RECIRCULATION FLOW BIOREACTOR FOR THE SYNTHESIS OF NITROXIDE AND N-OXIDE PHARMACEUTICALS

by
Douglas P. Lischick

A Thesis
Submitted to the Faculty of the Graduate Division of the
New Jersey Institute of Technology
in partial fulfillment of the requirements for the degree of
Master of Science in Chemical Engineering
1989

APPROVAL SHEET

Title of Thesis: Recirculation Flow Bioreactor for the Synthesis of Nitroxide and N-Oxide Pharmaceuticals

Name of Candidate: Douglas P. Lischick
Master of Science in Chemical Engineering, 1989

Thesis and Abstract Approved: ---------- 5-12-89

Sam Sofer, Professor
Sponsored Chair in
Biotechnology

Date

---------- May 12, 1989

Piero Armenante,
Assistant Professor
Dept. of Chem. Eng.,
Chem. and Envi. Sci.

Date

Signatures of other members of the thesis committee.

---------- May 12 1989

DeKang Shen,
Research Professor
Dept. of Chem. Eng.,
Chem. and Envi. Sci.

Date

VITA

Name: Douglas P. Lischick

Permanent Address:

Degree and Date to be conferred: M.S., 1989

Date of Birth:

Place of Birth:

Secondary education: Northern Valley Regional High School,
Old Tappan, N.J.

Collegiate institutions attended	Dates	Degree	Date of Degree
New Jersey Institute of Technology. Newark, N.J.	Sept. 1987 to May 1989	M.S.	May 1989
New Jersey Institute of Technology. Newark, N.J.	Sept. 1983 to May 1987	B.S.	May 1987

Major: Chemical Engineering

ABSTRACT

Title of Thesis: Recirculation Flow Bioreactor for the Synthesis of Nitroxide and N-Oxide Pharmaceuticals

Douglas Lischick: Master of Science in Chemical Engineering, 1989

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

An investigation of the properties of calcium alginate immobilized hog liver microsomes, rich in FAD-containing mixed function oxidase, was performed. Studies involving catalyst storage, immobilization of cofactors within the matrix, and alginate gel concentration were performed using a 1.8 ml mixed flow micro assay reactor. A recirculation flow reactor was designed and used for the biochemical production of 2,2,4,4-tetramethyl-1,3-oxazolidine-1-oxyl, a potentially useful MRI contrast agent.

The activity of the immobilized microsomes was found to increase over time when stored in $CaCl_2$ or in buffer at 4-8 ^0C. The increase in activity is a result of the removal of inherent inhibitors present in the microsomal preparation. The cofactors $NADP^+$ and G6P and coenzyme G6PDH can be immobilized within the alginate matrix, however

significant losses will result from diffusion of the cofactors during the catalyst preparation. The rate of diffusion through the matrix is a function of molecular weight, $NADP^+$ being the largest cofactor is retained the most. The alginate gel produces a uniform resistance to mass transfer between 0.5 and 1.5 % gel. The rate of oxygen consumption is found to be a linear function of the enzyme concentration. The immobilized enzyme has an effective V_{max} of 10.4 nmole/min * mg protein.

A recirculation flow reactor was designed for the use of immobilized microsomes. The nitroxide 2,2,4,4-tetramethyl-1,3-oxazolidine-1-oxyl was produced enzymatically using 2,2,4,4-tetramethyl-1,3-oxazolidine as substrate. The product was compared to the same compound synthesized organically, using UV/VIS and HPLC. The formation of the nitroxide free radicals is verified by ESR spectroscopy.

ACKNOWLEDGEMENTS

I wish to express my sincere gratitude and appreciation to my supervising advisers, professor Sam S. Sofer and Dr. Dekang Shen. Their guidance and moral support has been an invaluable aid to my research.

I am grateful to professor Piero Armenante for serving as a member on my Masters Thesis Committee.

I am also grateful to Dr. Ziegler from Univ. Texas at Austin for gift of the microsomes, to Dr. Potenza from Rutgers Univ. for the help in the ESR analysys and to the State of New Jersey for the funding of this project through the Sponsored Chair in Biotechnology.

I wish to thank all the member of the Biotechnology Laboratory for their help and suggestions in conducting this investigation. Special thanks going to Ioannis Valvis for his advice and help in conducting the experiments.

I would also like to thank my parent, family and Cynthia for all the support and encouragement which made this thesis possible.

TABLE OF CONTENTS

Chapter		Page
I.	INTRODUCTION	1
	1.1 Application	3
	1.2 Kinetic Mechanism	6
	1.3 Endogenous Rate	10
	1.4 Enzyme Activity	12
	1.5 Cytocrome P-450 Monooxygenase	12
II.	OBJECTIVE	15
III.	MATERIALS AND EXPERIMENTAL METHODS	16
	3.1 Immobilized Enzyme Catalyst Preparation	16
	3.2 Protein Determination	17
	3.3 Micro Assay Technique	18
	3.4 Verification of the Major Oxidative Product	20
	3.5 Bead Storage	21
	3.6 Immobilization of Cofactors	21
	3.7 Organic Synthesis	23
	3.7 (i) Synthesis of 2,2,4,4-Tetramethyl-1,3-Oxazolidine	23
	3.7 (ii) Synthesis of 2,2,4,4-Tetramethyl-1,3-Oxazolidine Nitroxide	26

3.8 Bioproduct Analysis..... 30

3.9 Instrumentation..... 30

VI. RESULTS AND DISCUSSION..... 31

4.1 Increase in Rate During Storage Time..... 31

4.2 Cofactor Immobilization..... 38

4.3 Enzyme Loading..... 43

4.4 $NADP^+$ Saturation..... 47

4.5 Methimazole and n-Octylamine Saturation..... 47

V. RECIRCULATION REACTOR..... 52

VI. CONCLUSIONS AND RECOMMENDATIONS..... 64

BIBLIOGRAPHY..... 66

LIST OF FIGURES

Figure		Page
1.1	Catalytic Cycle of FMO	8
3.1	Micro Assay Reactor Setup	19
3.2	NMR Spectrum of 2,2,4,4-Tetramethyl-1,3-Oxazolidine	25
4.1	Effect of Storage Time in $CaCl_2$ on Activity	33
4.2	Effect of Storage Time in TRIS Buffer on Activity.	34
4.3	UV/VIS Spectrum of the Storage Medium	36
4.4	Immobilization of $NADP^+$	40
4.5	Immobilization of G6P and G6PDH	41
4.6	Immobilization of G6P, G6PDH and $NADP^+$	42
4.7	Effect of Alginate Gel on Activity	45
4.8	Effect of Enzyme Loading on Activity	46
4.9	$NADP^+$ Saturation Requirement	49
4.10	Methimazole Saturation Requirement	50
4.11	n-Octylamine Saturation Requirement	51
5.1	Recirculation Flow Reactor Scheme	53
5.2	CPZ N-Oxidation in the Recirculation Flow Reactor	57
5.3	Rate versus TOX Concentration	58

5.4 Biochemical N-Oxidation of TOX Followed by HPLC..... 59
5.5 UV/VIS Comparison of Bioproduct and Organic Product..... 61
5.6 HPLC Comparison of Bioproduct and Organic Product. 62
5.7 ESR Comparison of Bioproduct and Organic Product.. 63

LIST OF TABLES

Table		Page
1.	Column Chromatography of Organic Synthesis.....	28
2.	ESR Spectroscopy of Nitroxide Products.....	29
3.	Weight Loss of Beads During Storage.....	35
4.	Protein Balance During Storage.....	37

CHAPTER I

INTRODUCTION

The liver is sometimes referred to as the detoxification center of the body. It is within the liver that the metabolism of many xenobiotics and pharmaceutical drugs begins [1]. For many of these compounds, the first step of metabolism is an oxidation process. Once a compound is oxidized, a hydrolysis step can easily occur resulting in a more water soluble form of the compound. The water solubility of the metabolite facilitates the removal of the compound by excretion through the urinary tract. However, even without an additional hydrolysis step the oxidized compounds are inherently more hydrophilic due to an increase in their dipole moment and polarity.

Aside from hydrogen and carbon, nitrogen is the most common element found in xenobiotics and pharmaceuticals [2,3]. The metabolism of many of these nitrogen bearing compounds begins with the oxidation of the nitrogen atom. The site for N-oxidation lies within the smooth endoplasmic reticulum of the liver cells.

The endoplasmic reticulum of hepatic cells are made up of a network of ribosome-like and smooth membrane, existing in the form of channels. Viewed under an electron

microscope they appear as parallel arrays of "rough" membrane and scattered "smooth" vesicles respectively. After removing and homogenizing the liver tissue, the membranes are separable from the bulk tissue through differential centrifugation. These membranes separated from the cellular organelles and soluble components are referred to as "microsomes". The rough and smooth membranes can be further separated by ultracentrifugation techniques. Once separated ultrasonication of the microsomes also enables these elements to be subfractioned even further.

In a procedure developed by Ziegler and Poulsen, FAD-containing mixed function oxidase (FMO) has been isolated and purified to homogeneity from pig liver microsomes [4]. FMO has been identified as being the responsible enzyme for the oxidation of a variety of tertiary, secondary and aryl amines, some primary amines and a variety of phenothiazine drugs, in addition to a variety of sulfur containing compounds [3]. Although present in other tissues such as the lung and kidney of many mammalian species, the FMO activity is much greater in the liver and in unusually high concentrations in hog and human liver [5]. The monooxygenase is one of the more abundant flavoprotiens of the liver, accounting for 3-4% of the total liver microsomal protein from an adult female hog [5].

Although the use of isolated enzymes displays faster

reaction rates, isolation and purification of the enzyme is costly and can lead to deactivation when the enzyme is removed from its natural environment. The use of microsomes is a much more feasible approach. Through immobilization, the flexibility and potential applications of the microsomes are greatly enhanced. The immobilization of enzymes on or within a support serves not only the purpose of recovery from reaction mixtures, but it can also slow enzyme deactivation by inhibiting protease attack, by reducing shear in a stirred reactor and by minimizing interfacial temperature or solvent denaturation [6].

One of the greatest advantages of the immobilization of FMO is the increase in half-life of the enzyme. Although this phenomenon is not fully understood it has been reported by several investigators [7,8]. The half-life of purified hog liver FMO immobilized on glass beads is extended to several hours while the half-life of the soluble enzyme is in the order of 15 minutes [9]. With an extended half-life, the immobilized enzymes are expected to have higher product yields. This result has been demonstrated by Camp in his work using calcium alginate immobilized hog liver microsomes [7].

1.1 APPLICATION

The potential applications of hepatic microsomal

enzymes are presented in an earlier review [9]. One of the most promising uses of immobilized enzymes is in their role as metabolic agents in the transformation of foreign compounds into useful bioproducts. Enzymes, known for their specificity in catalyzing unique reactions, can serve as an indispensable aid to the organic or pharmaceutical chemist. With the use of enzymes, new compounds, or compounds which are difficult or thought impossible to synthesize chemically can be produced biochemically.

The production of metabolites, such as N-oxides or glucuronides, as new drugs is one illustration of enzyme utilization. The metabolites of various phenothiazine drugs have been produced using microsomal enzymes [9]. These metabolites can also prove useful as drugs themselves. In fact, the metabolite can have more favorable properties than the parent compound. For example, a metabolite of imipramime, imipramine N-oxide, has been reported to have similar therapeutic effects to the parent compound, however the side effects, decreased blood pressure and toxicity, are reduced [10]. Although N-oxides of many pharmaceuticals can be synthesized via standard practices, some N-oxides are very difficult to prepare. One such compound is the N-oxide of the tranquilizer prochlorperazine [9]. In this case, the enzyme system is ideal.

The microsomal FMO found in hog liver is also known to

catalyze the production of stable nitroxide free radical compounds [11]. Nitroxides are paramagnetic organic molecules containing one unpaired electron in the N-O group and are known to stimulate the proton relaxation of the water environment in which they are dissolved [12]. The ability of nitroxides to alter the relaxation state of water makes them useful as Magnetic Resonance (MR) contrast agents. The utility of stable nitroxide free radicals as MR imaging enhancers to strengthen the image intensity of tumors, soft tissue inflammation, and defects in the blood brain barrier has been demonstrated [13]. They are also currently being used as probes to monitor biological membranes and describe the contour of macromolecular surfaces [14]. Nitroxides have several advantages over other image enhancement techniques such as altering the hydration state of tissue and other inorganic paramagnetic ions. Nitroxides demonstrate many characteristics of clinically useful NMR contrast enhancers including: 1) stability at varying pH and temperature, 2) a prolonged shelf life, 3) chemical flexibility for enhancement of non specific and of specific systems or processes, and 4) longer spin relaxation times and lower toxicity than metallic ions [14,15]. Another advantage is that nitroxides are readily metabolized and excreted by the kidney [15].

The nitroxide compounds are mainly prepared by

classical organic synthesis techniques. The procedures involved are often difficult to carry out without producing unwanted by-products especially when several oxidation sites appear on the molecule. This can lead to laborious separation methods and poor product yield. Once again an enzymatically catalyzed reaction would prove to be beneficial.

Another interesting phenomenon of FMO is its product stereospecificity. The N-oxidation of nicotine by porcine liver FMO shows stereoselectivity in the formation of its diastereomeric N-oxide [16]. Reactions involving the (R)-(+)-Nicotine result in the formation of only trans 1'R,2'R-N-oxide. The result may however be due to steric factors of the starting material which can effect the attack of the enzyme [16]. Nonetheless, stereoselectivity is a valuable asset in chemical synthesis.

1.2 Kinetic Mechanism

The steady state kinetic mechanism which FMO is believed to follow is an ordered ABC mechanism with an irreversible step between the AB and ABC enzyme bound complexes [3]. The order of the substrate addition is NADPH (Reduced Nicotinamide Adenine Dinucleotide Phosphate), O_2, and substrate, and the order of product release is the oxygenated substrate along with water and finally $NADP^+$.

The steps involved in the FMO catalyzed oxidation of nucleophilic sulfur or nitrogen containing compounds are illustrated in Figure I. G6P (D-Glucose-6-Phosphate) and G6PDH (Glucose-6-Phosphate Dehydrogenase) are used to regenerate $NADP^+$ back to the usable reduced form as NADPH.

Ziegler and Poulsen [3] have found that NADPH is capable of reacting with the oxidized form of the enzyme, however the binding of oxygen or the oxygenatable substrate to this same form is not detected. Therefore, it was concluded that NADPH must be the first substrate added. They have also shown, as well as other investigators [17], that $NADP^+$ is competitive with NADPH, both binding to the same form of the enzyme, and noncompetitive with oxygen and nitrogen bearing substrate. Thus, the first substrate added is NADPH and the last product to be released is $NADP^+$.

The work of Beaty and Ballou [17] has further supported this mechanism. They have spectrographically monitored the appearance and disappearance of a C(4a)-hydroperoxide intermediate in a stopped flow apparatus. Analysis of the kinetic mechanism has shown that there is a reversible connection between the enzyme forms that bind NADPH and oxygen. In addition they have been able to verify the order of addition of substrates. According to their experiments, if the substrate were to bind after NADPH and before O_2, the kinetic formation of the C(4a)-hydroperoxide

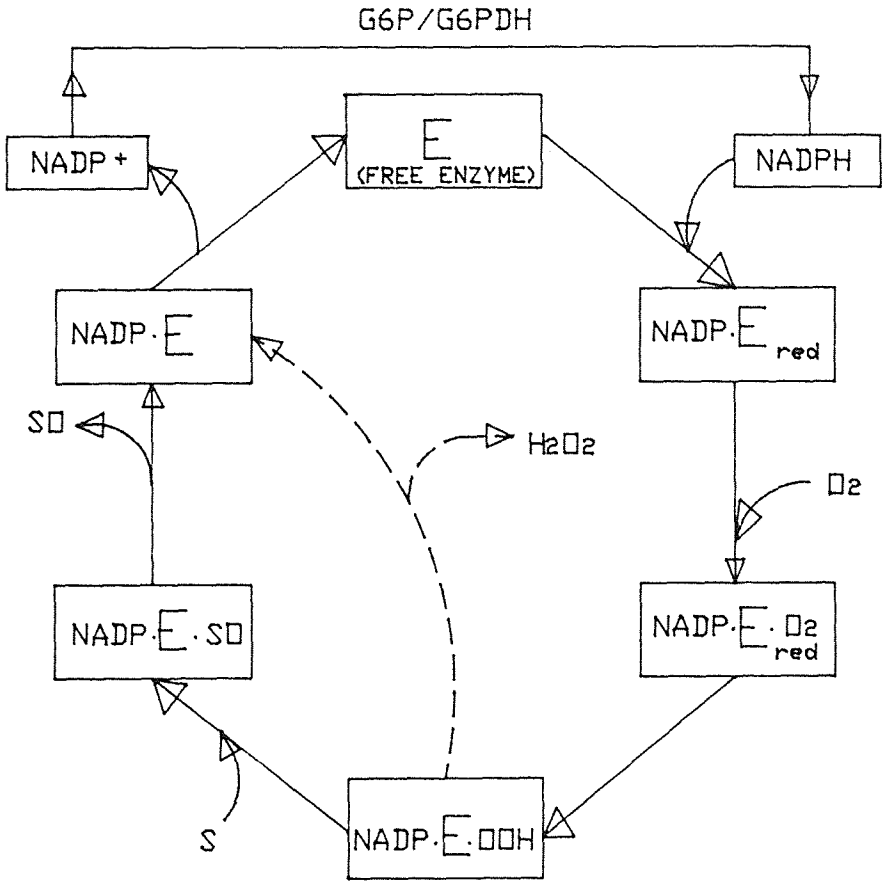


Figure 1.1 Catalytic cycle of FMO. S Stands for a nitrogen or sulfur containing substrate, and SO, the oxygenated substrate. The heavy line indicates an irreversible step in the formation of the C(4a)-hydroperoxide intermediate. The dashed line shows NADPH oxidation in the absence of substrate. $NADP^+$ is regenerated by G6P and G6PDH.

intermediate should be affected by the substrate additions, yet it was not. Furthermore, if the substrate were to bind prior to NADPH, the reduction of the enzyme would be expected to be affected and it was not.

