Fall 1996

Oxidative response of immobilized bovine blood to the model pollutant phenol

Michael George Nickey
New Jersey Institute of Technology

ABSTRACT

OXIDATIVE RESPONSE OF IMMOBILIZED BOVINE BLOOD TO THE MODEL POLLUTANT PHENOL

by
Michael George Nickey

The primary purpose of this work is to determine if bovine blood can be studied in an immobilized cell bioreactor to determine if a phenol induced oxygen releasing optimum exists. This method of study has the potential to be used to determine what effects xenobiotics have on the oxidative activities of blood.

Three blood fractions containing approximately 21, 44, and 70% red blood cells (RBCs) were immobilized and placed in a recirculating bioreactor. These fractions were exposed injections of 0 ml, 3 ml, or 10 ml amounts of 2000 ppm phenol. The most active fraction was the 21% red cell control, followed by the 44% control which was similar to those of the 70% 3 and 10 ml runs. The 44% 3 ml and the 70% control runs had similar responses. Doses of 20,000 ppm decreased the activity in the 44% runs.

In the whole blood microassay runs, 0.1 ml of 2000 ppm phenol proved to be the optimum. There proved to be a correlation between the oxidative activity of the immobilized blood and the initial and overall slopes in the microassay and recirculation reactors respectively.

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by
Michael George Nickey

A Thesis
Submitted To The Faculty of
New Jersey Institute of Technology
in Partial Fulfillment of the Requirements for the Degree of
Master of Science in Environmental Science

Department of Chemical Engineering, Chemistry and
Environmental Science

January 1997

APPROVAL PAGE

OXIDATIVE RESPONSE OF IMMOBILIZED BOVINE BLOOD TO THE MODEL POLLUTANT PHENOL

Michael George Nickey

11-11-96

Dr. Samir S. Sofer, Thesis Advisor — Date
Professor of Chemical Engineering
New Jersey Institute of Technology, Newark, NJ

11-19-96

Dr. Richard Trattner, Committee Member — Date
Professor of Environmental Science
Associate Chair for Environmental Science
New Jersey Institute of Technology, Newark, NJ

12-05-96

Dr. Marcus J. Healey, Committee Member — Date
Director New Jersey Technical Assistance Program
New Jersey Institute of Technology, Newark, NJ

BIOGRAPHICAL SKETCH

Author: Michael Nickey

Degree: Master of Science

Date: January 1997

Undergraduate and Graduate Education:

- Masters of Science in Environmental Science,
  New Jersery Institute of Technology, Newark, NJ, 1997

- Bachelor of Science in Environmental Science,
  Rutgers University, New Brunswick, NJ, 1995

Major: Environmental Science

This thesis is dedicated to my family

ACKNOWLEDGMENT

The author wished to express his sincere gratitude to his advisor, Dr. Sam Sofer, for his consistent friendship, guidance, fairness, patience, and cooperation without which this research would not have been possible. I would further like to express deep thanks to Cristin McKenna, whose friendship and insights have improved both this project and my graduate experience. Much thanks are also due to Jeong Seop Shim, who has helped me generously and without fail. Also I would like to thank Tim Roche for his patience and assistance. I would also like to thank Dr. Richard Trattner and Dr. Marcus Healey for serving as members of my thesis committee.

I would of course like to thank my family for their constant moral support throughout my educational and personal endeavors. And finally I would like to offer thanks to the one through whom all things are possible, for I know that "I can do all things through Christ which strengthen me" Philipians 4:13.

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CHAPTER 1

INTRODUCTION

1.1 Oxygen Release as an Immune Response

Human neutrophils often release small amounts of superoxide anions as part of the immune response. Although these anions play an important role in the immune response, they can damage tissues in high amounts [1]. While some chemicals can have the beneficial effect of controlling the amount of superoxide anions generated, others can potentiate the release of these anions [2]. Therefore a device such as a bioreactor which can screen contaminants for their effects on superoxide generation could prove useful. This would particularly be the case for occupational health studies undertaken to determine the effects exposures to workplace chemicals have on workers.

1.2 Observation of Oxygen Releasing Events in Blood

A device that has been used extensively in the NJIT Biotech Lab to study oxygen releases in free bovine blood is the microassay reactor. This device contains the blood in an oxygen deficient environment at a constant temperature. A probe in the reactor vessel monitors the amount of dissolved oxygen in the reactor fluid. A polaragraph attached to the probe allows for a graphical representation of the oxygen consumption and release by the blood. By comparing the dissolved oxygen level of the baseline with the reaction levels it can be determined if oxygen is being released by the blood.

