not within the scope of this work. The Lineweaver-Burke plot for TMPO biotransformation are shown in Figure 4.3.

In the presence of 0.78 mM n-octylamine a decrease of the TMPO-dependent NADPH oxidation rate by 20% was observed. This indicates that MFAO is not involved in TMPO's biotransformation.

In order to decide on the fate of the biotransformed TMPO we consider two pathways: (i) TMPO reduction by

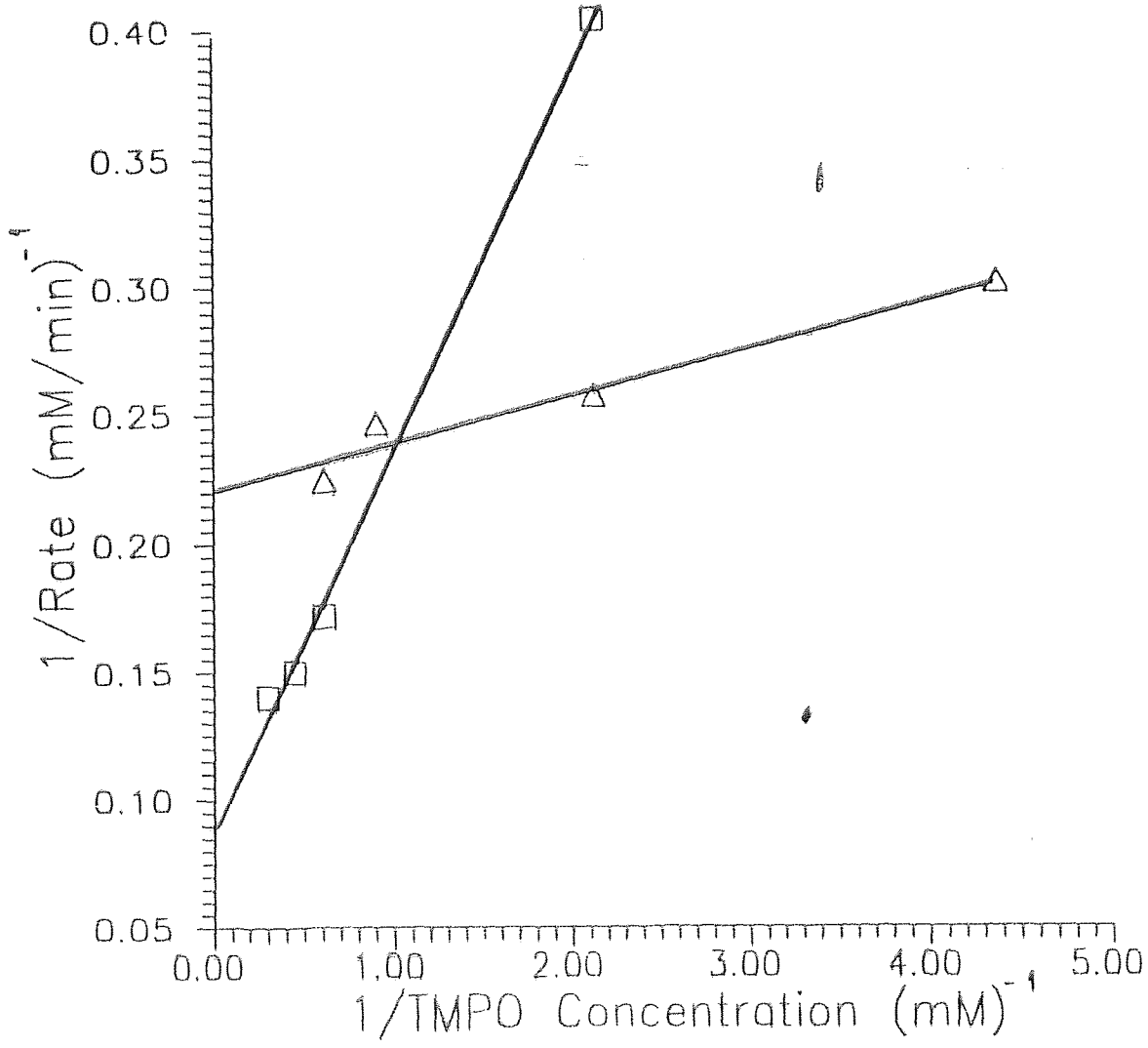


Figure 4.3 A Lineweaver-Burke plot for TMPO bioreduction. (-□-□-), immobilized microsomes; V_{max}=1.05 umoles/min/mg protein, K_m=0.0745 mM. (-△-△-), free microsomes; V_{max}=5.74 umoles/min/mg protein, K_m=1.63 mM.

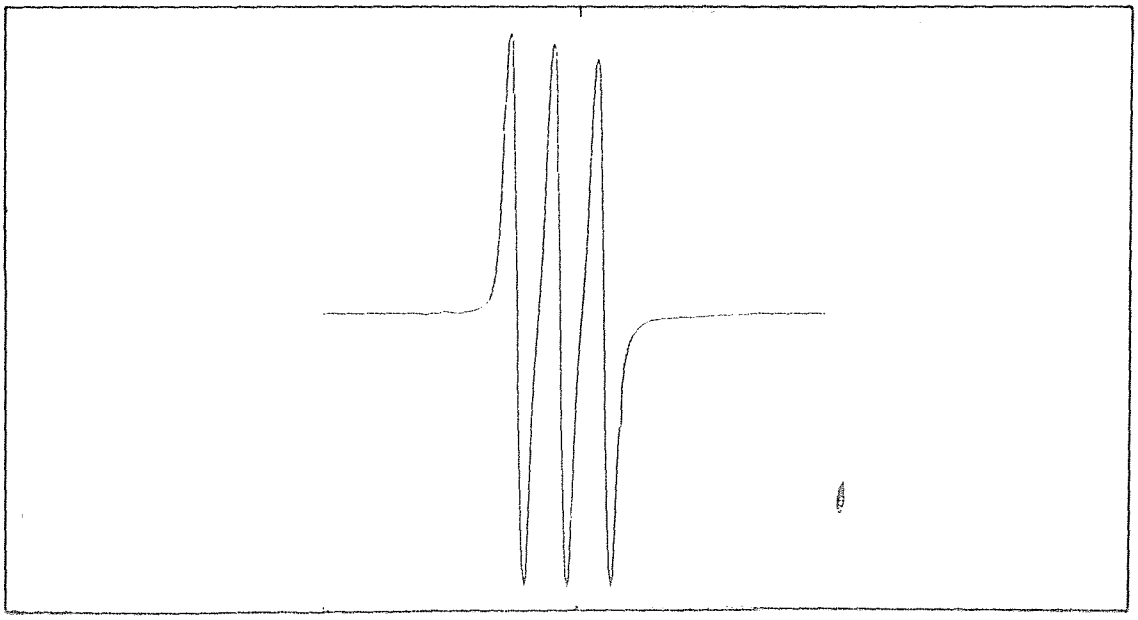
cytochrome P-450 in a reaction requiring oxygen, (ii) TMPO oxidation to 4-hydroxy -2,2,6,6-tetramethyl-1-oxopiperidinium ($N^{+}=O^{-}$) by MFAO. If the first hypothesis is correct n-octylamine should not facilitate the catalytic activity of cytochrome P-450. This was found to be true with the immobilized microsomes. Rauckman and Rosen reported (5,7) that the nitroxide undergoes an NADPH-dependent selective reduction to hydroxylamine by cytochrome P-450 and that this reduction is competively inhibited by cytochrome P-450 antagonists. As a result, the second hypothesis appears to be less likely to occur. In any case the biotransformation of TMPO to TMPOH was verified by TLC.

Swartz et al. (31), have reported that nitroxide reduction increases as a its concentration increases while the presence of oxygen decreases the rate of its reduction independently of the concentration. The data that were collected at oxygen saturation conditions indicated that the TMPO biotransformation increased as the TMPO concentration increased.

As a result, it is more likely for TMPO to become reduced by a non MFAO (i.e. cytochrome P-450) enzyme in the presence of NADPH and oxygen.

In conclusion, this study suggests that TMPO is reduced by cytochrome P-450 to TMPOH. TMP is found to be

oxidized by the MFAO enzyme of hog liver microsomes according to Figure 4.2, forming an intermediate whose breakdown is a rate limiting step on the TMP biooxidation, and it is enhanced by the presence of n-octylamine. As proof of the nitroxide formation the E.S.R. and HPLC spectra of TMPO obtained by enzymic oxidation of TMP, and TMPO standard purchased by Sigma Chemical Co. are shown in Figures 4.4, and 4.5, respectively. The biooxidation of TMPOH to TMPO is found to be the result of a superoxide mediator related to MFAO's catalytic activity.