An important step in the mechanism is the formation of the C(4a)-hydroperoxide flavin intermediate which is a unique property of this enzyme [18]. It is this form of the enzyme that is believed to be responsible for the oxygenation of substrates [18]. The hydroperoxy flavin binds to the substrate and transfers the oxygen to the substrate. Within the reaction vessel, the enzyme mainly takes the form of the intermediate [17]. The oxidation of amines by this mechanism is an ionic two-electron oxidation which will yield N-oxygenated products [19]. The oxygen transfer from the peroxyflavin intermediate is similar to the oxidation of amines by peroxides. Thus, many compounds that can be oxidized by organic compounds such as organic hydrogen peroxide or organic peracids are potential substrates for the enzyme. However, the ability of the enzyme to oxidize a compound may be greatly affected by the geometry and steric factors of the compound [18].

The rate limiting step of the FMO reaction mechanism is presumed to be either the breakdown of the pseudobase C(4a)-hydroxyflavin or the release of $NADP^+$ [17]. Since both of these steps occur after the addition of substrates

and the release of the products, one expects that all substrates would have the same value of V_{max}. A review of the literature shows that this is obviously not the case [20]. The V_{max} for lipophilic protonated tertiary amines is twice that of neutral substrates, however, the V_{max} of the latter can be increased to the former with the addition of a positive activator such as n-Octylamine (NOA). While this primary amine is not a substrate, it increases the rate limiting step to result in a constant value of V_{max} for virtually all substrates using purified hog liver microsomes [18]. This suggests that the V_{max} found for one substrate in conjunction with NOA can predict this kinetic constant for all substrates.

1.3 Endogenous Rate

In the absence of substrate, the FMO oxidase is capable of oxidizing NADPH to form $NADP^+$ and H_2O_2 in stoichiometric amounts [3].

$$NADPH + O_2 + H^+ \rightarrow NADP^+ + H_2O_2$$

This reaction is referred to as the "endogenous" reaction. However, the hydroperoxide intermediate reacts much faster with substrate (N,N-dimethylamine and methimazole) than it decomposes to give hydrogen peroxide [3]. The endogenous rate is barely detectable in crude preparations and progressively increases during isolation and purification.

This increase in the endogenous oxygen dependent uptake is an indication of the stability of the C(4a)-hydroperoxyflavin intermediate [20]. As the enzyme is purified it be comes more and more unstable and the endogenous rate approaches that of the substrate stimulated rate.

Cavagnaro et al. [2] have performed a detailed investigation on the endogenous rate. The oxygen uptake of the enzyme was monitored with and without substrate (N,N-dimethylamine and Methimazole) at various concentrations of rat hepatic microsomes. Various plots of the rate of oxygen uptake of the endogenous reaction versus enzyme concentration were examined and found to be nonlinear. However, the total of the endogenous plus the substrate stimulated rates was linear. The endogenous rate is a characteristic property of this enzyme and disappears either completely or partially in the presence of an exogenous substrate [2]. They were also able to validate this conclusion mathematically and show that there is no justification in the subtraction of the endogenous rate from the substrate stimulated rate.

The endogenous reaction rate of pig liver FMO does display a significant rate. However, in light of the above mentioned study and a similar study with pig liver FMO by Sabourin et al. [20], it can be concluded that at or near

enzyme saturation conditions of substrate the NADPH oxidation is due entirely to the oxidation of substrate.

1.4 Enzyme Activity

There are many factors about the animal from which the microsomes are obtained that have both positive and negative affects on the activity of FMO in the liver tissue. Individual variations of up to 2-fold in specific activity are not uncommon among adult hogs [5]. The major factors are: sex, physical size and health, breed and time of last feeding before slaughter [4,5].

Other factors affecting the activity arise during the microsomal and catalyst preparations. FMO is highly susceptible to postmortem inactivation. The liver should be removed and cooled to 4^{o}C within 10 minutes to prevent significant losses in activity [5]. FMO is also known to be very temperature and pH sensitive and should be handled under carefully controlled conditions.

1.5 Cytocrome P-450 Monooxygenase

The cytocrome P-450 dependent monooxygenase system, as well as FMO, must be taken into consideration when discussing the metabolic oxidation of a broad range of chemicals, drugs and pesticides. Earlier, the characterization of FMO the NADPH-dependent oxidation of

compounds was attributed to the cytocrome P-450 system [20]. It is now apparent that many nitrogen and sulfur containing compounds are oxidized by FMO. The cytocrome P-450 dependent monooxygenase system and its genetic control have extensively been studied and characterized in mice [20]. The metabolic pathway of the compounds may depend upon the relative amounts and the relative affinity for substrate of the two enzyme systems. However, the oxidation pathway by FMO and by cytocrome P-450 are different and may lead to different end products [19]. Oxidative N-dealkylation is catalyzed almost exclusively by cytocrome P-450 while N-oxidation is carried out by both systems [21]. However, N-oxidations catalyzed by cytocrome P-450 generate intermediate radicals and is restricted to a few substrates while N-oxidation by FMO follows an ionic mechanism without the formation of intermediate radicals [19].

Although purified FMO is very well understood, immobilization of hepatic microsomes raises new questions. With the immobilization of hog liver microsomes within a calcium alginate matrix an increase in the half life is observed [7], also an increase in the activity is seen over time [22]. The reasons for the changes in stability and activity are thought to be the result of a loss of inherent endogenous inhibitors or a physical realignment of the

enzyme during the immobilization process. However this phenomena still remains uncertain.

A second question which arises is the possibility of immobilizing the cofactors, G6P, G6PDH and $NADP^+$, within the matrix. Having the cofactors entrapped within the matrix may present several advantages over having the cofactors introduced in the reaction medium. Since the enzyme is held within the bead, the reaction takes place within the bead. If the cofactors are immobilized within the matrix they will not have to overcome the internal as well as external mass transfer resistance, thus reducing the requirement of a sufficient concentration gradient to force the cofactors inside the beads. This will also ease the separation of the final product from the cofactors.

These are the main areas of interest, as well as the investigation of operational parameters which are important in the development of a practical reactor design for the production of small quantities of pharmaceutical compounds using immobilized microsomes.

CHAPTER II

OBJECTIVE

The primary objective of this study has been to develop a recirculation flow bioreactor for the enzymatic synthesis of pharmaceuticals using immobilized hepatic microsomes.

The first step has been the investigation of the immobilized enzyme catalyst. The change in activity over time, when stored in $CaCl_2$ or in buffer, has been examined. A spectral analysis, a protein balance, and an analysis of the weight loss of the catalyst have been performed over time to detect losses of inhibitors from the immobilized matrix. The benefits of immobilizing cofactors within the matrix have been evaluated. Other operational parameters including enzyme loading and cofactor and substrate requirements have also been defined.

A recirculation flow reactor has been designed and used for the biochemical synthesis of 2,2,4,4-tetramethyl-1,3-oxazolidine-3-oxyl by the immobilized enzyme. The bioproduct has been compared to the same product synthesized chemically.

CHAPTER III

MATERIALS AND EXPERIMENTAL METHODS

Alginic acid, Tris buffer, cofactors $NADP^+$ and G6P, coenzyme G6PD and protein assay kit are obtained from Sigma Chemical Company, St. Louis, Missouri. 2-Amino-2-methyl-1-propanol, para-toluensulfonic acid, sodium sulfate anhydrous, sodium bicarbonate and 3-chloroperbenzoic acid were obtained from Fluka Chemical Corp. Ronkonkoma, New York. Chlorpromazine was obtained from Smith, Kline and French Laboratories. The hog liver microsomal preparation was a gift from Dr. D.M. Ziegler of the University of Texas. Acetone, benzene, ethylene dichloride and methylene chloride are of a suitable reagent grade.

3.1 Immobilized Enzyme Catalyst Preparation

Sodium alginate gel is made by adding 1.87 grams of NaCl and 100 ml of 0.01M Tris buffer, pH 7.4 at 37^{o}C, in a 250 ml beaker. While stirring the solution over a low heat slowly add 2.00 grams of alginic acid. Once the alginic acid is added, the mixture becomes thick and a glass stirring rod is used to help the mixing process. This mixture is autoclaved and stored at 4-8^{o}C.

The immobilized enzyme catalyst is prepared by mixing

equal volumes of the chilled sodium aglinate gel and the cold, prepared liver microsome mixture in a 20 ml vial. The mixture is stirred on ice with a glass rod to a homogeneous state. The mixture is then extruded through a 21 gauge syringe needle using a syringe pump set at a flow rate of 0.4 ml per min. The extruded material falls drop wise into an ice cold and gently stirred 0.1M $CaCl_2$ solution. When the drop hits the $CaCl_2$ an ionic replacement takes place between the sodium in the gel and the calcium in the solution to form a stable calcium-alginate matrix which entraps the enzyme. The mixture will produce on average 50 drops or "beads" per ml.

The beads are left to cure in the $CaCl_2$ solution for 10 hours at a temperature of 4-8^{o}C. After the 10 hours of curing time, the $CaCl_2$ is drained off and the beads are washed and stored in 0.01M Tris-buffer, pH 7.4 at 37^{o}C. The beads remain in the buffer 4 hours before using.

3.2 Protein Determination

The amount of protein contained in solution and in beads was determine using a protein assay kit based on Peterson's modification of the micro-Lowry method which uses sodium dodecylsulfate to facilitate the dissolution of relatively insoluble lipoproteins.

One bead is placed in a test tube and crushed with a

glass rod and diluted to 1 ml, the sample is then measured for protein content per bead. Similarly 1 ml of a solution is measured. A minimum of three trials are done and the results averaged.

3.3 Micro Assay Technique

The reaction vessel consists of a stirred 1.8 ml jacketed reactor, maintained at a temperature of 37^{o}C by a constant temperature bath. The activity of the enzyme is determined by monitoring substrate dependent oxygen consumption. The oxygen consumption is measured polarographicaly using a YSI 4004 Clark oxygen probe inserted in the vessel. The signal from the probe is sent to a strip chart recorder through a signal conditioner. The reactor setup is shown in Figure 3.1.

Prior to all assays, the vessel is rinsed several times with methanol, distilled water and buffer. The vessel is then filled with 1.65 ml of buffer and saturated with air (228 nmole/ml). Once saturation is complete the catalyst is added in the form of beads or as free organelles. The cofactors are added in two injections of 25 ul each of stock solutions of $NADP^{+}$ and G6P/G6PDH such that the vessel contains 3.0 mM $NADP^{+}$, 3.0 mM G6P and not less than 2 units/ml G6PDH. Within 30 seconds the endogenous oxygen consumption of NADPH will appear. After approximately

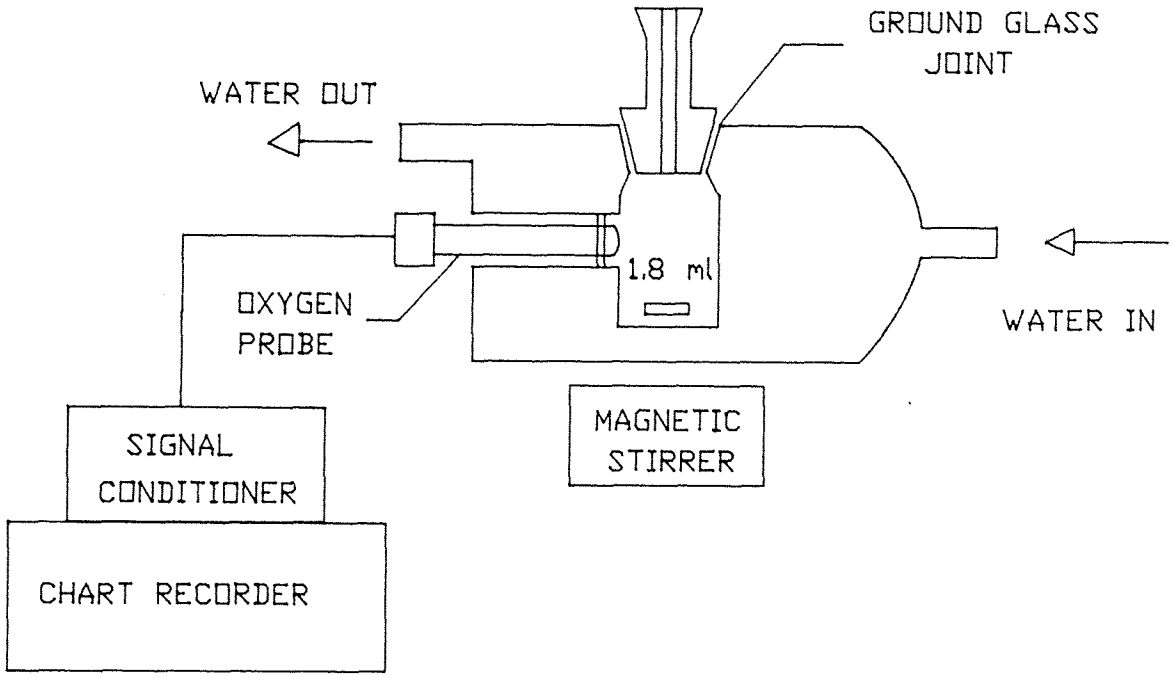


Figure 3.1 Micro Assay Reactor Setup.

2 minutes, the substrate is injected and a second change in slope appears, indicating substrate dependent oxygen consumption.

3.4 Verification of the Major Oxidative Product

Although FMO catalyzes the $NADP^+$ dependent oxidation of a variety of different compounds, activity is conveniently measured by following methimazole (MMI) dependent oxygen uptake polarographically [5]. It is shown that the oxidation of MMI is catalyzed exclusively by FMO from liver microsomes [24].

To verify that MMI is the major substrate, not the endogenous reaction, several control runs were performed. Using free enzymes in the micro assay reactor, at a 1 mM MMI concentration, the reaction was allowed to consume about 75% of the oxygen in the reactor after air saturation. This corresponds to roughly 325 nmoles of O_2 consumed. At this point an excess of catalase was added to the reaction mixture. Catalase breaks down H_2O_2 according to the following reaction;

$$\text{Catalase} + H_2O_2 \rightarrow 1/2\ O_2 + H_2O$$

After adding the catalase the amount of dissolved oxygen increased less than 10 nmoles.

The increase in oxygen most likely comes from the dissolved oxygen in the catalase solution, or it may come

from the breakdown of H_2O_2 resulting from the endogenous reaction during the time between substrate additions. In either case it can be concluded that the oxygen consumption rate is indicative of the substrate oxidation rate.

3.5 Bead Storage

In order to investigate the phenomena of the increase in rate during the storage period, the possibility of proteolytic species acting as inhibitors leaching out of the matrix was investigated. Under a constant ratio of 1 bead per ml storage medium a protein balance on the storage medium gain and bead loss was examined, a UV/VIS spectrographic analysis on the storage medium was done and the weight loss of the beads was monitored over time.

3.6 Immobilization of Cofactors

Immobilizing the cofactors along with the microsomes poses a problem. During the bead making process the beads remain in $CaCl_2$ for 10 hours and in buffer for a minimum of 4 hours. If the cofactors are immobilized they will diffuse out of the beads during this time. Being that they do diffuse out, it may be possible to make the beads in a solution containing the cofactor and allow them to diffuse into the bead while they are curing.

A comparison based on the total weight of cofactor

used was made in order to compare the results of immobilizing the cofactors in the bead with the results of injecting the cofactors into the reaction medium. The quantity of cofactors injected into the reactor using 10 beads was multiplied by 5 and immobilized in 1 ml of gel/microsomal homogenate (approximately 50 beads) and extruded into 50 ml of calcium chloride and transferred to 50 ml of buffer. A second batch of 50 beads were extruded into 50 ml of calcium chloride containing the same quantity of coefactors immobilized. A third batch of 50 beads were extruded and stored in 50 ml of buffer containing the same amount of cofactor. A fourth batch was prepared in the standard way maintaining a ratio of 1 bead to 1 ml of curing and storage medium. A total of three sets of the four above mentioned combinations were performed for $NADP^+$, G6P/G6PDH and all together $NADP^+$/G6P/G6PDH. The micro assay were carried out in the usual manor where the cofactor(s) not immobilized were injected.

Control runs were done using microsomal beads without adding the cofactors and using blank alginate beads adding the cofactors. The microsomal beads do show a small but negligible endogenous rate, while the blank beads did not result in any significant rate.

3.7 Organic Synthesis

The starting material used in the recirculation reactor experiments of the latter section and its nitroxide are not readily available and were chemically synthesized.

3.7 (i) Synthesis of 2,2,4,4-tetramethyl-1,3-oxazolidine

The starting material, 2,2,4,4-tetramethyl-1,3-oxazolidine (TOX) was produced according to methods outlined by Bordor et al. [25]. 23.7 Grams of 2-amino-2-methyl-1-propanol and an equimolar amount of acetone were added to a round bottom flask with 50 mg ground para-toluenesulfonic acid in 75 ml of dried benzene. The reaction mixture was stirred and heated to a gentle boil, 110^{o}C, to maintain a steady reflux. As the reaction proceeds water will be produced in a 1:1 ratio with 2,2,4,4-tetramethyl-1,3-oxazolidine. The water product of the reaction was separated using a Dean Stark Separator. The reaction was allowed to continue for 22 hours, at which time the theoretical amount of water was produced. The water was discarded and the solvent was evaporated in a rotary film evaporator under vacuum. The remaining clear yellow liquid, approximately 40 ml, was further purified by distillation.