1.3 Immobilization

Utilizing immobilized bovine blood for the study of oxygen concentration profiles has many advantages over using free blood. One important advantage of immobilization is that due to the slower diffusion of oxygen through the alginate matrix, oxygen consumption patterns can be viewed with more detail. Another advantage is that immobilization provides protection of the blood cells from the forces of shear. Blood cells can be readily immobilized within an alginate gel matrix. This method of entrapment provides additional protection from high concentrations of pollutant, as well as from microbial contamination.

1.4 Microassay vs. Recirculation Reactors

Microassay reactors are small reactors with liquid phase reactions. They generally have reactor vessel areas equal to or less than 2 ml. The contents of the reactor vessel are mixed by a small magnetic stir bar. The vessels are usually surrounded in a water jacket that allows the reactions to occur at a constant temperature. The oxygen probe is inserted directly into a portion of the reactor vessel. When studying a reaction in a microassay reactor the vessel is sealed with a ground glass cap to provide an oxygen deficient setting.

Recirculation reactors can vary in both the size of the reservoir and the reactor vessel. Therefore the reactor can be scaled up or down to observe reactions utilizing different amounts of blood or biomass. Unlike microassay reactors, recirculating reactors are open to the environment which allows oxygenation. Operating in an environment which allows oxygenation is beneficial because it prevents the possibility that an oxygen leak will be misinterpreted for an oxygen release.

Recirculating reactors do not depend upon magnetic stirrers to create mixing of the substrate and the blood. Therefore the potential interference of magnetic fields generated by the stir plate are absent. Without a stir bar the beads are also subjected to

less physical contact with a solid object. This extends the life of the beads and the period in which they can be studied.

1.5 On-Line Oxygen Measurement

Since oxygen is a co-substrate during aerobic respiration, monitoring dissolved oxygen levels in the reactor fluid can provide patterns of oxygen use. The increase of oxygen content in either of the reactor vessels or the recriculation reactor sump would be indicative of relative oxygen release. Oxygen content in the reactor vessel is being monitored constantly for the microassay runs. The dissolved oxygen content is being measured periodically between the reactor vessel and the sump in the recirculation reactor. The continuous measurement of dissolved oxygen prevents the possibility that an oxygen release could be missed which might result with periodic sampling.

1.6 The Basic Dissolved Oxygen Patterns

1.6.1 The Microassay Reactor

Figure 1 demonstrates a typical pattern of the dissolved oxygen concentration measured over a twelve hour time period. The initial portion of the pattern which appears to have little slope is the baseline. The baseline represents the saline in the reactor, without the cap, while air is being bubbled into the solution to saturate it with oxygen.

Non-control runs had the phenol added immediately after the beads were placed in the reactor. The sudden decrease in dissolved oxygen occurs immediately after the cap is placed on the vessel. The slope of this line indicating the rate of oxygen consumption eventually decreases as the amount of available oxygen diminishes. If a release is going to occur dissolved oxygen concentrations will level off then increase. Once the release has stopped the dissolved oxygen concentration decreases again.