E.S.R. spectra of bioproduct TMPO at 25 ^{o}C.
Scan Range: 400 G; Modulation Amplitude: 4 G;
Receiver Gain: 6.3; Microwave Power: 2 mW; Field Set: 3240 G;
Scan Time: 4 minutes; Microwave Freq.:9.07439 GHz.

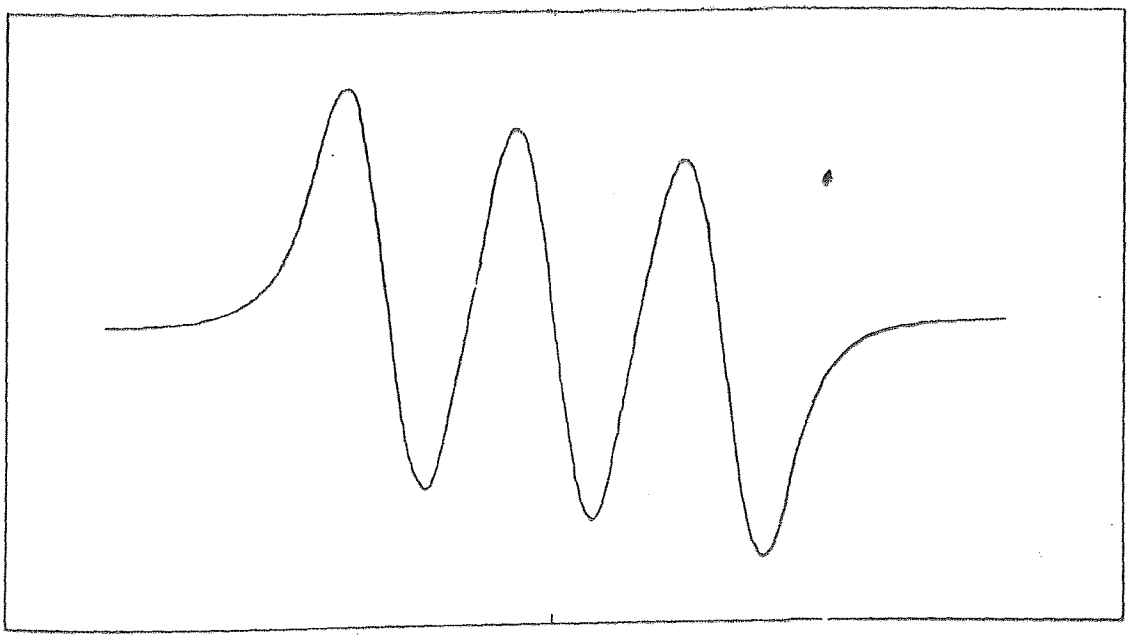


Figure 4.4 E.S.R. spectra of commercial TMPO (Sigma Co.) at 25 ^{o}C. Scan Range: 100 G; Modulation Amplitude: 4 G; Receiver Gain: 5; Microwave Power: 2 mW; Field Set: 3240 G; Scan Time: 4 minutes; Microwave Freq.: 9.07813 GHz.

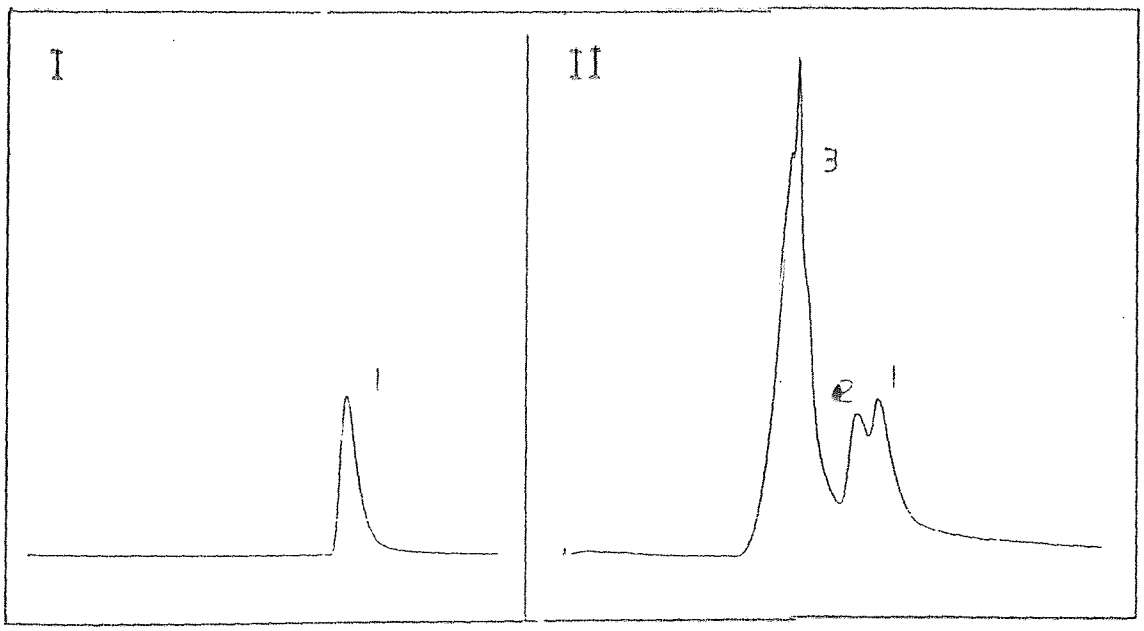


Figure 4.5 HPLC spectra of commercial, (I-1), (II-1), and bioproduct, (II-2), TMPO. Peaks (II-3) indicate the presence of the cofactors used and, possibly , any reaction intermediates.

CHAPTER V

BIOOXIDATION OF DIPHENYLAMINE BY HOG LIVER MICROSOMES

5.1 MATERIALS AND METHODS

5.1.1 MATERIALS

Alginic acid (grade IV), $NADP^+$, G-6-P, G6PDH, Tween-80 surfactant and Tris-buffer were obtained from Sigma Chemical Co. NADPH was regenerated from its oxidized form, NADP+, using G-6-P and G6PDH. Diphenylamine (reagent grade) was recrystalized from hexane, m.p. 53-54^{o}C [lit. (32) 54-55^{o}C]. Diphenylnitroxide (DPANO) was synthesized according to literature (33) by reacted nitrobenzene and phenylmagnesium m.p. 60-62 ^{o}C. Diphenylhydroxyl-amine (DPNOH) was synthesized by reducing DPNO with ascorbic acid in EtoAc (33). Hog liver microsomes were the generous gift of prof. D.M. Zeigler, University of Texas at Austin. The preparation of microsomes is described elsewhere (1).

5.1.2 BIOCATALYST PREPARATION

The preparation of biocatalyst is described in earlier chapters. The protein content of each bead ranged between 0.32 and 0.39 milligrams.

5.1.3 ANALYSIS

DPA oxidation rate was monitored by HPLC and oxygen uptake measurements. DPA oxidative products were determined by HPLC. Reversed phase columns: i) Altech, Econosphere 150x4.6 mm; ii) Altech, Econosphere (c18) 3u 50x4.6 mm were used. 254 nm UV detector was used for detection and 65/35 v/v Acetonitrial/water was used as eluent.

A Varian, E-12 EPR spectrometer was used for the analysis of diphenylnitroxide free radical.

A Clark-type D.O. probe [YSI 4004 Clark Oxygen Probe] was used to determine the content of oxygen in reaction medium.

5.1.4 PROCEDURE FOR BIOOXIDATION OF DPA

Diphenylamine (50 mg) was added to 10 ml 3% Tween-20 solution which was gently heated and stirred for one hour until a stable emulsion solution is formed.

A microassay bioreactor consisting of a 1.9 ml water jacketed reaction vessel with a small magnetic spin bar was used. The oxygen content in the reactants was monitored using a Clark-type oxygen probe. The installation of this reactor is shown in Appendix A.

The reaction assay began by adding 1.6 ml of 0.05 M Tris-buffer (pH 7.4) and beads, at 37^{o}C, to the reactor and saturating with air. For pure oxygen saturation, the temperature was kept at 25^{o}C for higher yield. Further additions to the reaction medium consisted of 0.1 ml G-6-P/G6PDH solution containing no less than 20 umoles/ml G-6-P and 20 units/ml G6PDH followed by 0.1 ml of 20 umoles/ml NADP+ solution. Depending upon the substrate concentration, the cofactors were kept in excess to avoid cofactor limitations on the oxidation rate of the substrate. After 5 minutes, DPA emulsion (10 ul, at the desired concentration) was injected into the bioreactor.