A series of two distillation processes were performed at atmospheric conditions. The first was a simple distillation where 50% of the liquid was collected as

overhead vapor within a range of 120-135^{o}C. The condensed vapor, a clear liquid, is distilled a second time using a packed column. Three fractions were collected; I < 128^{o}C 4.95 grams, II 130-133^{o}C 7.25 grams, and III 137-139^{o}C 5.28 grams.

The product, 2,2,4,4-tetramethyl-1,3-oxazolidine, was identified using Proton NMR. The NMR spectra of each fraction shows chemical shifts occurring at 3.8(S 2H, -CH_2-O-), 1.9(B 1H -NH), 1.4(S 6H -CH_3) and 1.3(S 6H -CH_3) which is in agreement with published values [26]. An additional peak occurs at a chemical shift of 7.8, aromatic hydrogen, indicating that these fractions contain a small amount of benzene (fraction I > II > III). The NMR spectrum of fraction III is shown in Figure 3.2. Fraction II will be used for further organic synthesis and fraction III will be used for biochemical synthesis.

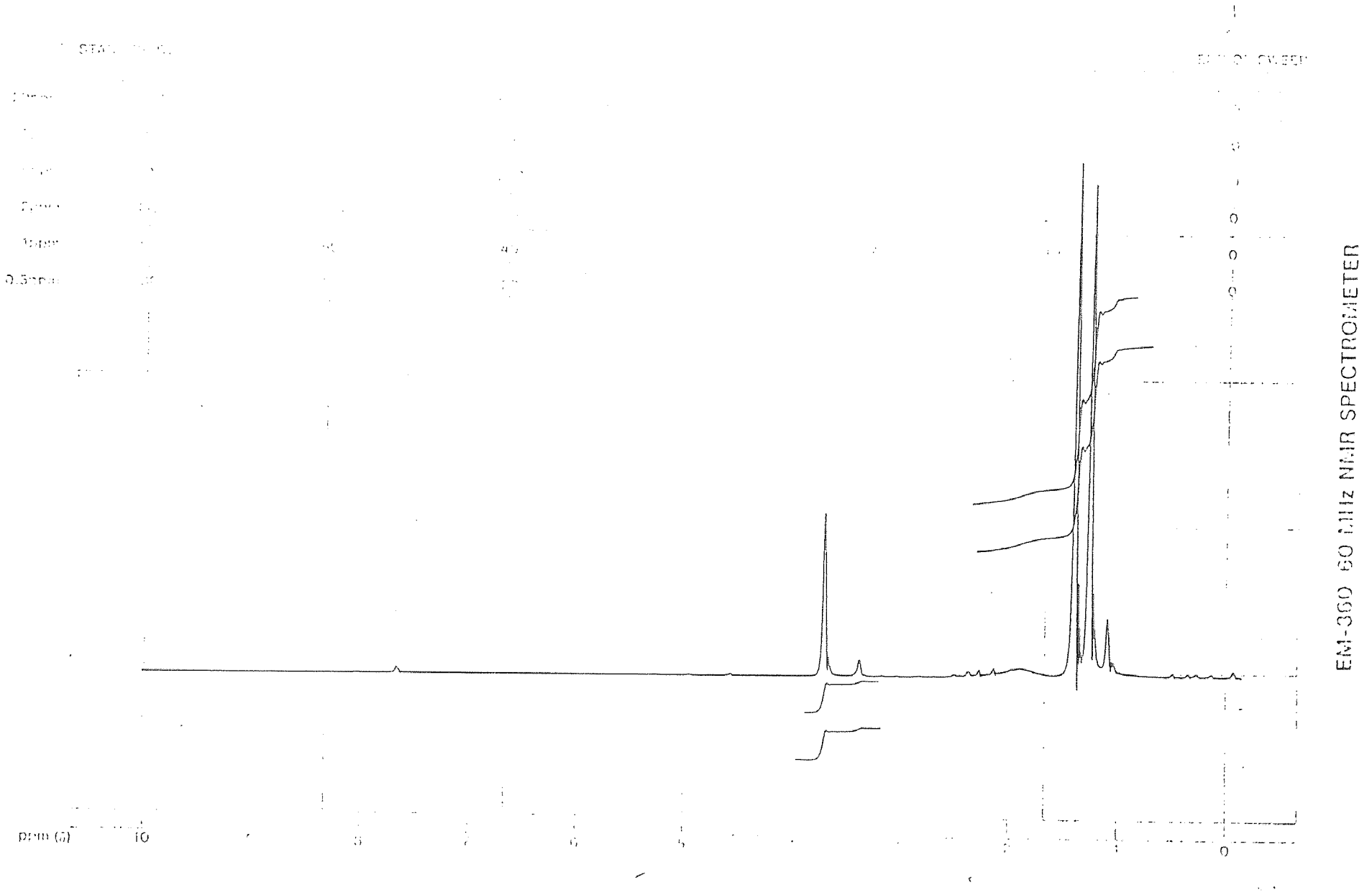


Figure 3.2 NMR of 2,2,4,4-Tetramethyl-1,3-oxazolidine

3.7 (ii) Synthesis of 2,2,4,4-tetramethyl-1,3-oxazolidine Nitroxide

The synthesis of 2,2,4,4-tetramethyl-1,3-oxazolidine-1-oxyl was carried out under mild conditions to prevent undesired side reactions. To a reaction medium of 125 ml of methylene chloride 2.6 grams of starting material, 2,2,4,4-tetramethyl-1,3-oxazolidine at 0-5^{o}C, 6.6 grams of 3-chloroperbenzoic acid dissolved in methylene chloride was added drop wise to the well-stirred mixture. After adding the 3-chloroperbenzoic the mixture took on a clear yellow-green color. The temperature was allowed to rise to room temperature after 1 hr. and left to stir for 24 hours.

Once the reaction was completed, the mixture was washed with 0.025 moles NaOH to remove the acid produced. Anhydrous sodium sulfate was added to remove water and the methylene chloride was removed using a rotary evaporator. 4.33 Grams of a green paste remained and was brought up with 40 ml of hexane and stirred for 1 hour. The green color residue was transferred to the hexane and a white solid remained. The liquid was allowed to evaporate resulting in 1.62 grams of a green oily residue.

The green residue was further separated and purified using a chromatographic separatory column containing 40 grams of 70-230 mesh silica gel. The residue was brought up in a 2:1 hexane/ethyl acetate solvent and eluted with the

same solvent system. Two distinct bands resulted. First a blue band and second a yellow band. The eluant was collected in eight separate flask over time. Thin plate chromatography using the same hexane/ethyl acetate solvent system was done on each of the fractions collected for product identification, refer to Table 1. The solvent was again evaporated off and the fractions combined according to the TLC results. Table 2 shows the results of ESR spectroscopy.

Table 1. COLUMN CHROMATOGRAPHY RESULTS OF ORGANIC SYNTHESIS

			TLC R_f			
Fraction	Color	Volume	0.8	0.6	0.5	0.4
1	Before Blue	20 ml	+			
2	Blue	25	+	+/-		
3	Blue/Yellow	10		+	+	
4	Yellow	20			+	
5	Yellow/Clear	25			+	+/-
6	Clear	30				+
7	Clear/Cloudy	20				+
8	Cloudy	20				+

After Combining and Drying

Fraction	Color	Weight
1,2	Yellow oil	0.14g
4,5	Yellow crystals	0.24g
6,7,8	Orange oil	0.35g
3	Yellow oil	0.06g

Table 2. RESULTS OF ESR SPECTROSCOPY OF NITROXIDE PRODUCTS

Sample	Peak Area	Gain	Peak Area/Gain	A_N (Gauss)	Conc. (mM)
Frac 1,2	26.0	$8x10^3$	$3.25x10^{-3}$	14.5	0.18
Frac 4,5	7.6	$4x10^2$	$1.84x10^{-2}$	14.5	1.08
Frac 6,7,8	11.6	$10x10^2$	$1.16x10^{-2}$	14.5	0.65

Concentrations were determined by makeing 1 mM samples and comparing them to a standard. A linear relationship between peak area/gain and concentration was assumed. Benzene was used as solvent.

3.8 Bioproduct Analysis

After a reaction has been completed both the beads and the reaction medium are collected and extracted using ethylene dichloride. Prior to the extraction the pH of the medium is raised to 10 with sodium bicarbonate to bring the oxidized product into the organic phase. The organic solvent is evaporated and the resulting weight noted.

(a) The product of Chlorpromazine is determined by TLC. Elution is done on silica gel plates developed in a 7:1:2 Acetone/Water/6 M Ammonia solvent. The product and starting material have R_f 0.2 and 0.8 respectively.

(b) The product of 2,2,4,4-tetramethyl-1,3-oxazolidine-3-oxyl is identified by EPR Spectroscopy.

3.9 Instrumentation

Varian E-12 EPR Spectrometer.

Perkin-Elmer Series 10 HPLC with a LC 135 Diode Array Detector with a 50:50 hexane/ethyl acetate mobile phase and a CRI C8 column.

Perkin-Elmer Lambda 3B UV/VIS Spectrometer.

CHAPTER IV

RESULTS AND DISCUSSION

4.1 Increase in Rate During Storage Time

Plots of the oxygen consumption rate versus storage time in calcium chloride and in buffer are shown in Figures 4.1 and 4.2. Although there is some degree of scatter among the data, both curves indicate an increase in rate. Storage in calcium chloride displays a steady increase while storage in buffer increases in stages. The rate, when stored in buffer, increases rapidly and reaches a plateau after approximately 4 hours and increases again after 50 hours.

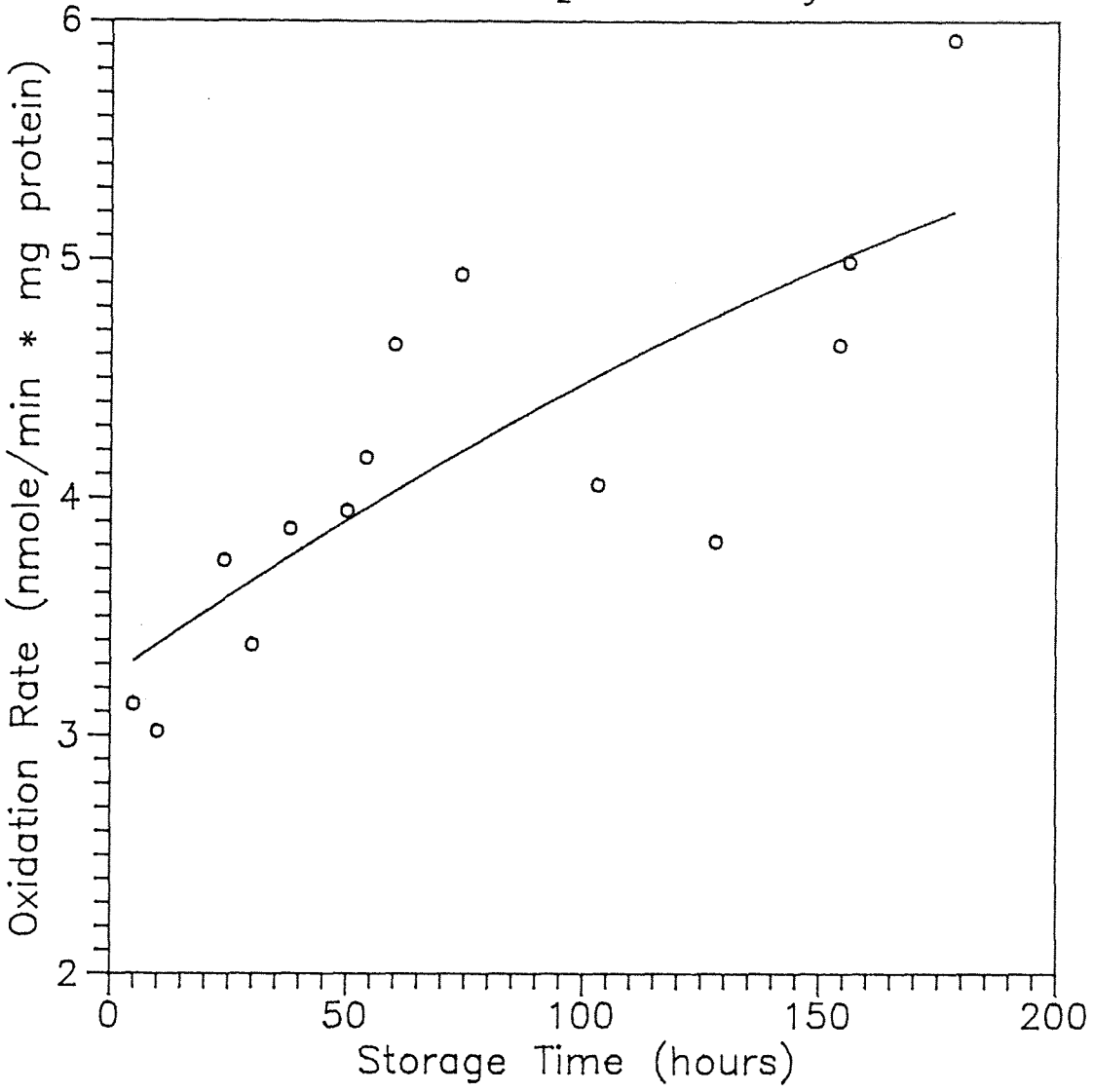
The initial increase in rate during the first 4 hours after the beads are transferred to buffer is most likely a result of the decrease in mass transfer resistance due to softening of the beads when removed from calcium chloride. However, the second increase can not be explained in the same manner. This may be the diffusion of endogenous inhibitors from the matrix or the contamination or growth of another microorganism. Contamination is unlikely because of controlled storage conditions and sterilization. The microsomal mixture in not purified and can contain many endogenous compounds, some of which are inhibitors [5]. The stages may be a division of species of different molecular

weights moving into the liquid medium. When the beads are in the calcium chloride the ionic linkage between alginate and calcium is at its greatest, maintaining a smaller pore size and stronger beads, slowing the diffusion of the inhibitors. This is believed to be the reason for the absence of visual stages when stored in calcium chloride.

It should be noted that repeated freezing and thawing can be used as a method of purifying the enzyme by breaking the vesicles allowing inhibitors to escape [4]. This type of purification may be taking place during storage at a temperature near $0^{o}C$. Also, as the enzyme becomes purified an increase in activity is observed [23].

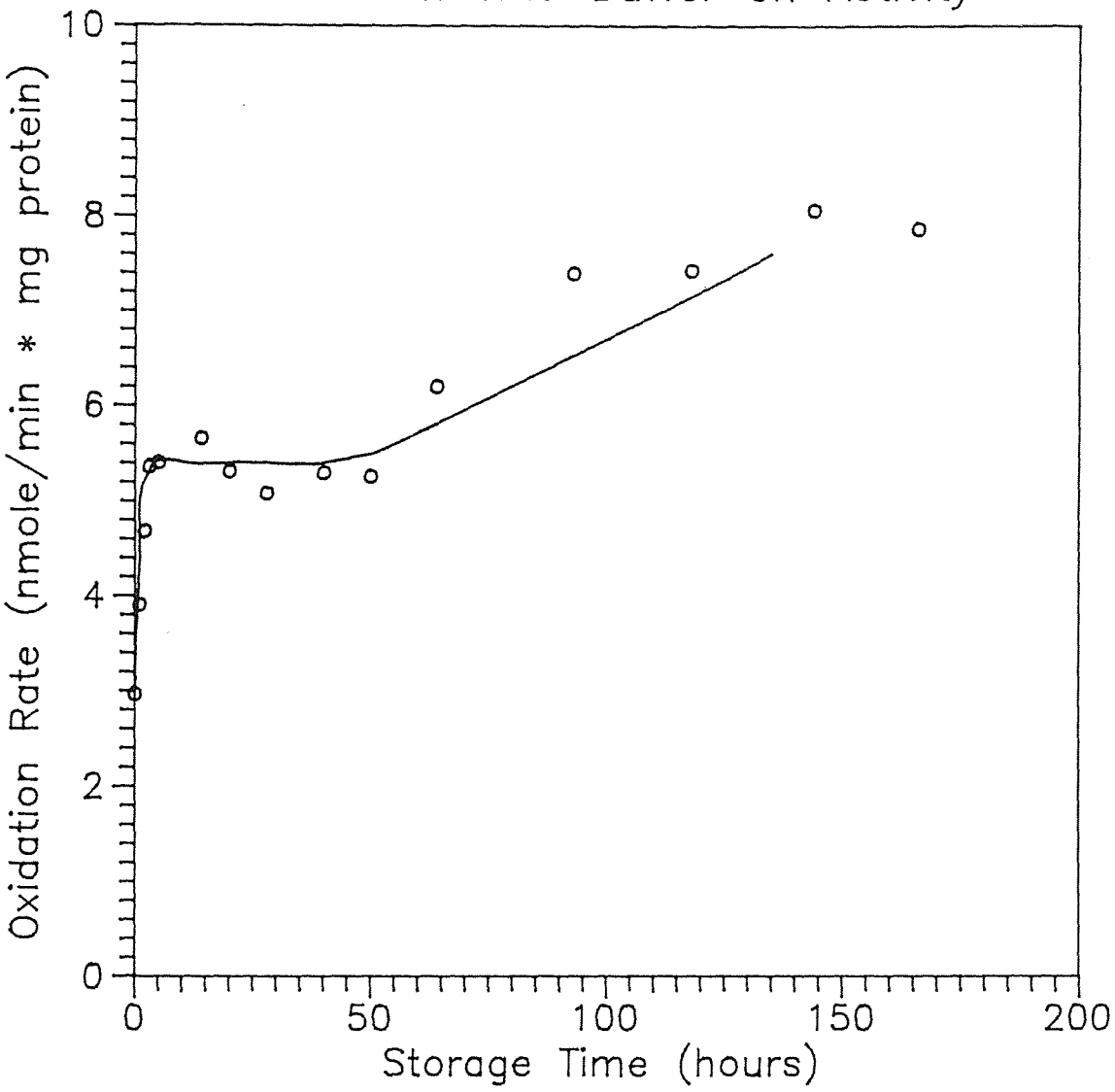
In order to identify the diffusion of protein from the beads to the storage medium, a spectral analysis, a protein balance on the beads and medium, and an analysis of the weight loss of the beads were performed. There is positive evidence of mass diffusion out of the beads shown by the weight loss of the beads over time, see Table 2. The spectral analysis, Figure 4.3, and the results of the protein balance, Table 3, indicate that protein is obviously diffusing into the storage medium and that UV/VIS spectrum of the protein is similar to that of the microsomal preparation. The nature of the inhibitors can not be determined by these tests. Ziegler [18] suggests that some forms of lipids can act as inhibitors. A diagnostic test for lipids would help to better identify the nature of the inhibitors.