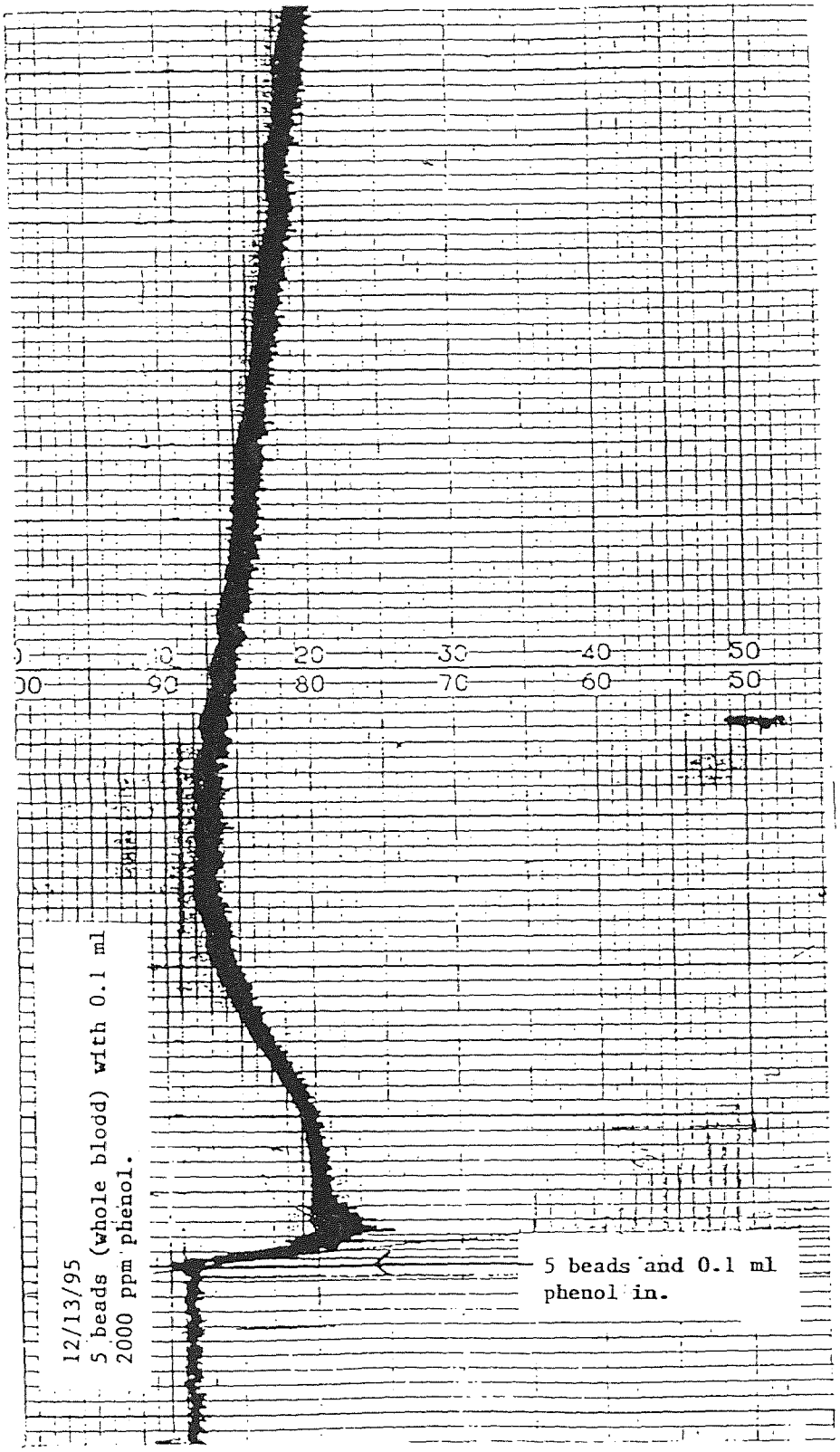

Figure 1 Example of a Typical Microassay Run

Occasionally the oxygen concentration continues to increase and levels off somewhat short of the baseline. Possible reasons for this include a weakening of the blood cells due to shear and the stress from the phenol. As the cells weaken and die their oxygen consumption decreases. Although the reactor is oxygen deficient during the reaction it is not anoxic. Therefore as oxygen comes slowly into the reactor without being consumed, the dissolved oxygen concentration increases. The pattern stops short of the baseline due to lingering consumption by the remaining viable cells.

1.6.2 The Recirculation Reactor

Figure 2 is a representation of a typical dissolved oxygen pattern measured over time in the recirculation reactor. The oscillating effect occurs because dissolved oxygen is being measured alternately between the reservoir liquid or inlet, and the sump or outlet. The initial "hill" part of the pattern preceding the "spike" is indicative of the sump or outlet of the reactor. The oxygen concentration increases in the sump during the sampling time (one half hour) due to the decreasing level of liquid. As the liquid decreases the surface area to volume ratio increases. This allows oxygen to diffuse into the liquid more easily.

The reservoir value has the spike appearance. Initially the reservoir value is high due to the higher dissolved oxygen valued in the reservoir. However when the sump is not being sampled it soon overflows into the reservoir during the period in which the reservoir is being sampled (one half hour). This liquid which comes out of the reactor has a lower dissolved oxygen content than the reservoir. This has the effect of decreasing the overall reservoir oxygen concentration.