Almost 100 beads were placed in the reactor. Since pure oxygen was used in this run, the concentration of cofactors added were increased by 5 fold over the ones used with the assays performed at air saturated conditions. The DPA emulsion added was increased by 2.5 fold. After the reaction was completed, the reactants were neutralized to pH 10; then, the oxidative products and unreacted DPA were extracted with EtoAc.

5.2 RESULTS AND DISCUSSION

Diphenylamine was found to be oxidized by hog liver microsomes to diphenylamine-nitroxide free-radical (DPNO) in a reaction requiring oxygen and NADPH. The radical formation was verified by EPR spectroscopy. Since no literature exists on the metabolic pathway of aromatic secondary amines, a preliminary investigation on the oxidative pathway of DPA is performed.

In this work, free as well as Ca-alginate immobilized microsomes, enriched in MFAO, were used. Due to its limited solubility in water, an emulsion of DPA in 3% Tween-80 surfactant was prepared. To minimize any possible deactivation of the microsomal enzymes, the minimum surfactant required to prepare a 30 mM DPA emulsion was used. A number of controlled experiments showed that at low concentration, Tween-80 does not inhibit the rate of reaction but rather facilitates in filtering inhibitors out of the microsomal preparations.

Based on reported tissue fractionation studies (34), detergents don't have inhibitory effects on enzymes, if used at low concentrations. In the case of microsomes, detergent solutions could cause solubilization of the lipoprotein barrier of the microsomal tissue. As a result,

low concentration detergent could cause the microsomal tissue to swell, allowing low molecular weight enzymes to leak out of the microsomes and increasing the substrate diffusion into the microsomes. In extracting oxidase from liver microsomes, M. Ziegler used low concentrations of Triton X-45 and Triton X-102 in order to avoid deactivation of the enzyme. Activity increase for mouse microsomes and whole liver homogenates in the presence of low concentration of detergents has been reported elsewhere (26).

In Figure 5.1, the extent of DPA diffusion into microsomal beads containing 50% v/v microsomes (curve A) and blank beads (curves B,C) is presented. Curve A suggests that the equilibrium concentration of the substrate into a blank bead is higher than in the case than in the case of a 50% v/v microsomal bead. In either case, euilibrium is essentially reached within 20-30 minutes for a variety of conditions. Figure 5.2 shows the HPLC spectra of DPA diffusion in microsomal beads. At the end of the reaction, unreacted DPA and oxidized DPA were extracted with EtoAc (Figure 5.2).

The rate of DPA biooxidation by immobilized microsomes as determined by HPLC is shown in Figure 5.3 and Figure 5.4. After allowing for diffusion equilibrium

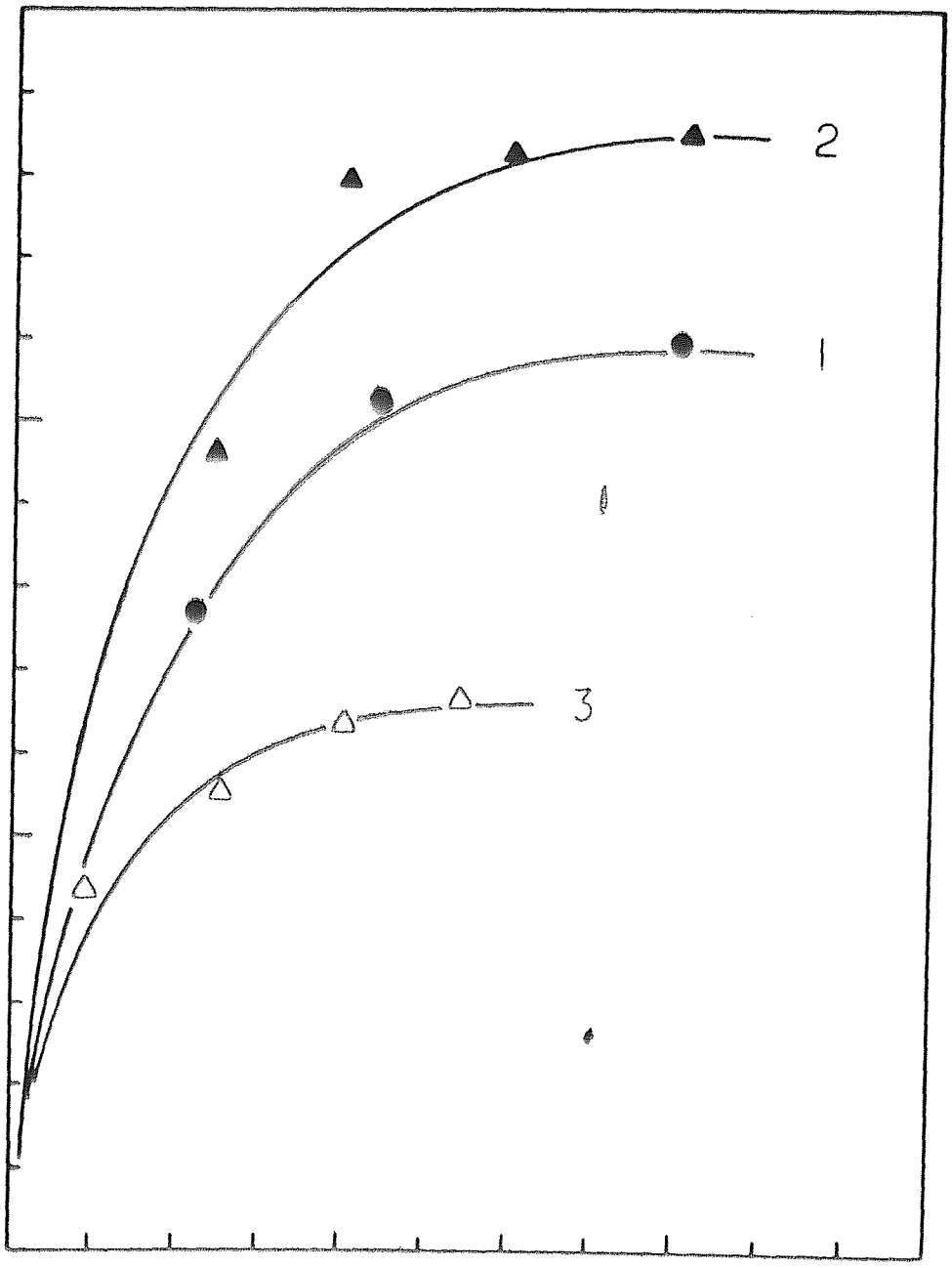


Figure 5.1 DPA diffusion into immobilized microsomes.
(1), 50 beads containing 50% v/v microsomes; 37°C;
DPA initial concentration: 235 nmoles/ml.
(2), 60 calcium alginate beads (blank); 37°C;
DPA initial concentration: 257 nmoles/ml.
(3), 60 calcium alginate beads (blank); 25°C;
DPA initial concentration: 129 nmoles/ml.
Reactant volume: 8 ml.