Figure 4.1 The Effect of Storage Time in $CaCl_2$ on Activity



Initial rates are plotted using 1mM MMI as substrate. The reaction rate steadily increases over time.

Figure 4.2 Effect of Storage Time in TRIS Buffer on Activity



Initial rates are plotted using 1 mM MMI as substrate. The reaction rate increases to a plateau after 4 hours and increases again at 50 hours.

Table 3. WEIGHT LOSS OF BEADS DURING STORAGE TIME

Time (hr.)	Dry Weight of 10 Beads (mg) T-1	T-2	T-3	Average
$CaCl_2$				
0	15.1	13.9	14.4	14.5
Buffer				
0 (10)	14.0	13.4	14.2	13.9
10 (20)	9.5	9.7	9.8	9.6
25 (35)	8.4	7.9	8.1	8.1
50 (60)	7.7	7.4	7.5	7.5
75 (85)	7.8	7.9	7.4	7.5
100 (110)	8.1	7.7	7.8	7.8

The numbers in parenthesis represent total storage time, in $CaCl_2$ plus buffer.

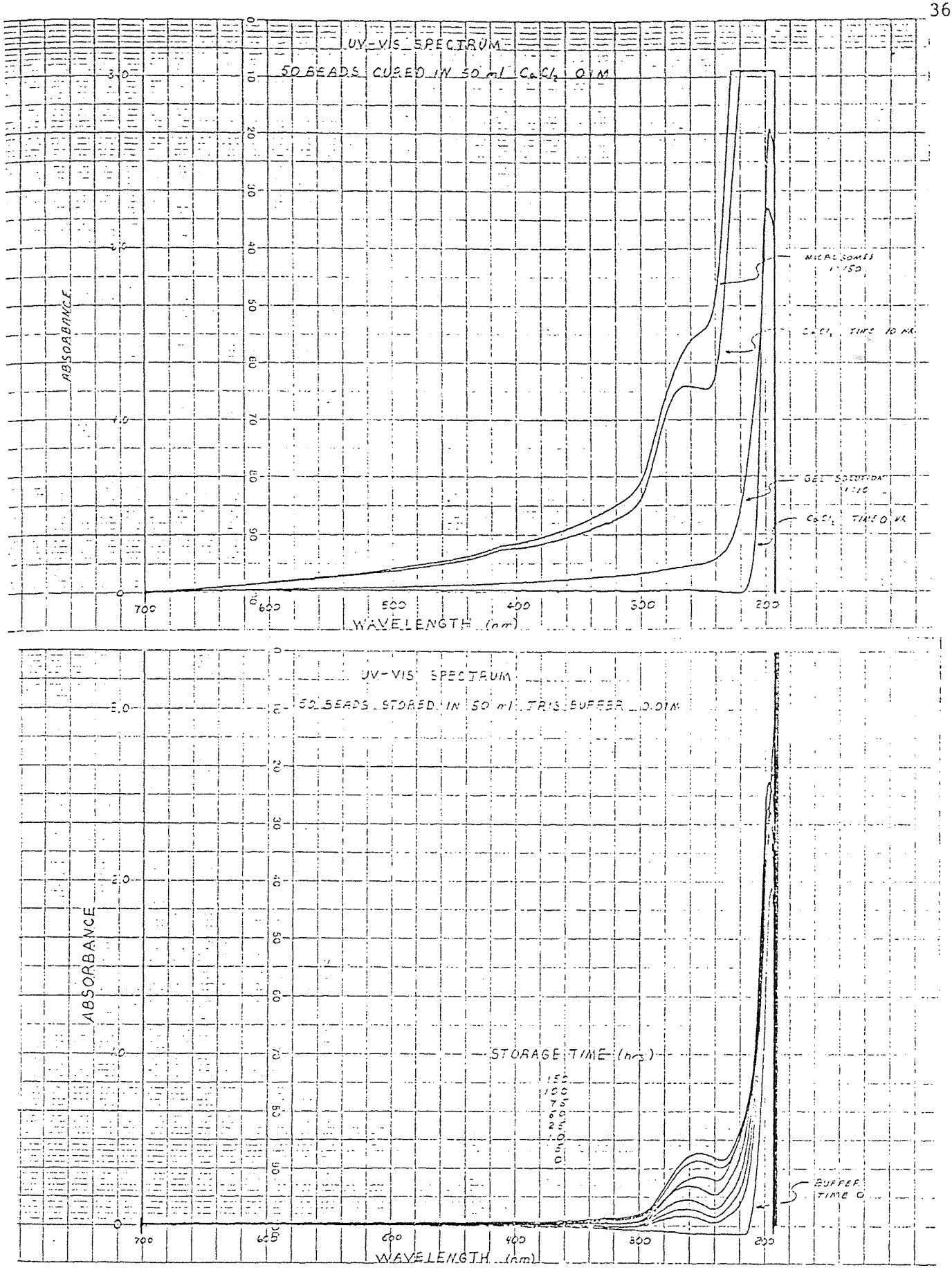


Figure 4.3 UV/VIS Spectrum of the Storage Medium

Table 4. PROTEIN BALANCE OF BEADS AND STORAGE MEDIUM DURING STORAGE TIME

Micrograms of Protein per Bead or per ml*

Time (hr.)			Total Protein
Gel/Microsome Homogenate	(Drops)		
0	303		303
	/ \		
$CaCl_2$	(Liquid)	(Beads)	
10	27.3	276	303
		/ \	
Buffer		(Liquid)	(Beads)
20		30	242	299
35		35.8	239	302
85		45.7	241	313
110		47.5	232	306

* The above values represent an average of 3 trials. The initial protein content of both $CaCl_2$ and buffer is 0.

4.2 Cofactor Immobilization

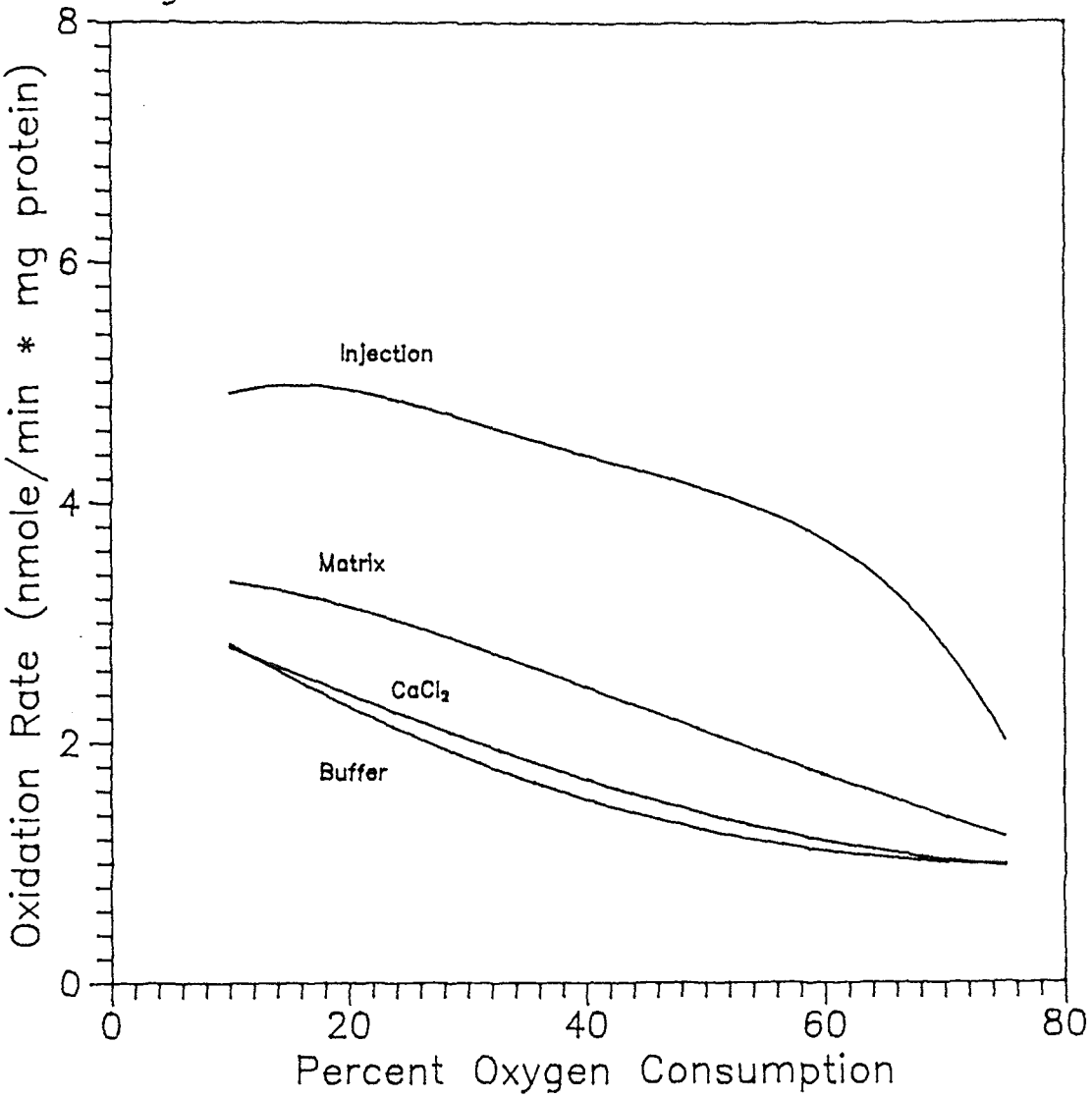
Figures 4.4, 4.5 and 4.6 show that there is a significant decrease in the rate of all cases of immobilization when compared to the standard method of introducing the cofactors. However, it is evident that a portion of the cofactors remain or diffuse into the beads when compared to pure calcium alginate beads. An interesting point is that in both cases when $NADP^+$ is immobilized within the matrix the reaction rate is approximately 45% of the standard procedure. In the experiment when only G6P/G6PDH is studied, the run where the cofactors are put in the buffer was the nearest to that of the standard procedure. This shows that $NADP^+$ is less mobile than G6P and G6PDH. G6P and G6PDH appear to be able to move in and out quite freely and no advantage is gained by their immobilization while $NADP^+$ is retained.

In this case the use of a macromolecular form of $NADP^+$ may be better suited for immobilization. A larger molecule will be more likely to be retained. If this can be immobilized and retained in the beads, the beads could be reused several times without additional $NADP^+$ requirement or separation.

In an effort to increase the amount of $NADP^+$ retained an investigation of different support matrixes may be beneficial. Examples of other potential supports would be

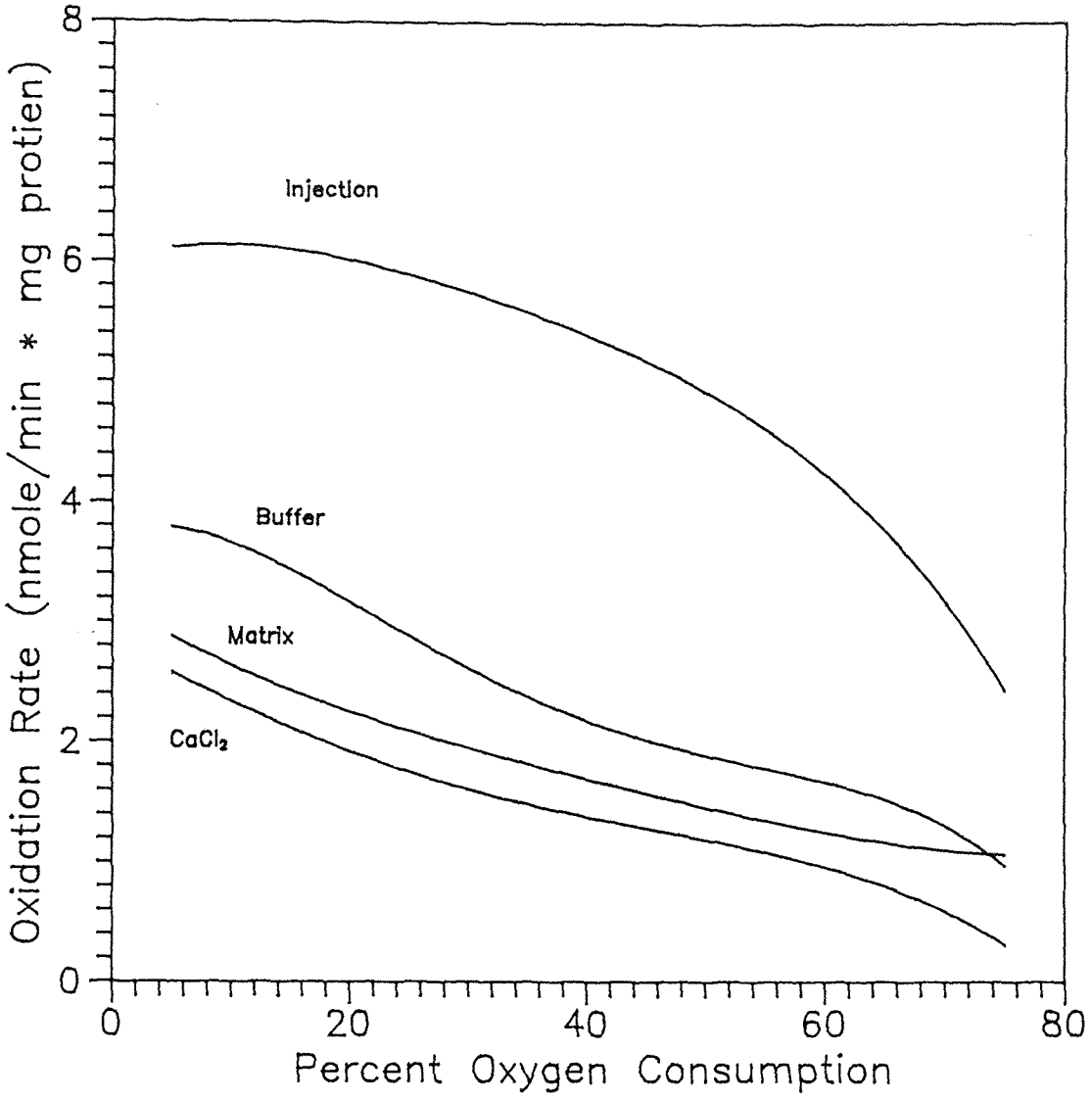
polyvinyl or polyamide gels. Here the attempt is to create a pore size large enough for substrate to enter but small enough to retain the cofactors. With this in mind, other methods such a polymer coating around the beads or a membrane enclosed system would also present possible avenues of investigation.