Figure 2 Representation of a Recirculation Run Output

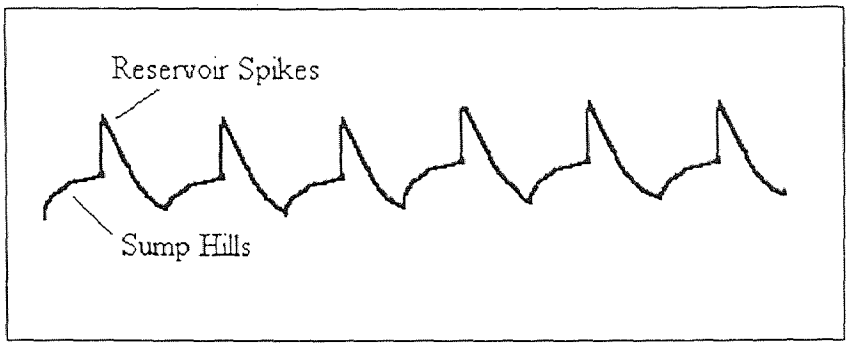

Figure 3 is a typical example of a recirculation reactor run. The initial portion of the pattern that occurs before the addition of the beads is considered the baseline. Once the beads are added the combined pattern of hills and spikes show a gradual decrease in the oxygen concentration of the reactor liquid. The pattern does not return to baseline during the time period utilized in this study due to the continued viability of the cells.