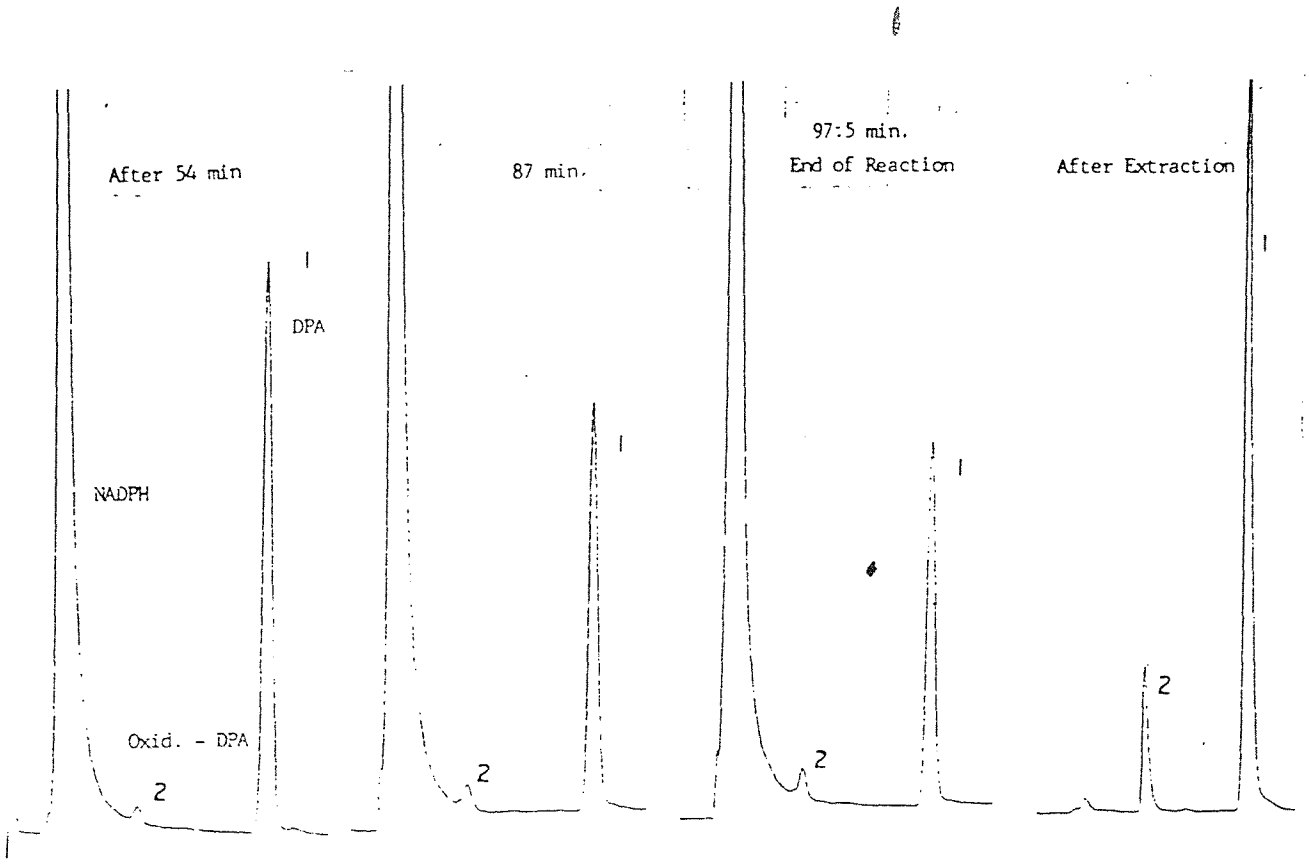


Figure 5.2 HPLC spectra of DPA diffusion into the immobilized microsomes. TMPO (2) can be seen more clearly after extraction from reaction medium).

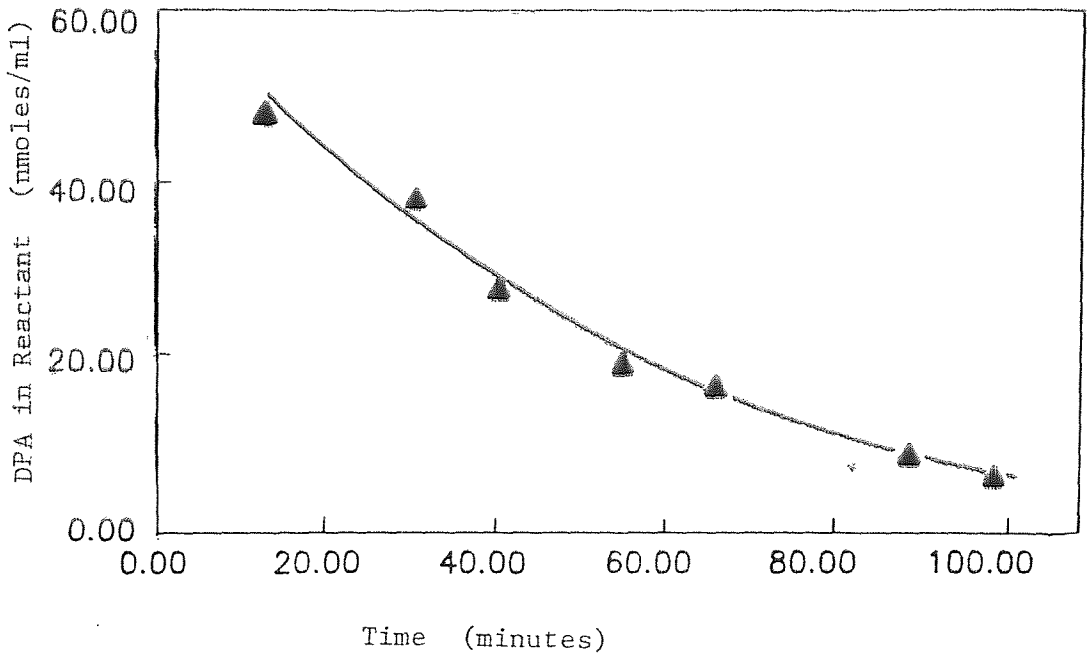


Figure 5.3 DPA biooxidation at 37°C.

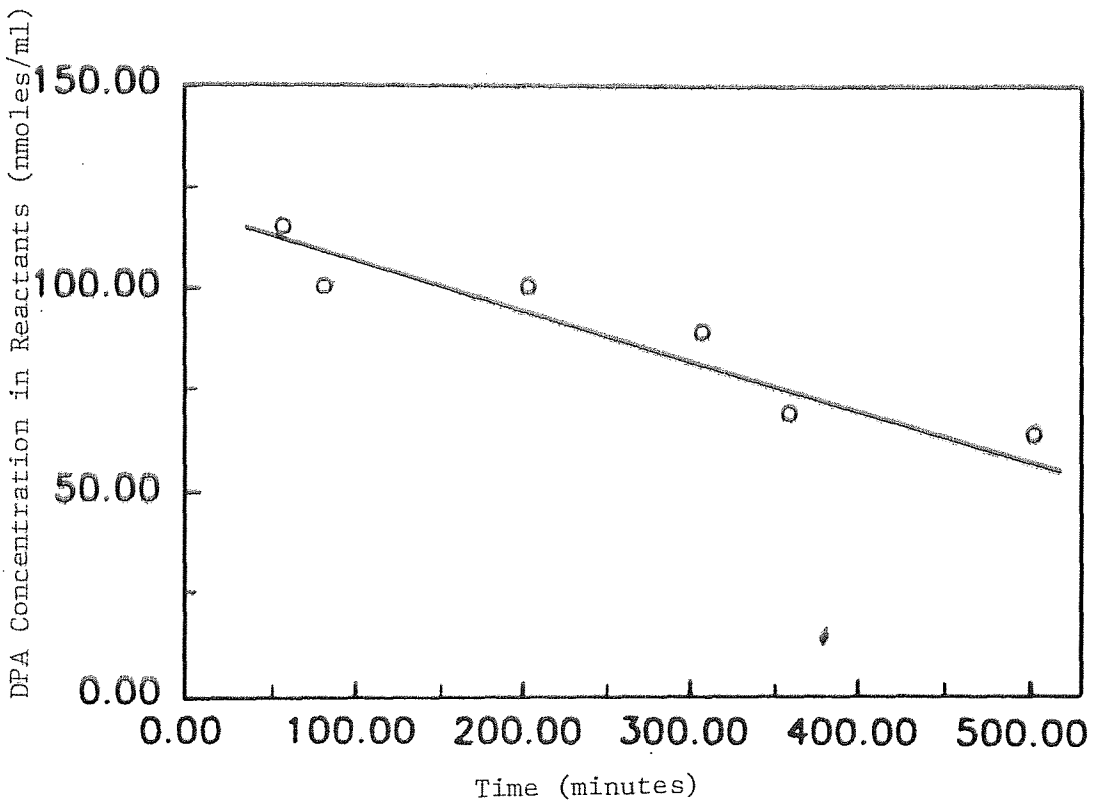


Figure 5.4 DPA biooxidation at 25°C.

to be established, the concentration of DPA in the reaction medium decreases according to the extent of reaction. The reaction in Figure 5.3 took place at 37^{o}C, 90% air saturation and with 60 beads in 8 ml solution. But in the other case, according to Figure 5.4, 50 beads in 11 ml solution at 25^{o}C,90% pure oxygen saturation, the relation of dissapearance of DPA in reactants versus the recation time it is shown to be essentaily a linear relationship for the regime of operation shown.

Table 5.1 Oxygen uptake rate for DPA oxidation rate (measured by oxygen probe) for equal amount of microsomes.