Figure 4.4 Immobilization of $NADP^+$



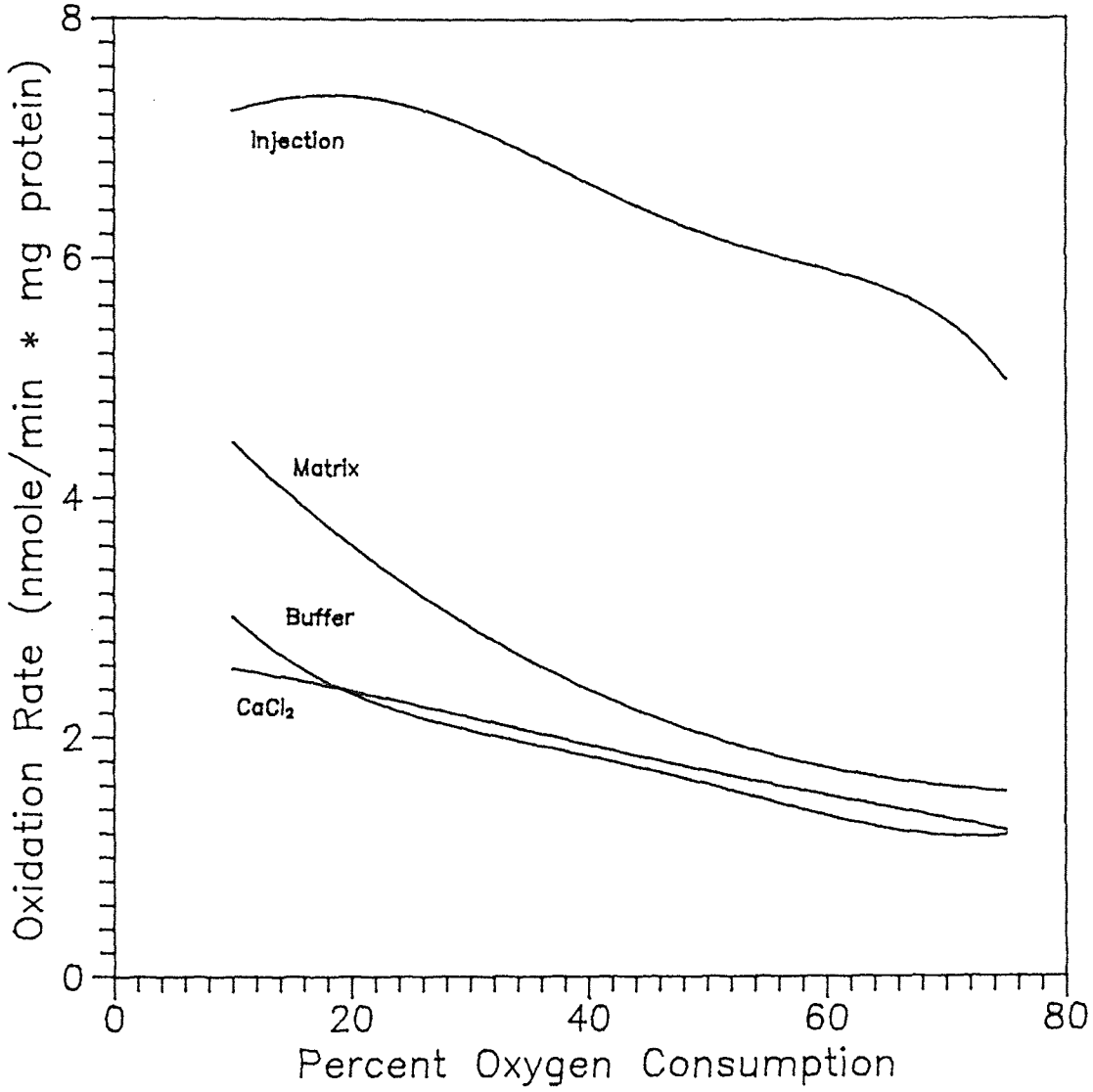
1 mM MMI is used as substrate.

Figure 4.5 Immobiliztion of G6P and G6PDH



1 mM MMI is used as substrate.

Figure 4.6 Immobilization of G6P, G6PDH and $NADP^+$



1 mM MMI is used as substrate.

4.3 Enzyme loading

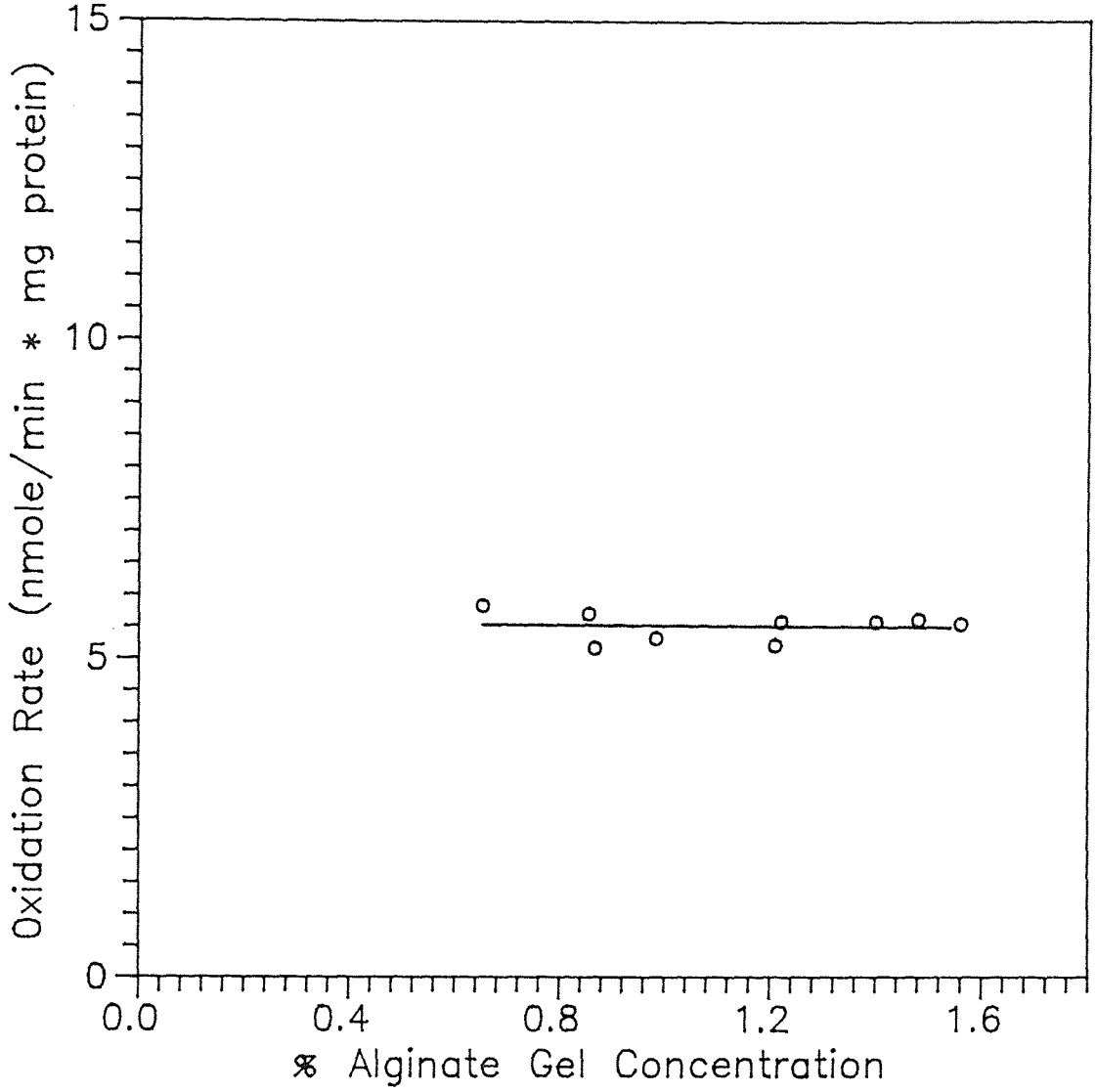
Figure 4.7 shows the effect of alginate gel on the reaction rate. Within the range studied, 0.5-1.5 % alginate, it appears that the alginate gel has little or no effect on the oxidation rate. The various alginate gel concentrations were produced by changing the ratio of gel to microsomes during the catalyst preparation. A study by Lakwala [27] using activated sludge immobilized in calcium alginate, showed the effect of calcium chloride concentration used to cure the beads also has little or no effect on the rate. Apparently, the alginate matrix produces consistent and uniform resistance to mass transfer (pore size) under these conditions.

An important consideration of the gel concentration is the bead stability. In the micro assay reactor, the beads are subjected to continuous pounding by the stirring mechanism. This drastically affects the physical integrity of the beads. At alginate concentrations below 0.5% beads do not form when the homogenate is extruded into the calcium chloride. At a gel concentration of 1% the beads will resist breaking for about 1 hour while at 1.5% they will last longer than 4 hours in the micro assay reactor.

In light of the above mentioned study the same data can be plotted as oxidation rate versus protein concentration, Figure 4.8. This plot indicates that the reaction rate

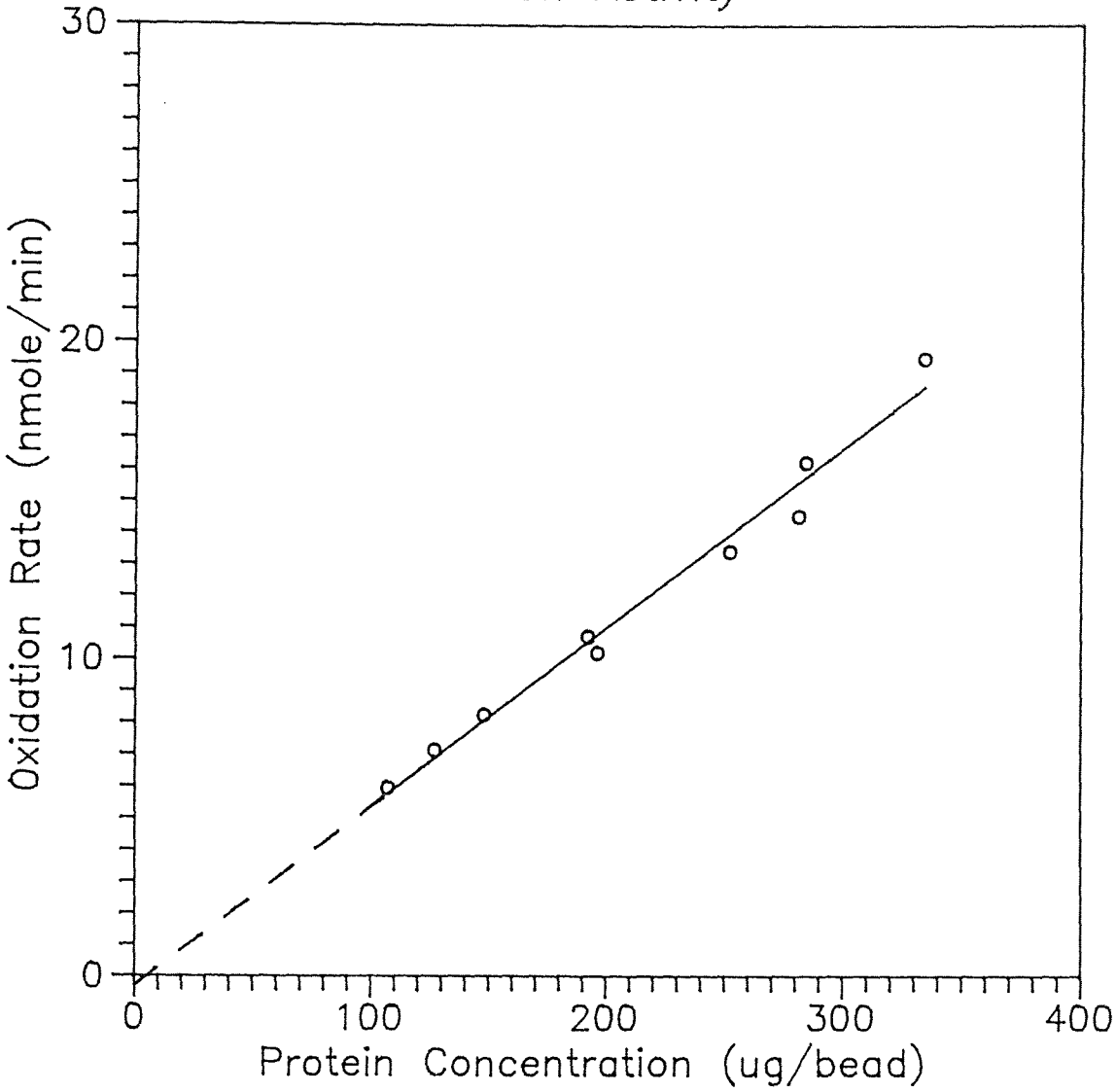
increases proportionally with an increase in protein. The highest possible enzyme loading is of course the most desirable condition. If the loading is too low the reaction will proceed at a very slow rate and if the loading is too high the beads will not form. A 1% gel concentration produces beads containing approximately 250 mg each. This number will vary depending on the protein concentration (enzyme purity) of the microsomal mixture used.

Figure 4.7 Effect of Alginate Gel Concentration on Acitivity



The reaction rate is uneffected by the alginate gel concentration.

Figure 4.8 Effect of Enzyme Loading on Activity



Within this region, the reaction rate increases linearly with respect to enzyme concentration. Beyond 350 mg/Bead, beads do not form in $CaCL_2$.

4.4 $NADP^+$ Saturation Requirement

The requirement of $NADP^+$ for free and calcium alginate immobilized microsomes was investigated. Free microsomes saturate at 3 mM while immobilized microsomes saturate at 5 mM, Figure 4.9. The sigmoidal appearance of the saturation curve suggests a "complex" or "abnormal" kinetics with respect to NADPH ($NADP^+$ is converted to NADPH by the regeneration system at a much higher rate than NADPH is consumed). This may be a result of using the oxygen consumption rate as an indication of the NADPH consumption, even though there is a 1:1 stoichiometric relationship between NADPH and oxygen. One other point of interest is that the curve does not appear to pass through the origin. The presence of endogenous substrate is the probable cause [4].

The catalytic function of enzymes may be affected and controlled by the interaction with ligands not only at or near the active site but also at allosteric sites. Such enzymes are labeled allosteric enzymes and do present "complex" kinetics. Allosteric enzymes are generally oligomers with several identical subunits and play an important role in metabolism. Allosteric effecters, usually structurally unrelated to substrate may be bound to the enzyme at an allosteric site, thus changing the catalytic activity. In many cases allosteric properties are lost as

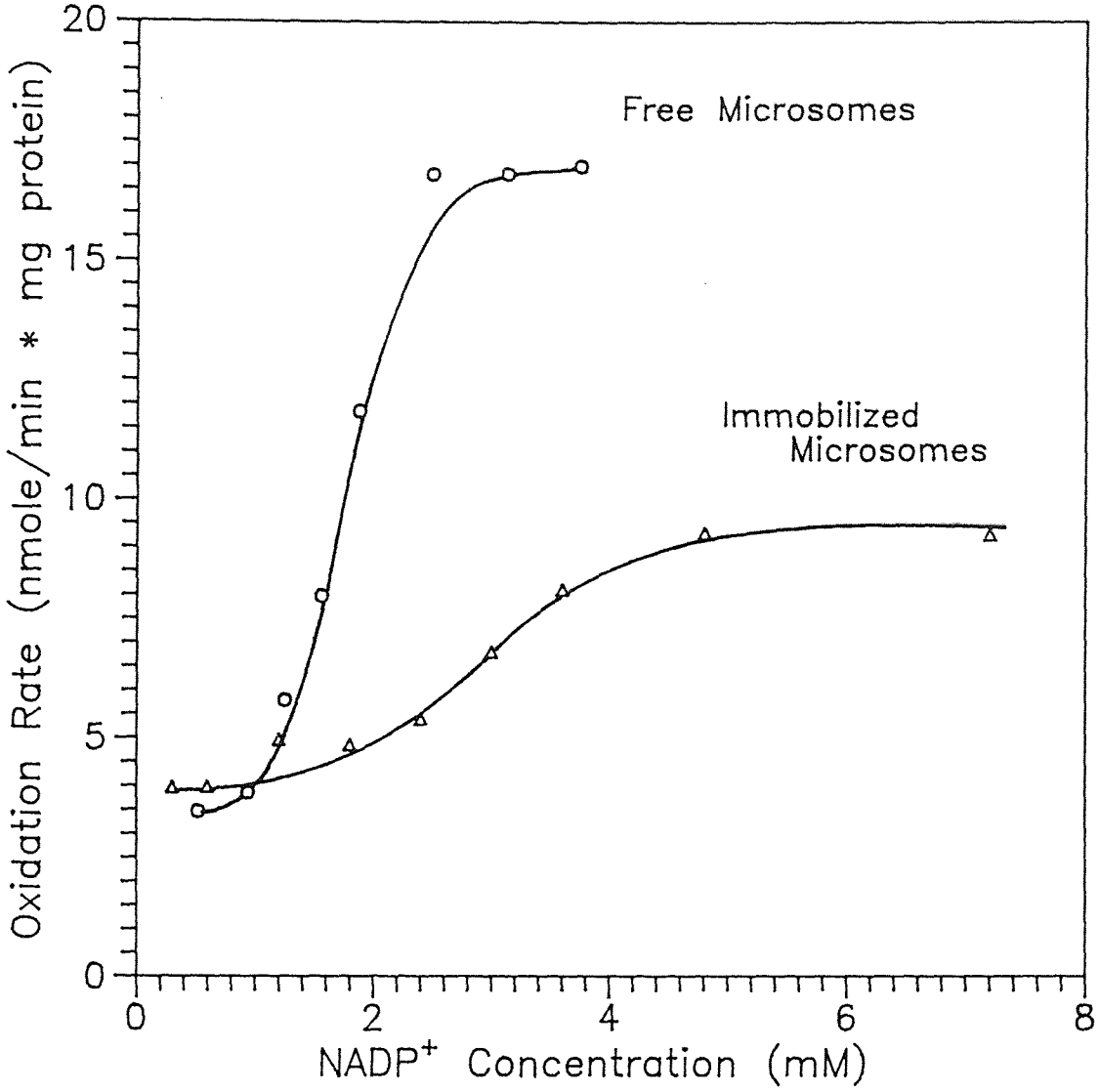
the enzyme is purified and will begin to exhibit Michaelis-Menten kinetics.

The higher saturation requirement of the immobilized microsomes is due to the mass transfer resistance of the matrix. The lower velocity is the result of the additional resistance becoming the rate limiting factor. It is possible to overcome the external limitation in a recirculation flow apparatus. Chien and Sofer [27] have demonstrated this using calcium alginate immobilized yeast cells in recirculation mode. They showed that the rate of ethanol production could be increased from 0.175 g ethanol/hour to 0.34 g ethanol/hour by increasing the flow rate.