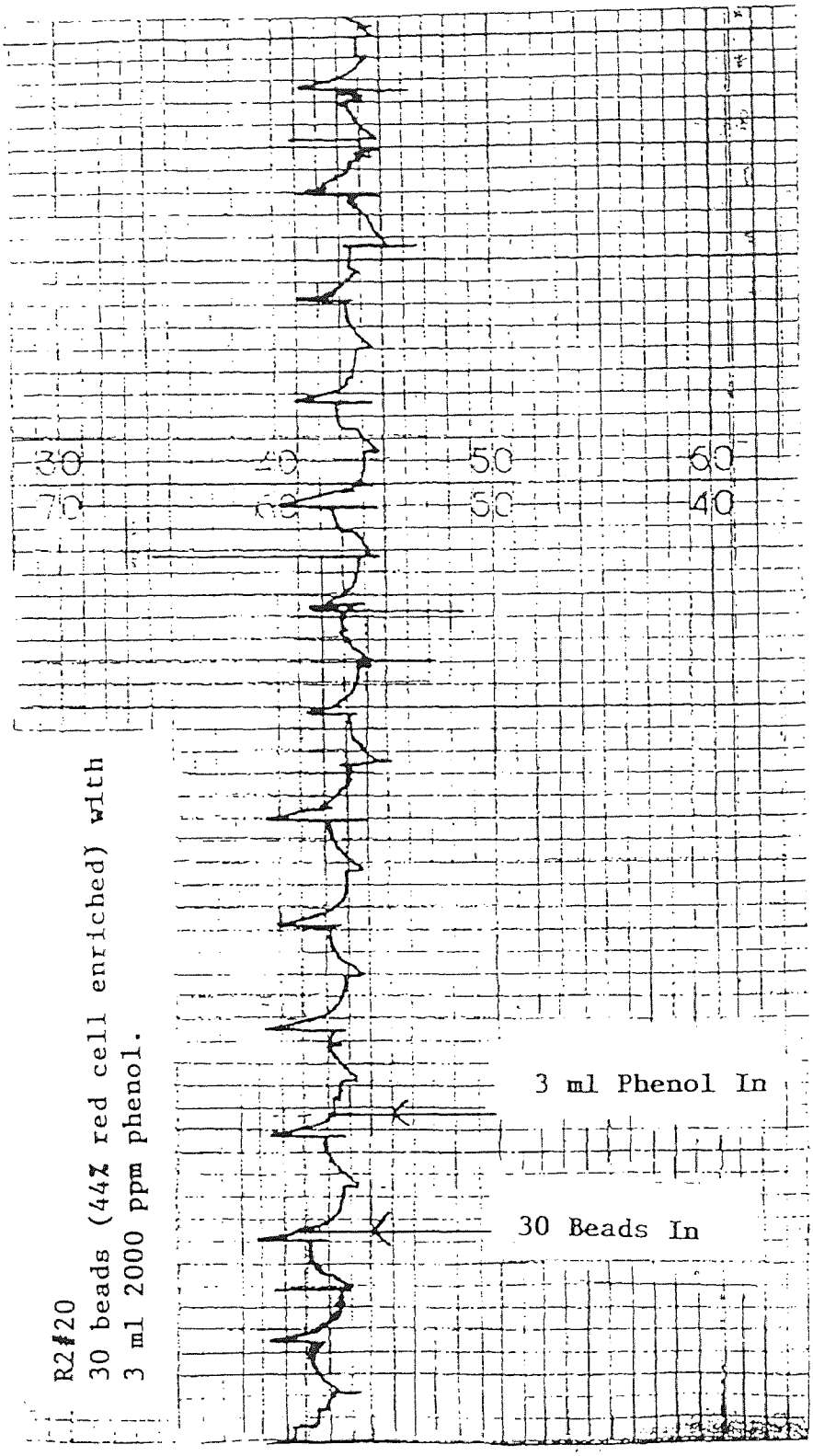

Figure 3 Example of a Typical Recirculation Run

CHAPTER 2

LITERATURE SURVEY

Simons [1] has shown that phenols inhibit the release of superoxide anion by human neutrophils, but leave the phagocytic capacity intact. This may be a means to protect the cells from damage inflicted by superoxide anions.

Scaccini [2] performed a study to determine if chemicals can potentiate a respiratory burst and a modification in low density lipoproteins. Of the chemicals tested Phorbol-12-myristate-13-acetate (PMA) had the greatest effect. PMA lead to a six fold increase in the generation of reactive oxygen species and a modification of LDL by 700%.

Heyneman [3] has shown that generation of reactive oxygen species by mature neutrophils may be of primary importance for microbial killing during the onset and recovery from mastitis in cows. Furthermore the ability of neutrophils to generate reactive oxygen species following stimulation with opsonized particles prior to infection was negatively correlated with severity of subsequently induced E. coli mastitis.

In a later study Heyneman [4] classified newly calved cows as moderate and severe responders to experimentally induced E coli mastitis based upon the reactive oxygen species (ROS) generating capacity of their blood neutrophils before infection. The two ROS groups also differed in blood and milk composition prior to infection. The initial classification was supported by the corresponding variation in clinical symptoms and in the responses by the calves to E coli. The animals with the higher capacity to generate ROS showed milder clinical symptoms. Heyneman suggests that evaluation of the ROS-generating capacity of blood neutrophils and blood and milk composition before infection might help to predict the cow's sensitivity to E coli mastitis.

Aust [5] has shown that chemical complexes such as cytochrome P450 reductase can catalyze one-electron reductions. Some reduce molecular oxygen by one electron to generate superoxide. Superoxide can cause toxicity against which superoxide dismutase is protective at certain levels. Therefore the benefits of superoxide generation can be achieved while superoxide dismutase protects the cells from the damaging effects of the oxygen radicals.

Mieyal [6] has shown that hemoglobin may have the ability to catalyze xenobiotic metabolism. Red blood cells contain many enzymes that are akin to those that catalyze xenobiotic metabolism in liver and other tissues. Hb can have the broad monooxygenase catalyst exhibiting properties of a monooxygenase enzyme. Although Hb mediated oxidase activity in erythrocytes is low relative to other sites of xenobiotic metabolism it may contribute to *in situ* activation of xenobiotics leading to increased oxygen consumption.

Chen [7] examined the ability of polymorphonuclear cells and monocytes of HIV infected individuals to generate superoxide anion (SO) and hydrogen peroxide. This study found that people who were HIV positive were defective in their ability to generate reactive oxygen intermediates when compared to non-HIV positive individuals. This deficiency may make HIV infected individuals more susceptible to infections.

Subrahmanyam [8] has shown that phenol, as a benzene metabolite, is a well studied toxicant. Benzene is a known human myletoxin and leukemogen. It has also been well established that benzene requires metabolism to phenol to induce its effects.

Lakhwala [9], of this laboratory, and others [11, 12] performed work comparing bioreactors treating phenol. One reactor used calcium alginate entrapped microorganisms and the other using free organisms. Lakhwala noted that immobilized microorganisms have great advantages over free ones. Included in these advantages are easy recovery and reuse of biomass, high biomass density, and increased biological activity due to better

mass transfer. Entrapment also has the advantage of offering more protection from high substrate concentrations.