O2 saturation (%)	DPA conc. (mM)	O2-uptake rate (nmoles/min/mg protein)	Enzyme form
13-17	0.156	4.7	Immobilized (20 beads)
13-17	0.156	2.4	Free (0.2 ml)

Table 5.2 DPA biooxidation rates (following the starting material's disappearance by HPLC) by Ca-alginate immobilized microsomes.

O_2 saturation (%)	DPA intl. conc. (mM)	Temperature (^{o}C)	Oxidation rate (nmoles/min/mg protein)
90	0.096	25	0.182
19	0.240	37	0.79

Table 5.1 shows the reaction rate of oxygen uptake for DPA oxidation. For the case that the initial concentration of DPA in the reaction medium is 0.156 nmoles/ml, at 37^{o}C, and oxygen saturation ranges between 13 and 17%, the rate of oxygen uptake is 4.7 nmoles/min/mg protein. Under the same conditions, using the same amount of free microsomes instead of immobilized microsomes, the oxygen uptake is reduced to 2.4 nmoles O_2 /min/mg protein. this result agrees with earlier work on chlorpromazine oxidation (13).

In table 5.2, the biooxidation rate of DPA is shown. At 25^{o}C and at oxygen saturation of 90%, the oxidation rate of DPA equals 0.182 nmoles DPA/min/mg enzyme protein. However, in a lower oxygen saturation percentage of 19%, the rate is reduced to 0.079 nmoles/min/mg protein. As a result higher oxygen concentration in the reactant increases the oxidation rate of DPA.

Comparison of the oxygen uptake (decrease in oxygen concentrtaion) and DPA oxidation rate, measured by product formation on HPLC, shows the effective DPA oxidation rate to be much smaller. More than a single molecule of oxide of oxygen is consumed per molecule of oxide formed. This implies that for each mole of oxygen consumed, either a small fraction causes oxidation of the substrate in the

presence of enzymes or, the oxidation of substrate follows a slightly different pathway.

A recirculation bioreactor installation, as shown in Appendix A, was used for the biooxidation of DPA. One hundrend bead (equal to 3.2 mg protein) of immobilized liver microsmes were placed in the reactor, with 22 ml reaction solution. recirculated by the pump. The initial concentartion of DPA was 0.4 mM; a total of 9 nmoles DPA was added as substrate, at 37^{o}C. After 5 hours recirculation, the conversion of DPA was equal to 85% .

Biooxidation product (DPNO) was determined by HPLC. Figure 5.5 shows the HPLC spectra of DPNO bioproduct obtained during a batch run, respectively. The spectra show the peak which was identified as diphenylnitroxide (DPNO) by comparison to DPNO synthesized chemically as described in the experimental section. Biooxidation product was determined by electron spin resonance spectrometer as well. A diphenylnitroxide free radical was detected from the extractant obtained from the reaction mixture of DPA. The E.S.R. spectrum of DPNO, in Fugure 5.6, shows a characteristic three line nitroxide free radical structure.

Two principal oxidative enzymes (1) are in the liver microsomes; mixed function amine oxidase (MFAO) and

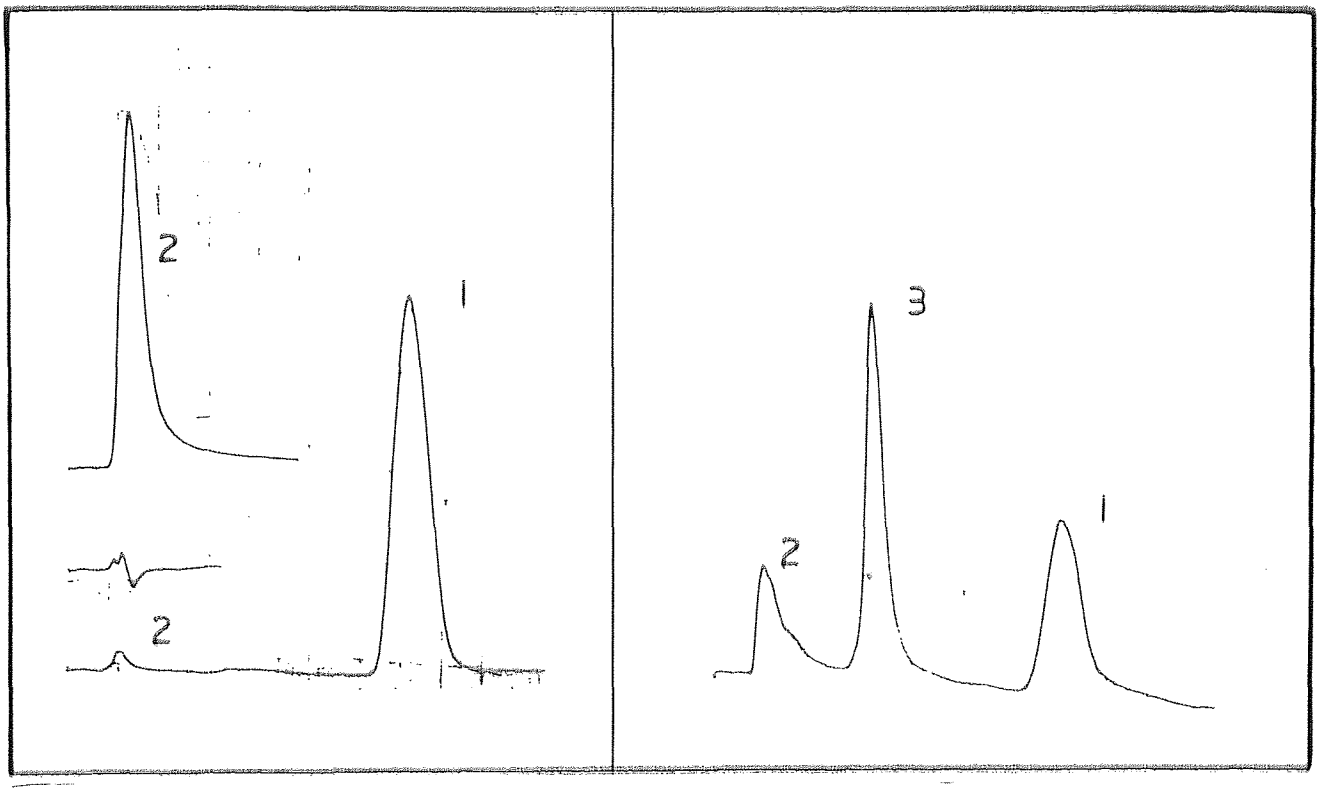


Figure 5.5 Identification of bioproduct DPNO by HPLC. (1), DPA; (2), cofactors; (3), bioproduct DPNO.

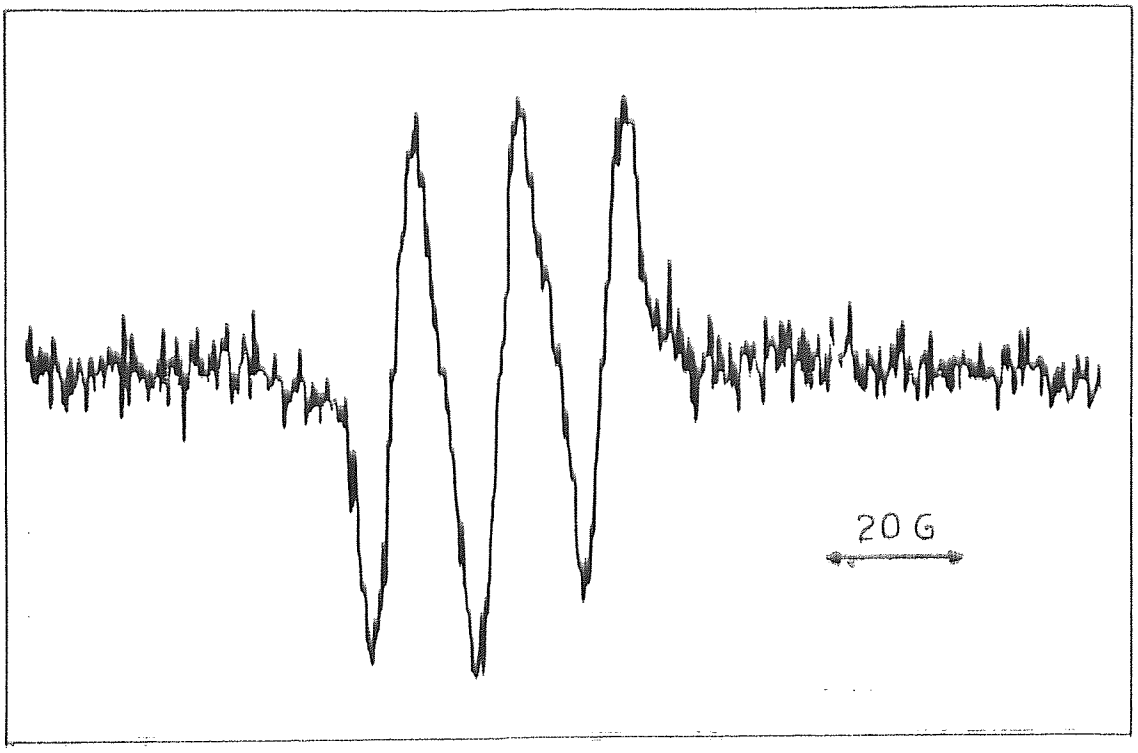
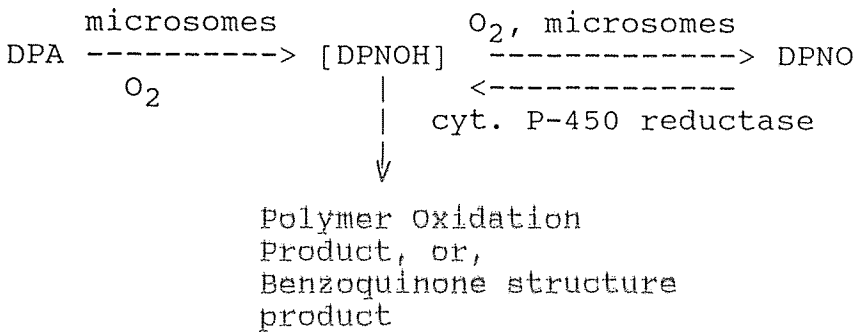