4.5 Methimazole and n-Octylamine Saturation Requirements

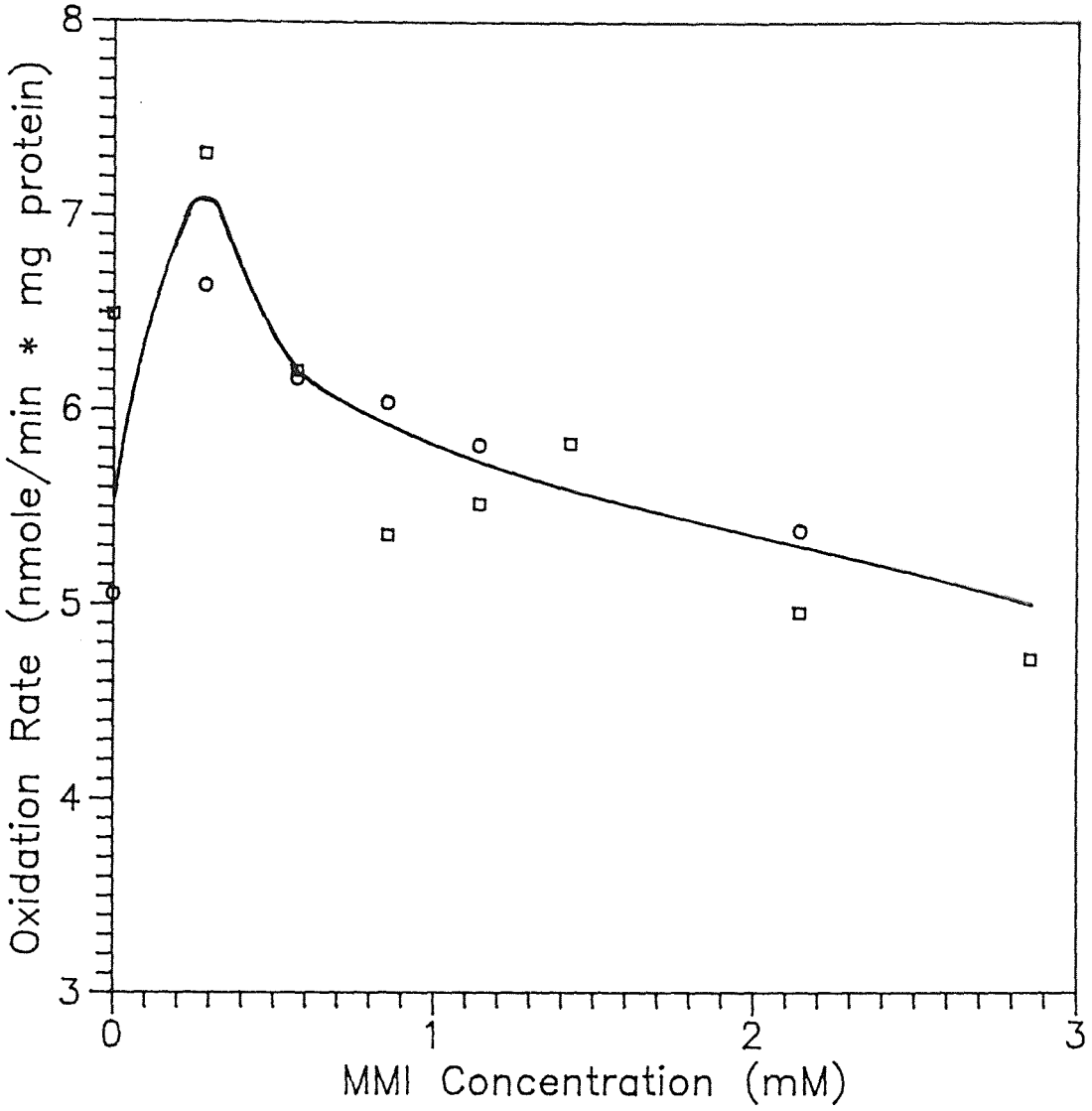
The saturation curve of MMI, Figure 4.10, is found to be 0.3 mM and indicates substrate inhibition. At the saturation concentration of MMI several runs were made to find the saturation of n-octylamine (NOA), Figure 4.11. From the standpoint of the argument presented earlier regarding the rate limiting step of the catalytic cycle, the apparent V_{max} for the immobilized enzyme can be found. Assuming Michaelis-Menten kinetics, a reciprocal plot gives a value of V_{max} = 10.4 nmoles/min * mg protein.

Figure 4.9 $NADP^+$ Saturation Requirement



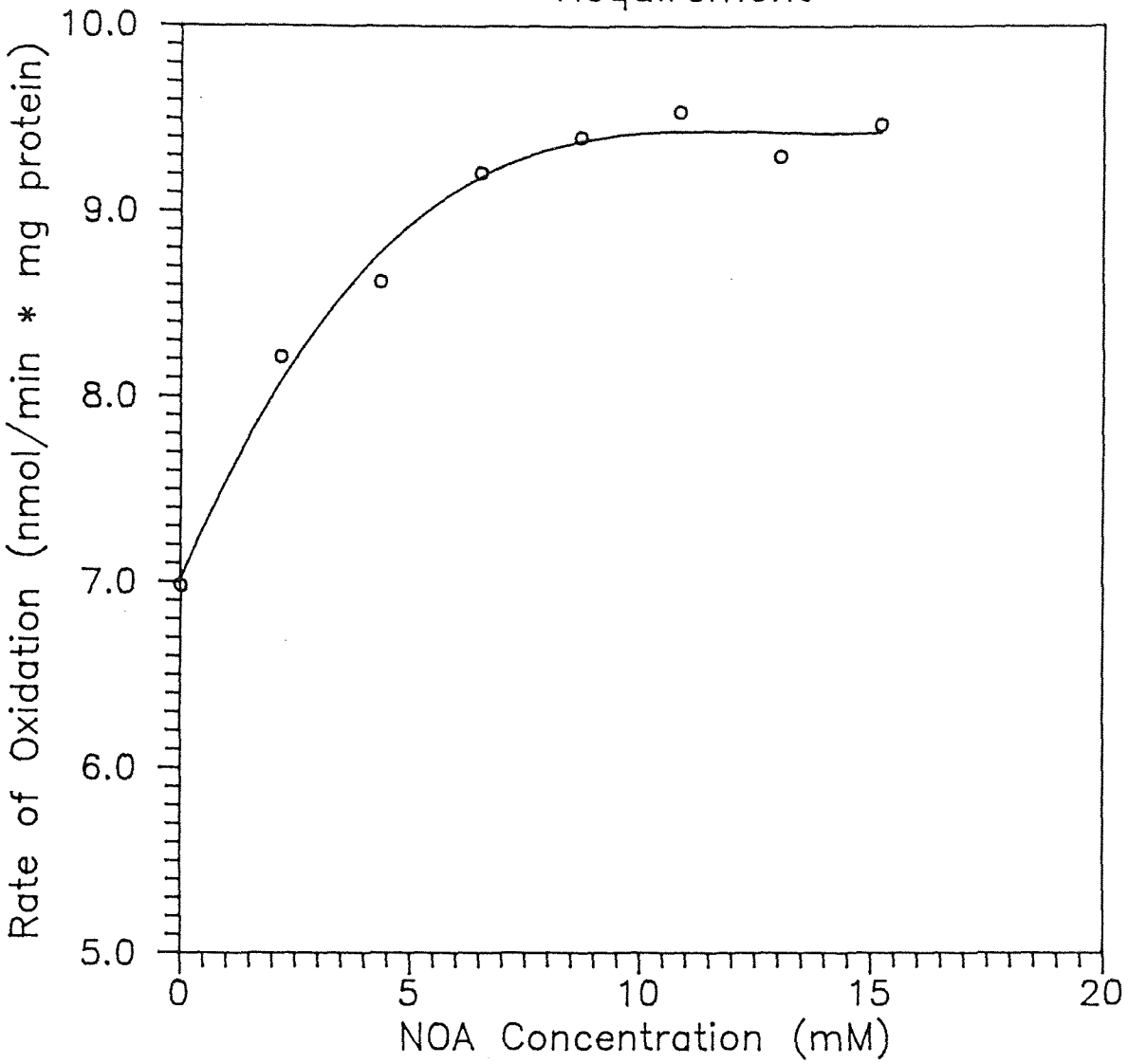
Free microsomes (3.5 mg total) saturate at 3 mM $NADP^+$ while immobilized microsomes (3.2 mg total) saturate at 5 mM $NADP^+$. The matrix has a strong effect on the saturation concentration.

Figure 4.10 Methimazole Saturation Requirement



MMI saturates the immobilized enzyme at a concentration of 0.3 mM. At higher concentrations substrate inhibition is observed.

Figure 4.11 n–Octylamine Saturation Requirement



At substrate saturated conditions (0.3 mM MMI), NOA saturation occurs at 10 mM. A Lineweaver–Burke plot results in a V_{max} of 10.4 nmole/min * mg protein.

CHAPTER V

RECIRCULATION REACTOR

The micro assay reactor is a useful tool for preliminary studies involving kinetic studies on immobilized organisms but it is limited in its practical application. A recirculation flow configuration rather than a mixed flow reactor is a more suitable setup for practical applications as well as a tool for obtaining inherent reactor design parameters for immobilized microorganisms.

The presence of external diffusional gradients of the immobilized biocatalyst will distort the kinetic reaction and often result in misleading kinetic parameters such as V_{max} and K_m. In a mixed flow configuration such gradients often exist at low agitation speeds. Higher agitation speeds will lead to deactivation and often the destruction of the biocatalyst due to the continuous pounding of the stirring mechanism. In a packed bed bioreactor immobilized bacterial alginate beads have been in continuous operation for several months in the NJIT Biotechnology Lab while even the strongest beads will only last a few hours in a mixed flow apparatus.

The design scheme of the recirculation bioreactor is shown in figure 5.1. The reaction medium is circulated between a temperature controlled reservoir and a packed bed

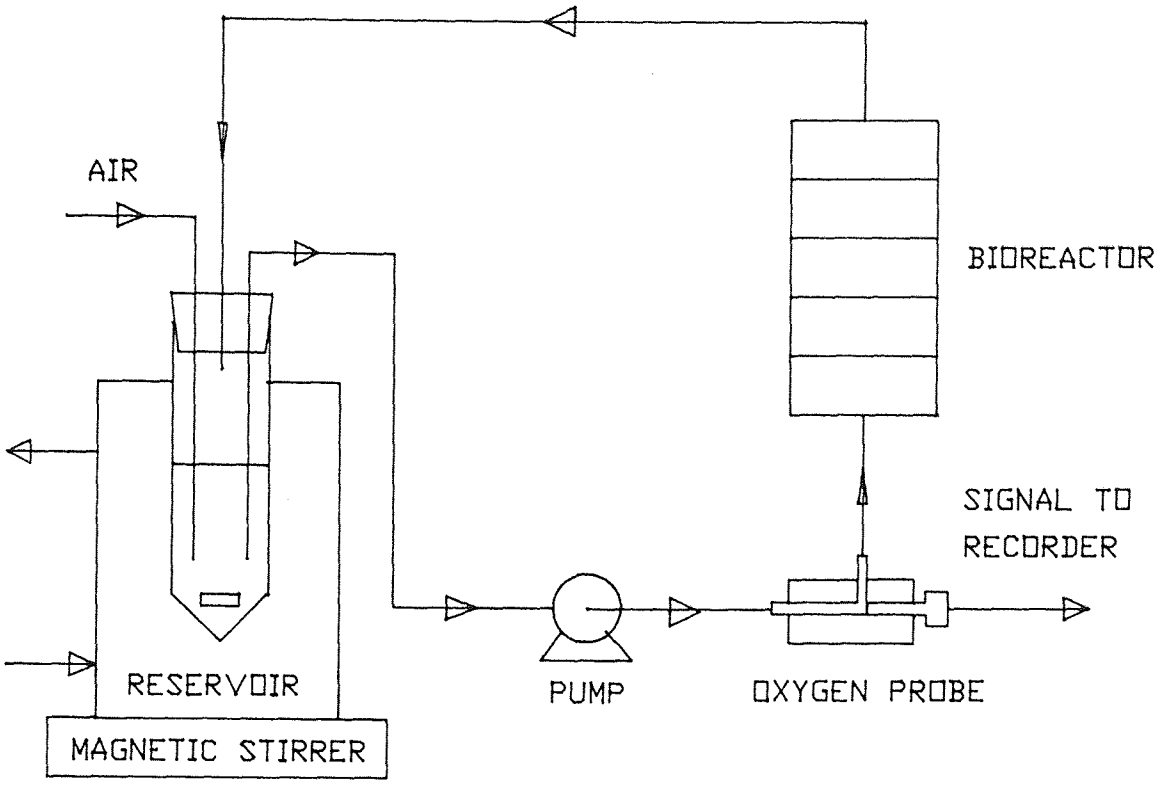


Figure 5.1. Recirculation Flow Reactor.

reactor using a peristaltic pump. A Clark-type impingement dissolved oxygen probe is used to monitor the oxygen level of the reservoir. Oxygen is supplied to the system by sparging either pure oxygen or a mixture of oxygen and nitrogen to achieve a desired oxygen level.

A similar recirculation configuration has been used by Ford et al. [29] for the kinetic studies of glass immobilized Tryspin used to catalyze the hydrolysis of BAPA (N-benzoyl-DL-arginine-P-nitroanilide) and ALME (N -acetyl-L-lysine methyl ester). Instead of monitoring the oxygen consumption of oxygen the disappearance of substrate is measured using a UV/VIS in line spectrometer. This configuration is designed to determine an operating regime with respect to flow rate to minimize mass transfer resistance. In a low-flow regime the substrate concentration at the surface of the bead is lower than the bulk medium. With increased flow rate it is possible to eliminate the external film resistance to mass transfer. This phenomena has also been demonstrated for alginate immobilized yeast using a recirculation reactor [27].

The design of the bioreactor was based on the design parameters from Chien and Sofer [27] and other experiments performed in the NJIT Biotechnology Lab using immobilized bacteria. The reactor was sized to provide 7.58 ml/min * cm^2 at a flow rate of 40 ml/min (100 ml/min maximum of pump).

The packing height was determined to be 1 cm through experiment. Values larger than 1 cm will cause too great a pressure drop and force the beads through the packing supports at high flow rates. Based on a packing factor of 1.3 for spheres and a bead volume of 0.018 ml (55 beads/ml) each stage could hold up to 120 beads. This value was kept at 100 to allow for the swelling of the beads over time.

The liquid is pumped in through a distributor at the bottom of the column to prevent channeling and damage to the beads by the feed stream. The column was designed to have variable sizes allowing for 1 to 5 stages. A maximum of 500 beads or 125 mg of protein (250 ug/bead) can be used.

In the recirculation mode the number of large scale runs was limited by the high cost of cofactors.

Chlorpromazine (CPZ) was used as the substrate to test the performance of the recirculation flow reactor, under pure oxygen saturation conditions (1085 nmole/ml) at 37^0C and pH 7.4. A scale up of 15 times that of the micro assay reactor was run.

Upon the addition of CPZ, substrate dependent oxidation was observed by a change in rate from 14.5 nmoles/min to 24 nmoles/min. The initial concentration of CPZ in the reaction medium was 0.3 mM. Figure 5.2 shows the progress of the run over a 5 hr period. TLC was used to verify the final product.

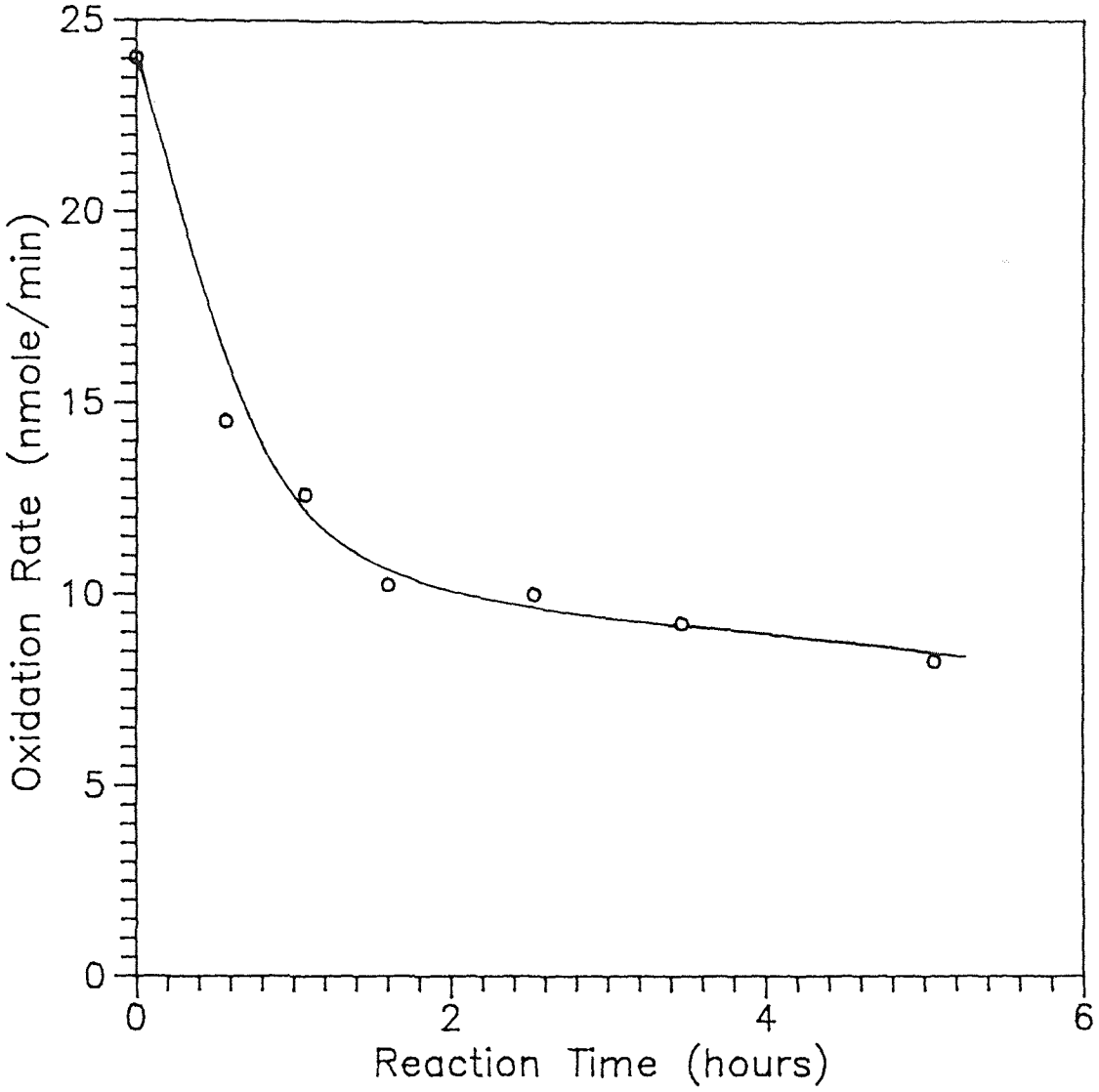
During this run it was noted that the rate of oxygen

removal by diffusion is significant above air saturated conditions, thus distorting oxygen consumption measurements. For this reason air saturation was used in the proceeding experiments even though higher levels of oxygen may yield higher reaction rates.