Figure 5.6 E.S.R. spectra of bioproduct DPNO.

cytochrome P-450. Previous work has suggested that immobilization leads to the purification of crude microsomal preparation from natural inhibitors (10). The nature of the inhibitors is believed to be lipids of low molecular weight. In this case, immobilization could lead to partial deactivation of cytochrome P-450 as an oxidative enzymic system. Lu and Levin (11) discuss the crucial role of the lipid requirements by the cytochrome P-450 during hydroxylation reactions. As a result of the above arguments, the MFAO could be responsible for the oxidation of DPA to form N,N-diphenyl-hydroxylamine (DPNOH); then DPNOH is oxidized further to form diphenylnitroxide (DPNO). In the presence of cytochrome P-450 reductase, DPNO will be reduced back to regenarate DPNOH. DPNOH is active in the presence of oxygen, forming the macromolecule's oxidative product. Although further research is required to explain the mechanism of DPA biooxidation, the following generalized scheme is proposed:


microsomes
O_2, microsomes
DPA
[DPNOH]
DPNO
O_2
cyt. P-450 reductase
Polymer Oxidation
Product, or,
Benzoquinone structure
product

CHAPTER VI

CONCLUSIONS AND SUGGESTIONS

6.1 CONCLUSIONS

Biosynthesis of amine oxides using calcium alginate immobilized hog-liver microsomes was found to be a successful process. Mixed function amine oxidase (MFAO) was found to be the primarily responsible microsomal enzyme for the biooxidation of, chlorpromazine (CPZ) to its N-oxide, and 4-hydroxy-2,2,6,6-tetramethylpiperidine and diphenylamine to their nitroxide free radical metabolite. All bioproducts were identified.

With respect to the performance of the immobilized microsomes as biocatalyst, the following conclusions were drawn:

1) Immobilized microsomes can consume 1650 nmoles of oxygen in a period of 3.7 hours. Based on this result it was calculated that 1 mg of microsomal protein is needed to convert 1.7 mg of CPZ (MW:355.5) to its N-oxide. The stoichiometry of the CPZ's biooxidation by immobilized microsomes was: 1 mole of atmospheric oxygen is consumed for every mole of CPZ that is converted.

For the biooxidation of TMP this ratio was 1:0.18, while for the biooxidation of DPA this ratio was approximately 1:25.

2) Decreasing the bead size by 60% and maintaining the same amount of enzyme, the rates obtained by the smaller beads were 5 times greater.

3) NADPH was found to be a limiting factor in the biooxidation of substrates by immobilized microsomes. The minimum requirement of $NADP^+$ for maximum CPZ biooxidation rate is 1.5 mM.

4) The maximal velocity, V_{max}, and the apparent half saturation constant K_m for the biooxidation of CPZ to its N-oxide were found to be 1.91 nmoles/min/mg protein and 0.37 mM, repectively. The corresponding values for the bioreduction of TMPO were calculated to be 1.05 umoles/min/mg protein and 0.0745 mM, respectively.

5) Refrigerated drying of immobilized microsomes was found to be a potential storage method for extended storage periods. Storage in 0.1 M $CaCl_2$ followed by several washings in 0.05 M Tris-buffer was found to be an adequate storage method for a 5-day use. In general, washing beads in buffer enhances their activity. The reason for this was the washout of contaminating enzymes.

6) The recirculation flow configuration can offer higher yields over the batch type configuration.

Oxygen uptake measurements are not feasible when immobilized microsomes are used as biocatalyst and pure oxygen sturation conditions are used. If we were to make this possible, it would be at the expense of biocatalyst and cofactors or, at the expence of enzyme purification.

7) The microassay reactor can be successfully used to study metabolic pathways and obtain valuable data for preliminary reactor design.

8) The use of n-octylamine, a known MFAO activator, was found to enhance the activity of immobilized microsomes up to 2-fold.

6.2 SUGGESTIONS

Further studies on refrigearted drying of beads as a potential storage method is recommended.

If the recirculation flow configuration is to be used for large scale production of amine oxides, immobilization of the coenzyme and cofactors should be attemted. This should be an invaluable help in lowering production cost.

The use of HPLC, connected on line with the recirculation reactor, would be a better approach in monitoring the biotransformation processes occuring in the reactor at all times and at all reacting conditions. For this the proper filtration precautions should be taken.

Lastly, the use of lipophilic coating to protect the alginate matrix should be tried. This could decrease of enzyme leaking in the reactor and make possible the use of solvent medium for the biooxidation of lipophilic compounds with lower solubity in water.

APPENDIX A

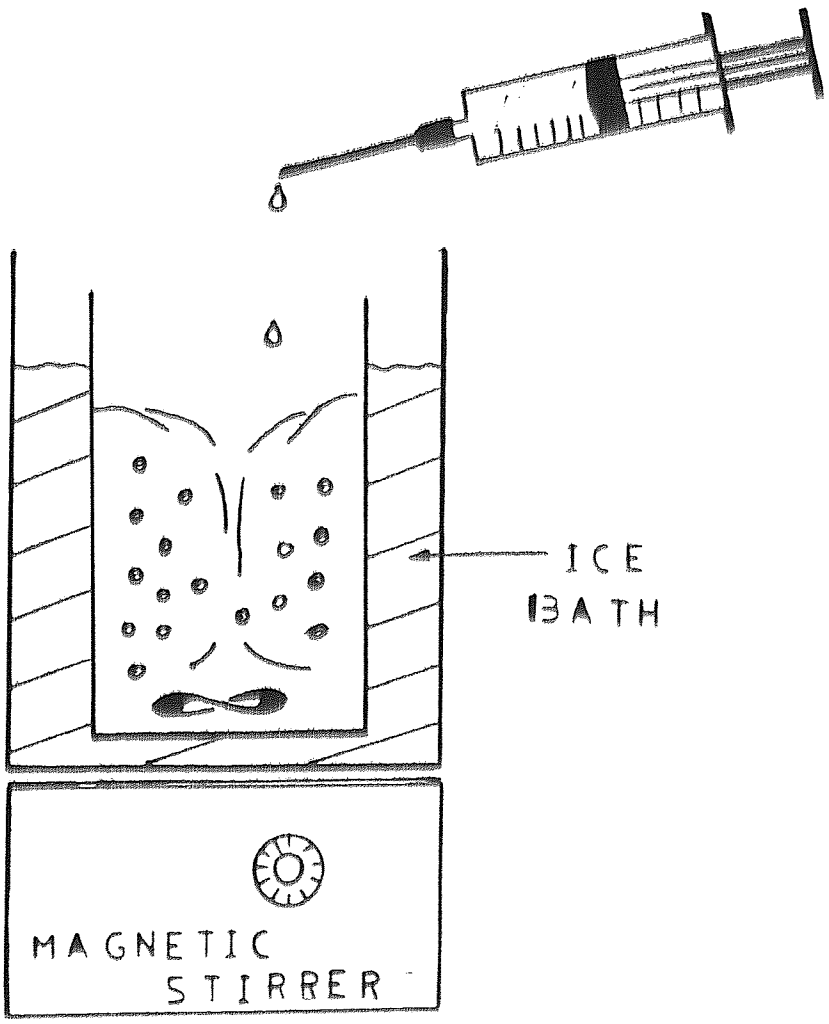


Figure A.1 Bead making process. An alginate-microsome mixture is extruded into a 0.1 M $CaCl_2$ solution.

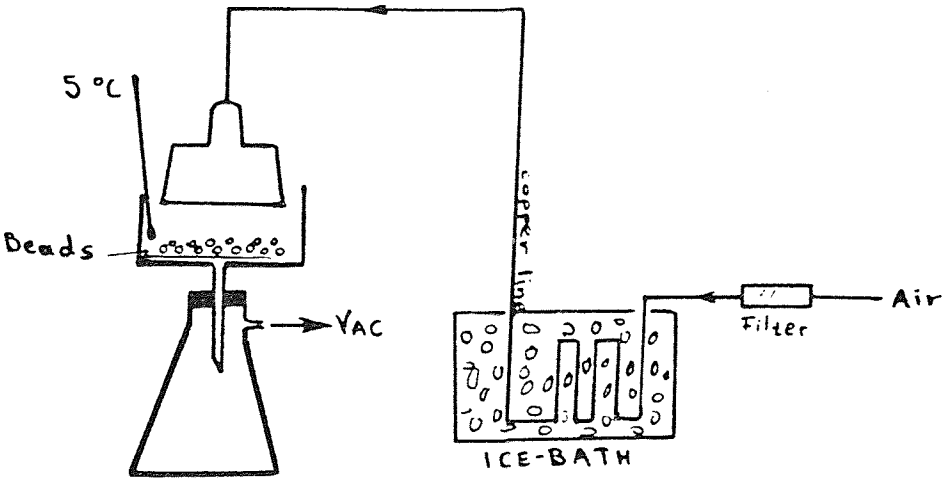
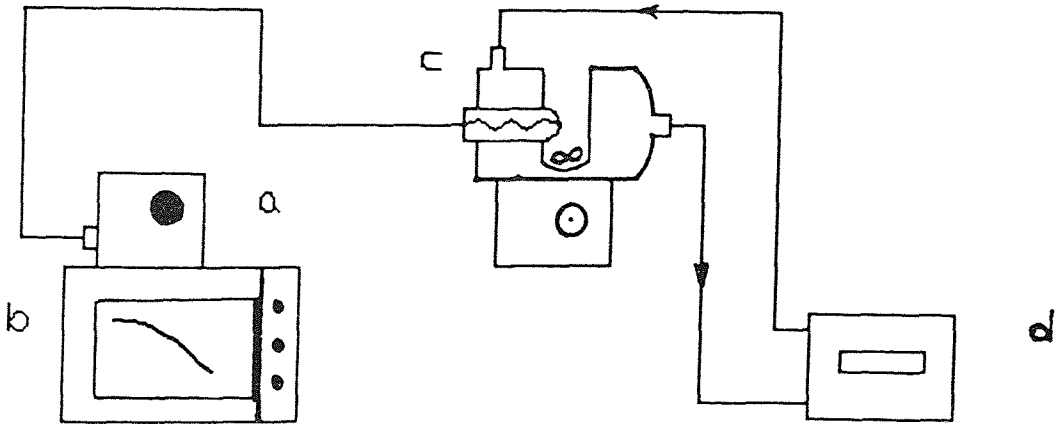
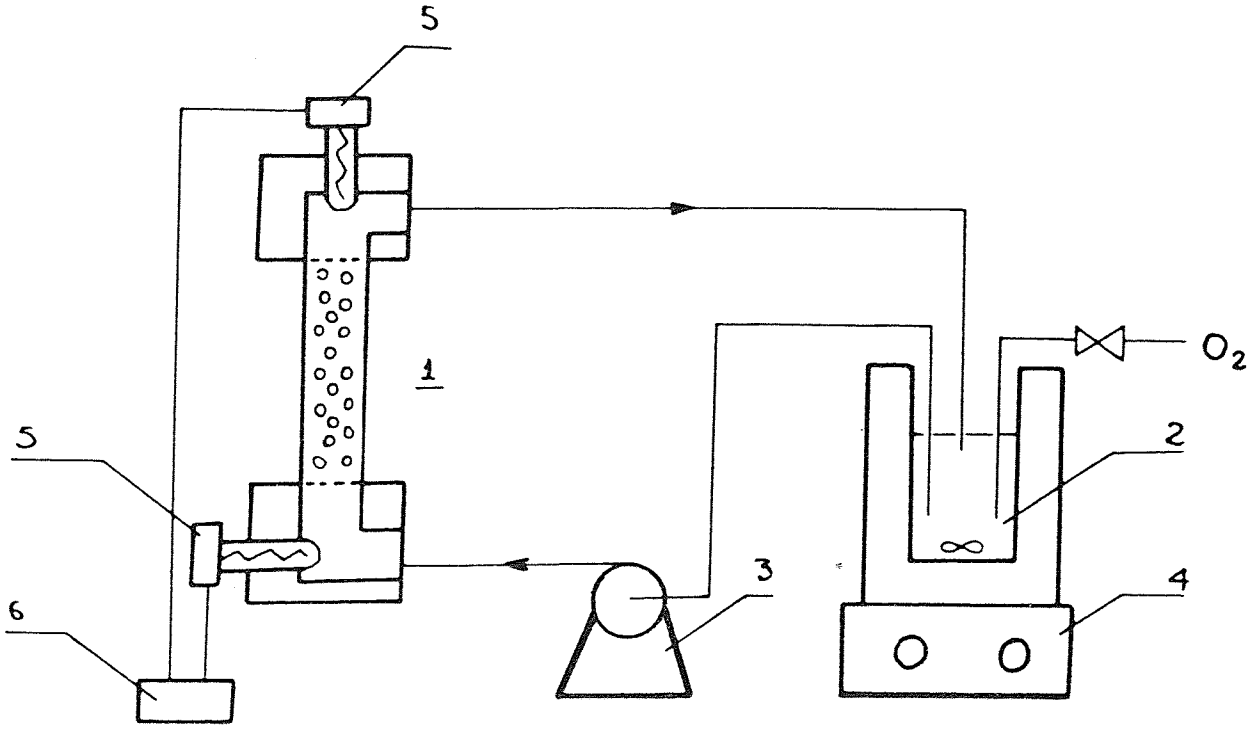


Figure A.2 Refrigerated drying of beads.



(a) Amplifier, (b) Chart recorder,
(b) Microassay reactor (1.9 ml), (d) Constant temperature bath.

Figure A.3 A microassay reactor consists of 1.9 ml water jacketed vessel with an 0.1 ml magnetic stirring bar. Dissolved oxygen is monitored by means of a Clar-type D.O. probe. All assays are conducted at pH 7.4 and 37°C.



(1) Fluidized bed reactor, (2) Reservoir,
(3) Recirculation pump, (4) Magnetic stirrer,
(5) Clark-type impigement D.O. probes,
(6) Amplifier box.

Figure A.4 Recirculation flow reactor system.