The recirculation reactor was next used for the N-oxidation of TOX, at a scale up ratio of 15 times the micro assay reactor. Prior to this run the saturation concentration of TOX was determined in the micro assay reactor. Although at higher concentrations the initial rate is higher, the total product yield at lower concentrations is higher, see figure 5.3. If one looks at a time period beyond 20 minutes the optimum concentration is 11 mM. The pH may be influencing the reaction at high concentrations of TOX.

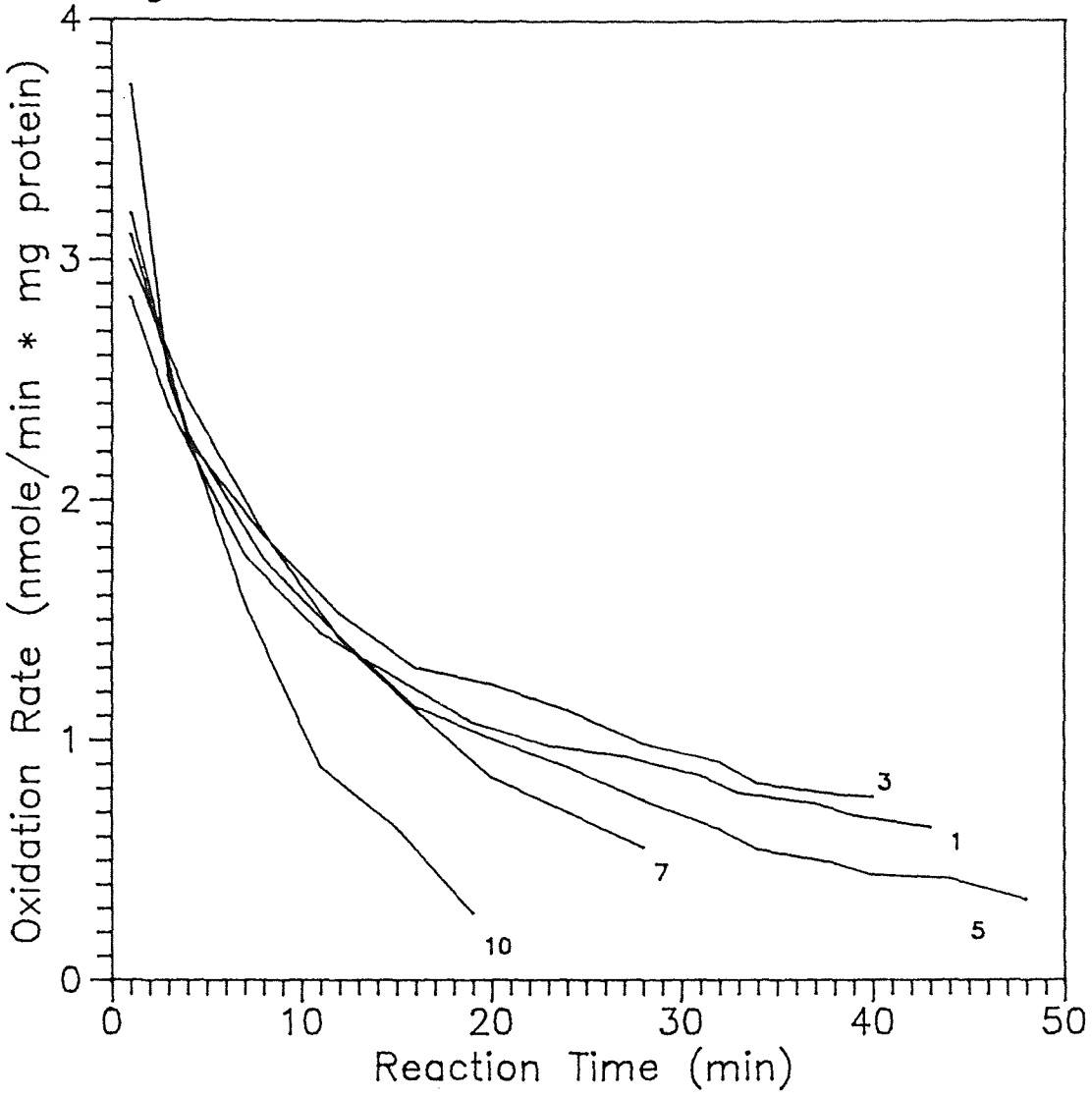
Oxygen consumption measurements as well as HPLC, figure 5.4 were used to follow the reaction. After the addition of substrate there was no noted increase in the oxygen consumption. At this point sample #1 was taken. After 3 minutes a sudden drop in the oxygen level was observed (13 nmole/ml) and sample #2 was taken. A large peak was formed, this peak is believed to be an intermediate. As time progressed with no change in the consumption rate an additional injection of TOX was made, shortly after which a small drop the oxygen level was noted (3 nmole/ml) and sample #3 was taken. In comparison of sample #2 with #3, it appears

Figure 5.2 CPZ N-Oxidation in the Recirculation Flow Reactor



Reaction was run under oxygen saturated condition at an initial concentration of 0.3 mM CPZ. Rates were measured at 60% oxygen saturation.

Figure 5.3 Rate vs. TOX Concentration



The numbers at the end of each curve indicate the amount of TOX injected (1 ul = 6.6 umoles). Saturation occurs at a concentration 11 mM in the reactor.

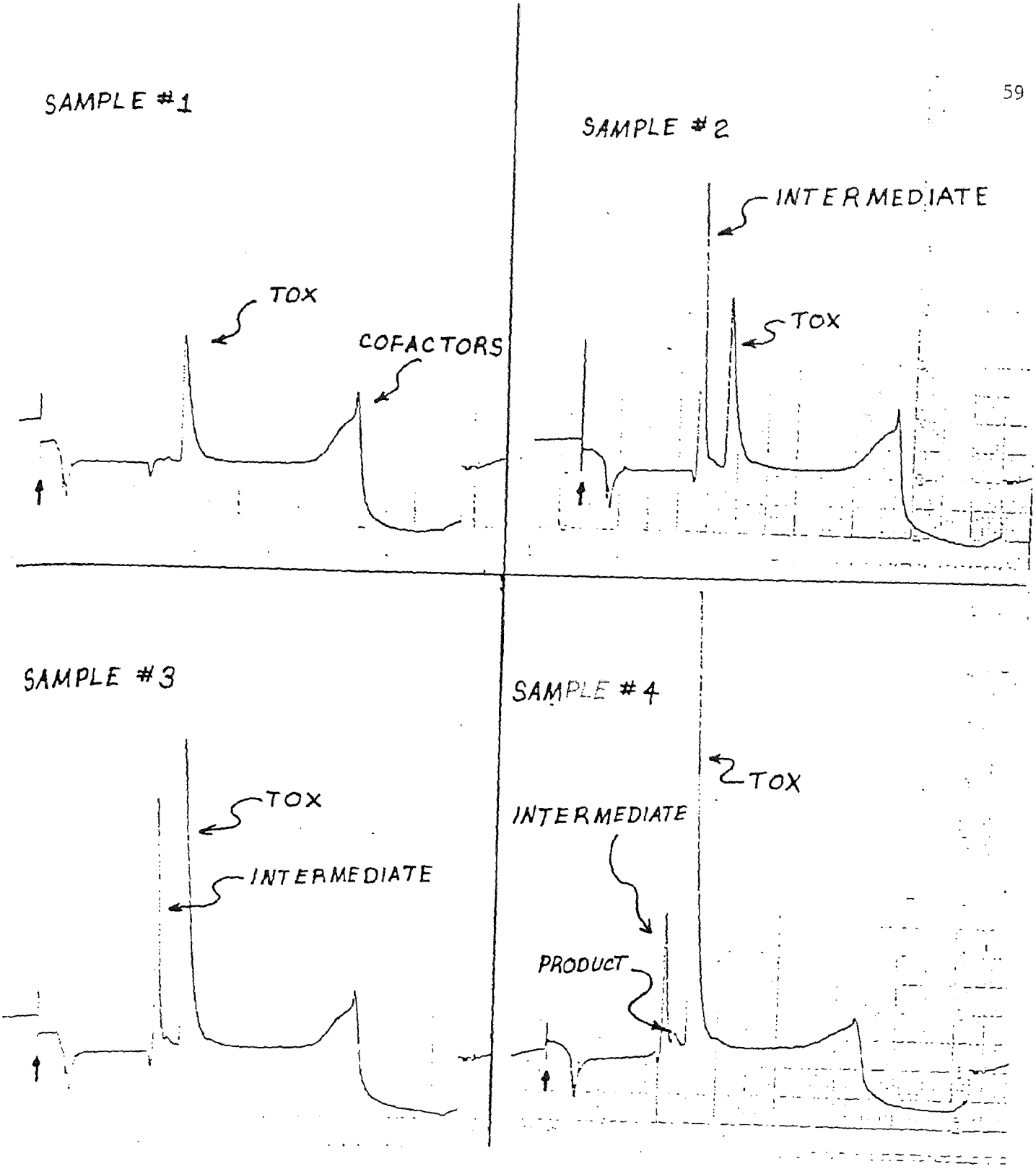


Figure 5.4 Biochemical N-oxidation of TOX followed by HPLC

that the intermediate is decreasing and the final product is appearing. A third injection of TOX was made but the oxygen level remained constant. A fourth sample shows a further decrease in the hydroperoxy intermediate and an increase in the final product. A repeat of this run was made, unfortunately without favorable results.

Isolation of the intermediate peak would be very helpful in identifying the nature of the intermediate. The isolation could also lead to the development of a new and versatile oxidizing agent. However such a task is beyond the scope of this work.

The final test for the end product is identification by ESR. This is a clear test for the presence of a stable free radical. A value of 15 Gauss for A_N is indicative of a nitroxide. The bioproduct disolved in benzene results in an A_N = 15.5 Gauss.

A comparison of the bioproduct with the synthetic product is made using UV/VIS spectroscopy, HPLC and ESR shown figures 5.5, 5.6 and 5.7 respectively. Both the bioproduct and the synthetic product appear to be the same.

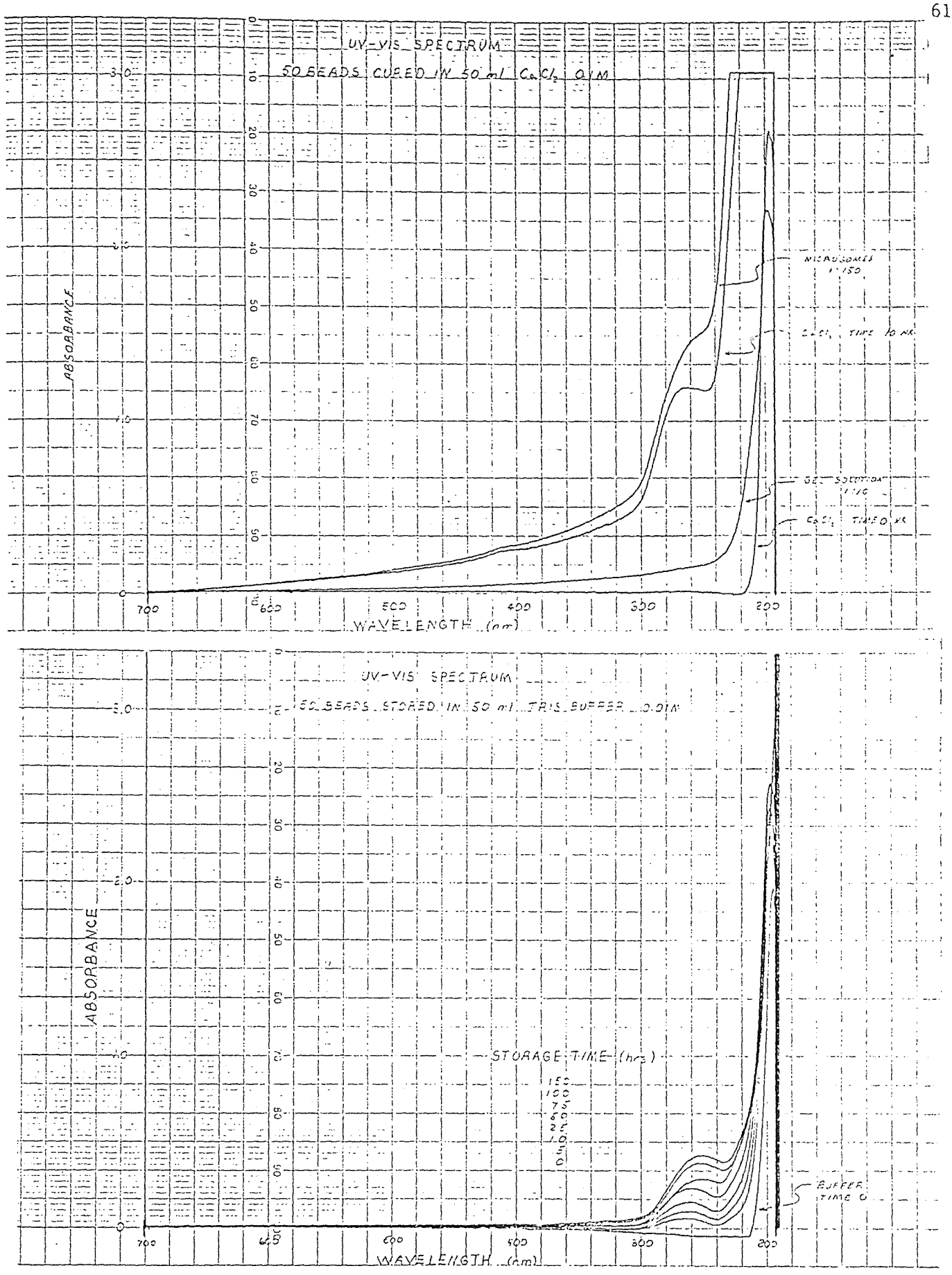
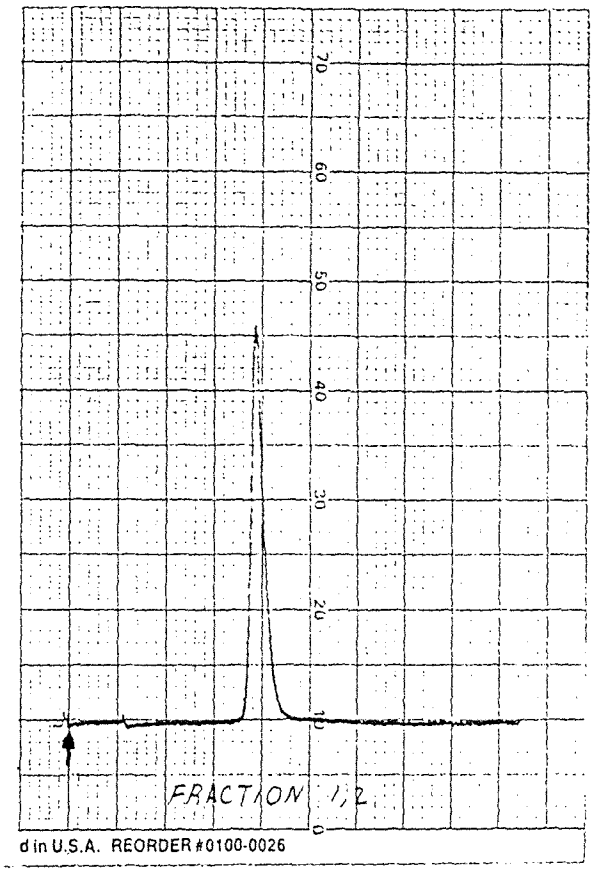
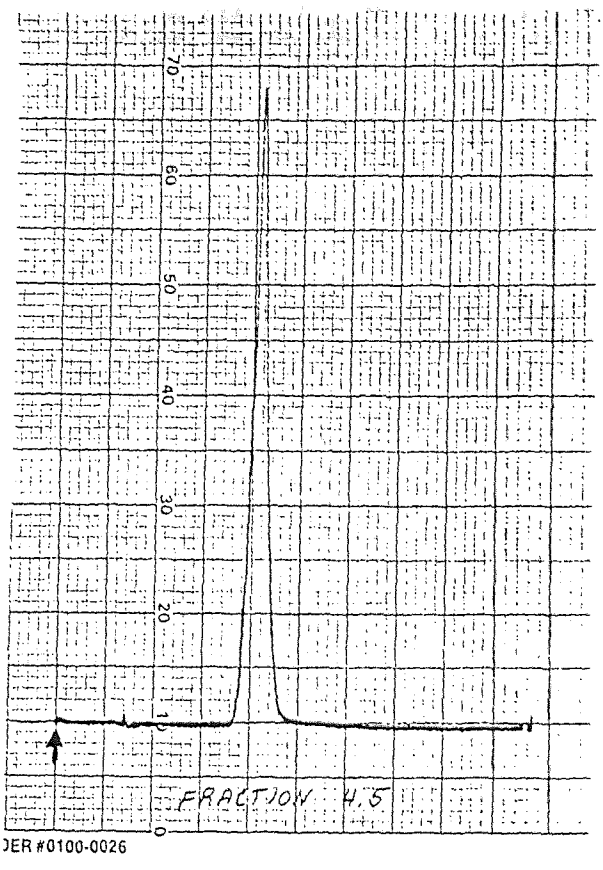
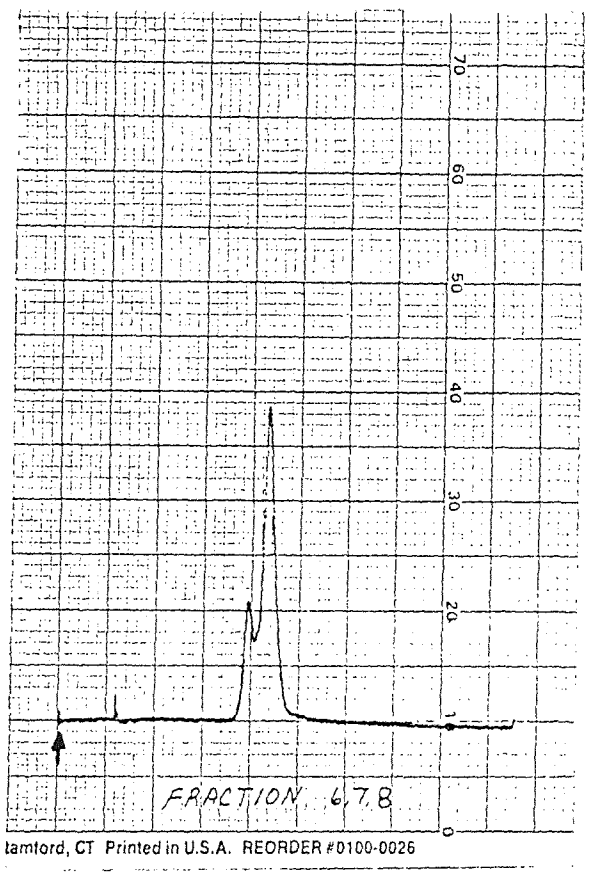


Figure 5.5 UV/VIS Comparison of bioproduct and organic product







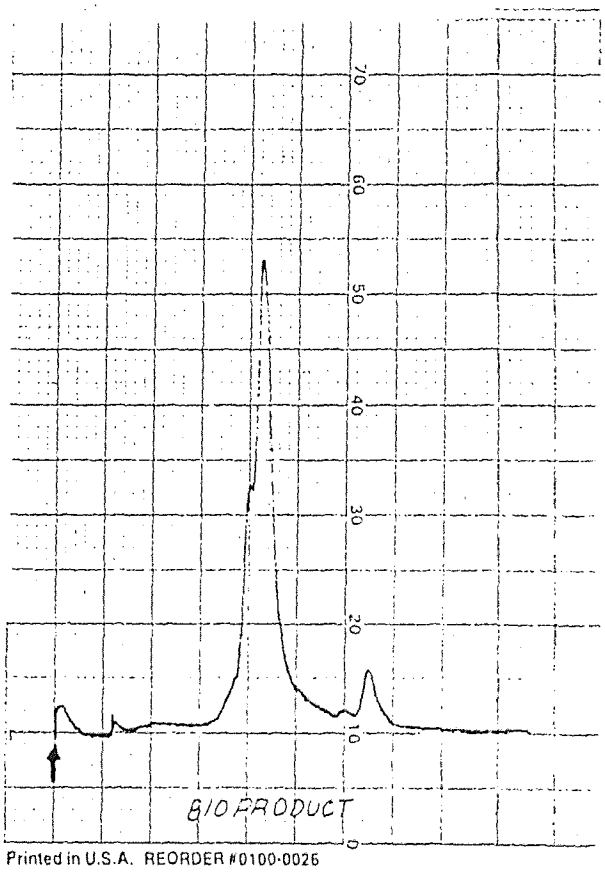


Figure 5.6 HPLC Comparison of bioproduct and organic product

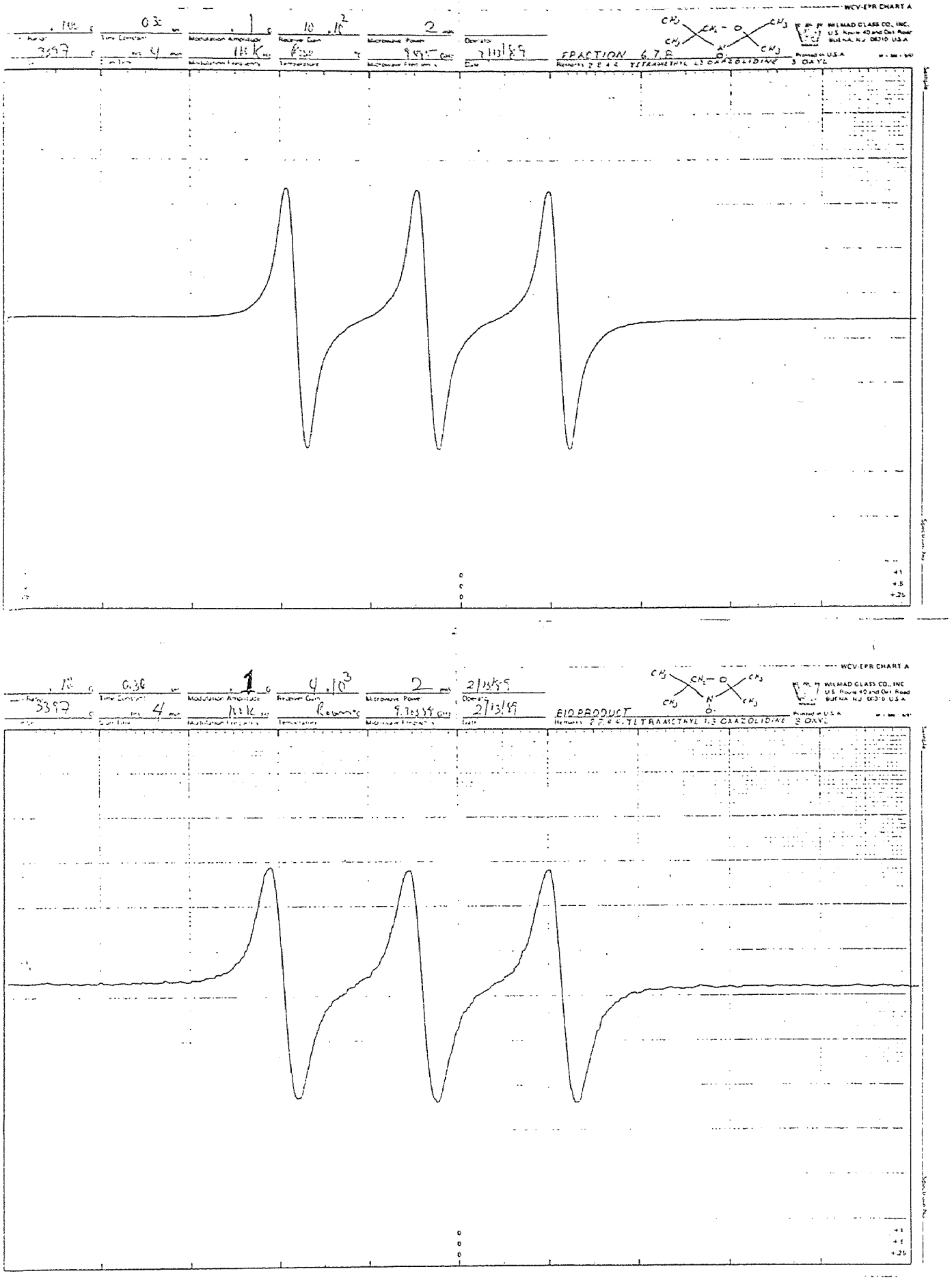


Figure 5.7 ESR Comparison of bioproduct and organic product

CHAPTER VI

CONCLUSIONS AND RECOMMENDATIONS

The study has shown that the oxygen consumption rate of immobilized microsomes increases over time due to the removal of inhibitory species. Storage in buffer or $CaCl_2$ acts as a purification process by allowing endogenous inhibitors to be removed from the microsomes. By washing the beads several time with fresh buffer, it may be possible to accelerate this process. A very convincing study would be to see the influence of the inhibitors on the activity of the soluble enzyme by reintroducing them in the reaction medium.

The cofactors $NADP^+$, G6P and coenzyme G6PDH can be immobilized with the microsomes in the alginate gel, yet there is a significant loss in the effectiveness when compared, on a weight basis, to introducing them through the reaction medium. The cofactors will diffuse out of the beads during the curing and storage time. The use of a macromolecular form of $NADP^+$ should be investigated for improved retention within the bead. Also, the possibility of other support matrices should be investigate, as well as other methods of entrapment, for a minimization of the cofactor requirement.

Alginate gel provides a uniform pore size at various

concentrations. The resistance to mass transfer by the matrix is constant over a range of 0.5-1.5 % alginate gel. The oxygen consumption rate increases linearly with enzyme loading (protein concentration). The microsomal beads will retain physical integrity at gel concentrations below 1 %. After curing the beads in $CaCl_2$ and transferring them to buffer, a constant rate (resistance to mass transfer) is obtained after 4 hours and is maintained up to 50 hours.

The recirculation flow reactor is a practical method of producing large scale runs for longer time periods. However, process parameters such as flow rate, pH and temperature should be optimized for maximum product yield.

Both the organic and the bioproducts, have the same products. At present the productivity of organic synthesis is higher than enzymatic synthesis. Without the optimizing the recirculation reactor the two can not be compared.

An interesting topic for future study would be on the ability of the enzyme to oxidize several nitrogen sites on one molecule. If this is possible the paramagnetic properties would be further enhanced.

BIBLIOGRAPHY

[1]. Feinman, L., Rubin, E. and Liber, C.. "Adaptation of the Liver to Drugs" Liver and Drugs edited by F. Orlandi and A.M. Jezequel. Academic Press, N.Y. 1972.

[2]. Cavagnaro, J., Rauckman, E. J. and Rosen, G. M.. "Estimation of FAD-Monooxygenase in Microsomal Preparations" Analytical Biochemestry. Vol 118, pp 204-211, 1981.

[3]. L. L. Poulsen and D. M. Ziegler, "The Liver Microsomal FAD-containing Monooxegenase Spectral Characterization and Kinetic Studies" The Journal of Biological Chemistry Vol. 254, No. 14, pp. 6449-6455, 1979.

[4]. Ziegler, D. M. and Pettit, F. H., "Microsomal Oxidasees. I. The Isolation and Dialkylarylamine Oxegenase Activity of Pork Liver Microsomes" Biochemistry., Vol 5, No 9, pp 2932-2938, 1966.

[5]. Ziegler, D.M. and Poulsen, L.L., "Hepatic Microsomal Mixed-Function Amine Oxidase" Methods Enzymol., Vol.52, 1978.

[6]. Polak, A., Blumenfeild, H., Wax, M., Baugn, R.L. and Whiteside, G.M.. "Enzyme Immobilization by Condensation Copolymerization into Cross-Linked Polyacrylamide Gels" J. Am. Chem. Soc. Vol 102, pp 6324-6336, 1980.

[7]. Camp, Carl E. and Sofer, Sam S.. "Calcium Alginate-

Immoblilized Hepatic Microsomes: Effect of NADPH Cofactor on Oxidation Rates" Enzyme Microb. Technol., Vol. 9, pp 685-689, 1987.

[8]. Wills, R.A., "The Glucuronidation of 7-Hydroxychlorpromazine by Insolubilized Hog Liver Microsomes", M.S. Thesis, Univ. of Okla. 1976.

[9]. Sofer, S. S.. "Hepatic Microsomal Enzymes: Potential Applications" Enzyme Microb. Technol., Vol. 1, pp. 3-8, 1979.

[10]. Theobald, W. et.al.. C. Med Pharmacol. Exp. Vol.15, p.187, 1966.

[11]. Rosen, G. M., Finkelstien, E. and Rauckman, E. J.. "A Method for the Detection of Superoxide in Biological Systems" Archives of Biochem. and Biophys. Vol. 215, No. 2, pp 367-378, 1982.

[12]. M. F. Tweedle, H.G. Brittain, W.C. Eckelman, G.T. Gaughan, J.J. Hagan, and P.W. Wedeking. "Principles of Contrast Enhancement with Paramagnetic Metal Complexes" The Squib Institute for Medical Research Runge, V.M. New England Medical Center. 1988.

[13]. Ehman, et al."Diradical Nitroxyl Spin Lable Contrast Agents for Magnetic Resonance Imaging a Comparison of Relaxation Effectiveness Investigative Radiology Vol. 21, No. 2 pp 125-131, 1986.

[14]. Griffeth, L. K., Gerald, M. D., Rosen, G. M., Rauckman,

E. J. and Drayer, B. P. "Pharmacokinetics of Nitroxide NMI Contrast Agents". Investigative Radiology Vol. 19, No. 6 pp 553-562,1984.

[15]. Afzal, et al. "Nitroxyl Spin Label Contrast Enhancers for Magnetic Resonance Imaging Studies of Acute Toxicity and Mutatgenesis" Investigative Radiology Vol. 19, No. 6 pp 549-552, 1984.

[16]. Damani, L. A., Pool, W. F., Crooks, P. A., Kaderlik, R. K. and Ziegler, D. M. "Stereoselectivity in the N-Oxidation of Nicotine Isomers by Flavin-Containing Monooxygenase" Molecular Pharmacology. Vol.33 pp 702-705, 1988.

[17]. N. B. Beaty, and D. P. Ballou, "The Oxidative Half-reaction of Liver Microsomal FAD-containing Monooxygenase" The Journal of Biological Chemestry Vol. 25, No. 9 pp. 4619-4625, 1981.

[18]. Ziegler, D. M., "Flavin-Containing Monooxegenases: Catalytic Mechanism and Substrate Specificities". Drug Metab. Rev., Marcel Decker, Inc. 1988.

[19]. D. M. Ziegler, "Molecular Basis for N-Oxidation of Sec- and Tert- Amines", Biological Oxidation of Nitrogen in Organic Molecules - Chemistry, Toxicology, and Pharmacology. (J. W. Gorrod and L. A. Damani, eds.) Ellis-Norwood, Chichester, England, 1985

[20]. P. J. Sabourin and E. Hodgson, "Haracterization of the

purified Microsomal FAD-containing Monooxygenase from Mouse and Pig Liver" Chem.-Biol. Interactions, 51, pp. 125-139 1984

[21]. Hlavica, P. and Kehl, M., "Mechanism of N-Oxide Formation by Hepatic Mixed Function Oxidases", Biochim. Biophys. Acta. 1972.

[22]. Valvis, I, "Biosynthesis of Amine Oxides Using Calcium-Alginate Immobilized Hog Liver Microsomes", M.S. Thesis, NJIT 1989.

[23]. Poulsen, L.L., Nagata, T. and Taylor, K.L.. "Lipid Stabilization of the Flavinhydroperoxide in the Microsomal Flavin-Containing Monooxegenase" Flavins and Flavoprotiens Walter de Gruyter & Co., Berlin. 1987.

[24]. Poulsen, L.L., Hyslop, R.M. and Ziegler, D.M., Biochm. Pharmacol. Vol.23, p.3431 1974.

[25]. Border et al., "3-Chloro-Tetrahydro-1,3-Oxazines or Oxazolidines" United States Patent #3,897,425, July 29, 1975.

[26]. J. J. Kaminski, N. Bodor and T. Higuchi, "N-Halo Derivatives IV: Synthesis of Low Chlorine Potential Soft N-Chloramine Sysems" Journal of Pharmaceutical Sciences Vol. 65, No. 12 1976.

[27]. Chien, N. K. and Sofer, S.,"Flow Rate and Bead Size as Critical Parameters for Immobilized-Yeast Reactors"

Enzyme Microb. Technol., Vol.7 p.538 1985.

[28]. Lakhwala, F.,"Bead Design for Biodegradation of 2-Chlorophenol using Microorganisms Entraped in Alginate Gel" M.S. Thesis, NJIT 1988.

[29]. Ford, J. R., Lambert, A. H., Cohen, W. and Chambers, R. P., "Recirculation Reactor System for Kinetic Studies of Immobilized Enzymes" Biotechnol. & Bioeng. Symp., No.3, p.267 1972.