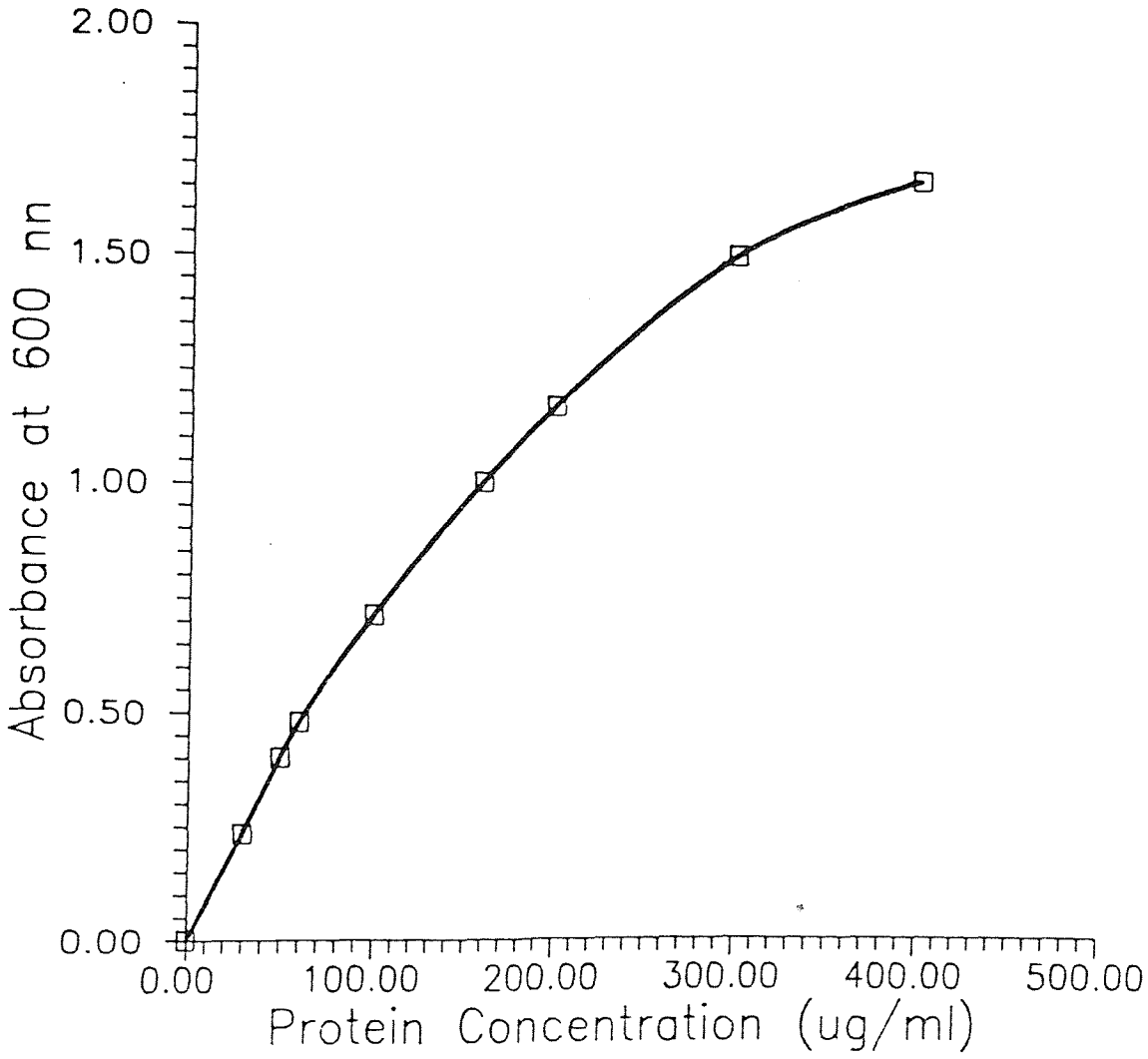


Figure A.5 Calibration curve for protein determination using the "Lowry method".

APPENDIX B

B.1 REACTION KINETICS CONSIDERATIONS FOR A RECIRCULATION FLOW REACTOR.

In a recirculation reactor system, the concentration change of substrate per pass can be amplified by increasing the recirculation rate (#passes/unit time). Thus, a measurable conversion can be obtained. This can be shown by the following material balance:

$$Q(\Delta S) = rW \qquad (1)$$

where:

- Q is the volumetric recirculation flow rate
- ΔS is the substrate concentration change across the differential reactor
- r is the rate of reaction in moles per unit time per weight of immobilized protein
- W is the weight of immobilized protein

The concept of recirculation can be utilized in two formats: first, as a batch recirculation system (BRS) and second, as a continuous flow recirculation reactor system (CFRRS).

For the first case, a material balance can be written:

$$V_f(-dS/dt) = rW$$

$$r = V_f(-dS/dt)/W \qquad (2)$$

where:

V_f is the fluid volume of the system

$-dS/dt$ is the decrease in substrate concentration per unit time

Combining equations (1) and (2), the fractional conversion of substrate per pass, S/S can be obtained:

$$\Delta S/S = V_f(-dS/dt)/QS \qquad (3)$$

where:

S is the substrate concentration in the system

For the second case, the material balance considers the inlet and outlet volumetric flow rates around the reactor, S_o, S:

$$q(S_o-S) = rW$$

$$r = q(S_o-S)/W \qquad (4)$$

where:

q is the inlet and outlet volumetric flow rate

S_o is the inlet substrate concentration

Combining equations (1) and (4), the per pass conversion can be obtained:

$$\Delta S/S = q(S_o-S)/QS \qquad (5)$$

In both cases low per pass conversion is required in order to obtain a gradient free operation. This can be accomplished by operating at sufficiently high flow rate, Q, such that the calculated per pass conversion becomes very small.

In general, there are two categories of gradients: first, concentration, flow, and temperature gradients within the reactor as a whole, and second, concentration and temperature gradients in the immediate vicinity of or within the biocatalyst.

With respect to the reactor as a whole, an axial concentration gradient, similar to a plug fow reactor, can be assumed under ideal conditions. Such ideal conditions would account for the causes of non-ideality, such as the axial dispersion due to mixing between the

biocatalyst particles, the liquid-solid contacting, thermal effects, and pressure drop in the reactor bed. In reducing the effect of the previously mentioned non-ideality sources, the volume of the reactor in the system should be decreases. Specifically at the points at which a 0%-2% conversion per pass exists, the reactor operates closer to an ideal differential reactor. In this case, it makes very little difference whether a small radial flow rate gradient or a high axial dispersion coefficient exist, since the biocatalyst reacts the same in the vicinity of inlet or outlet of the reactor.

With respect to concentration gradients in the vicinity or within the biocatalyst, temperature gradients are usually insignificant relative to diffusional problems; however by eliminating the diffusional problems, problems due to temperature gradients get eliminated. Concentration gradients external to the biocatalyst are have been shown by Lilly (35) to have a significant effects on experimental rates. To eliminate the effects Wilson and Geankoplis (36) have studied external transport in packed beds at low Reynolds numbers. They experimentally determined and correlated the mass transfer coefficient, k_c, for $0.0016<N_{Re}<55$. Using their results Ford et al. (37) gives an equation for the maximum rate

of external mass transfer per unit volume of immobilized enzyme, r_D:

$$r_D = \frac{6k_cS}{d_p} = \frac{6.54D^{2/3}Q^{1/3}S}{cd_p^{5/3}} \quad (6)$$

where:

- D is the diffusivity of water in substrate in water (in our case buffer medium)
- Q is the recirculation flow rate
- c is the external void fraction
- d_p is the biocatalyst diameter

From the above equation, it can be clearly seen that by decreasing the biocatalyst size the rate can be significantly increased.

At this point, it should be noted that for simple Michelis-Menten kinetics, the effect of external diffusion is greater in the first order region ($S<<K_m$). To test for external, one can vary the recirculation flow rate, Q, while operate at low substrate concentrations. Based on the above equation, the rate is proportional to the the recirculation flow rate raised in the 1/3 power. However,

if the rate of diffusion is faster than the rate of reaction the observed rate, r_D, will be independent of the recirculation flow rate.

The effects of internal diffusion are not as easy to to eliminate. Generally, the internal diffusion increases as the Thiele modulus increases (38).

To test for the presence of internal diffusion, simple experiments such as, particle size test, enzyme loading and substrate concentration test can be used.

If by varying the particle size no effect on the experimental rate is observed, internal diffusion effects are absent. If by varying the enzyme content of the biocatalyst change the experimental rate linearly proportionally, no internal diffusion effects exist. Lastly, in the absence of internal diffusion effects, for an immobilized enzyme system obeying Michelis-Menten kinetics, a maximum rate is obtained at concentrations larger than the K_m value (zero order region). On the other hand, the influence of internal diffusional effects will cause the rate change proportioanaly to the concentration by a second order form.

Lastly, combining the mass balance equation describing a plug flow configuration (equation (7)) and the kinetic constants (from Michelis-Menten model,

equation (8)), the design equation for a plug flow reactor (i.e. recirculation reactor) can be obtained:

$$\text{Mass Balance:} \quad dV/QS_o = dx/r \qquad (6)$$

$$\text{Michelis-Menten model:} \quad r = \frac{V_{max}S}{K_m + S} \qquad (7)$$

After combining equations (7) and (8), and after integrating, we can get the following design equation:

$$V_p = Q/V_{max} \left(S_o(x_o - x_i) + K_m \ln\frac{(1-x_i)}{(1-x_o)} \right) \qquad (8)$$

where:

V_p is the volume of the reactor

x is the conversion at the inlet, x_i, and at the outlet, x_o

Q is the flow rate

S is the concentration

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