Yang [10] of this laboratory, studied the performance of immobilized cells in the treatment of 2-chlorophenol in a batch recirculation reactor. Among his findings were that the recirculation reactor allows for the determination of an accurate operating window, while optimizing with respect to the reaction rate. He also noted that the configuration of the batch reactor enables continuos measurement of the rate of dissolved oxygen consumption.

CHAPTER 3

OBJECTIVES

The primary objective of this study has been to observe the various oxidative profiles of immobilized bovine blood fractions in a recirculation bioreactor. Additionally oxidative effects have been observed in an oxygen deficient microassay reactor. The specific objectives are:

1. To create a bioreactor capable of sustaining immobilized bovine blood fractions for a period of at least 24 hours.
2. To observe and compare the oxidative effects of phenol on three different blood fractions in the bioreactor.
3. To observe and compare the oxidative effects of different amounts of phenol on immobilized whole blood cells in a microassay setting.
4. To determine if there is a link between initial slope and overall oxidative activity in a microassay reactor.
5. To determine if there is a link between the average slope and overall oxidative activity in a recirculation reactor.
6. To provide general suggestions for the operation of the immobilized blood bioreactor based on experiments done during this study.

CHAPTER 4

MATERIALS AND EXPERIMENTAL METHODS

4.1 Bovine Blood and Immobilization

For this study, bovine blood was obtained from Green Village Packing in Green Village, New Jersey. The blood was collected from a healthy cow at the time of slaughter and mixed with an anticoagulant consisting of 250 ml distilled water, 12.1 g dextrose, 10.9 g sodium citrate anhydrous, and 3.95 g citric acid. For cell enriched beads the whole blood was spun in a bench top centrifuge before immobilization. The suspended proteins and plasma were then removed, and replaced with ISOTON® II blood group buffered saline, purchased from Curtin Matheson. The cells were then resuspended and spun once again. The saline and remaining suspended proteins were then removed. This techniques yields a red cell content in the range of 40 - 44% . This fraction was considered the primary fraction of interest.

To achieve the denser 70% cell enriched fraction the 40% fractions were spun down further for an additional twenty minutes at 2300 RPM in a specially designed centrifuge [13,14]. The bottom third of the reactor vessel had on average a concentration of 70% red cells. The 21% fractions were created using whole blood and did not involve either cell washing or the use of the centrifuge. Red cell content was verified using a standard analog microhematocrit.

The immobilization technique combines 20 ml of the individual blood fraction with a gel matrix consisting of 0.19 g alginic acid (or its sodium salt), 0.13 g NaCl, and 3.5 ml distilled water. The blood fractions and the gel were mixed thoroughly to form a fairly homogenous mixture. This mixture was extruded through a 30 ml syringe into a 0.1

Molar $CaCl_2$ solution, kept at 20 degrees Celsius. Approximately 140 beads (or 7 beads per ml of blood cells) were created with each immobilization .

4.2 Experimental Set Up

4.2.1 Microassay Reactor

Figure 4 shows a schematic of the microassay reactor configuration. The capacity of the reservoir is 1.8 ml and is surrounded by a water jacket. This water is kept at a constant 42 degrees Celsius. The saline and the blood in the reactor are mixed via a magnetic stir bar.

4.2.2 Recirculation Reactor

Figure 5 shows a schematic of the recirculation reactor configuration. The reactor vessel is held upright on a standard clamp stand. The capacity of the reservoir is 150 ml and is kept immersed in a constant temperature bath at 42 degrees centigrade. The pump recirculates the reservoir liquid into the bottom of the reactor. A small glass beaker collects the output from the outfall at the top of the reactor. This container is added to facilitate flow rate and assists in the dissolved oxygen measurements. A steady stream of oxygen is provided by the output of the liquid from the sump to the reservoir. Injections were accomplished by injecting the phenol directly into the reservoir. The reservoir was then mixed to ensure a fairly homogeneous concentration of phenol.

4.3 Dissolved Oxygen Measurements

4.3.1 Microassay Reactor

The method of monitoring the oxidative action of the blood involves the use of a dissolved oxygen probe and chart recorder. The DO probe is in direct contact with the fluid in the reactor. The output is constantly recorded on a chart recorder.

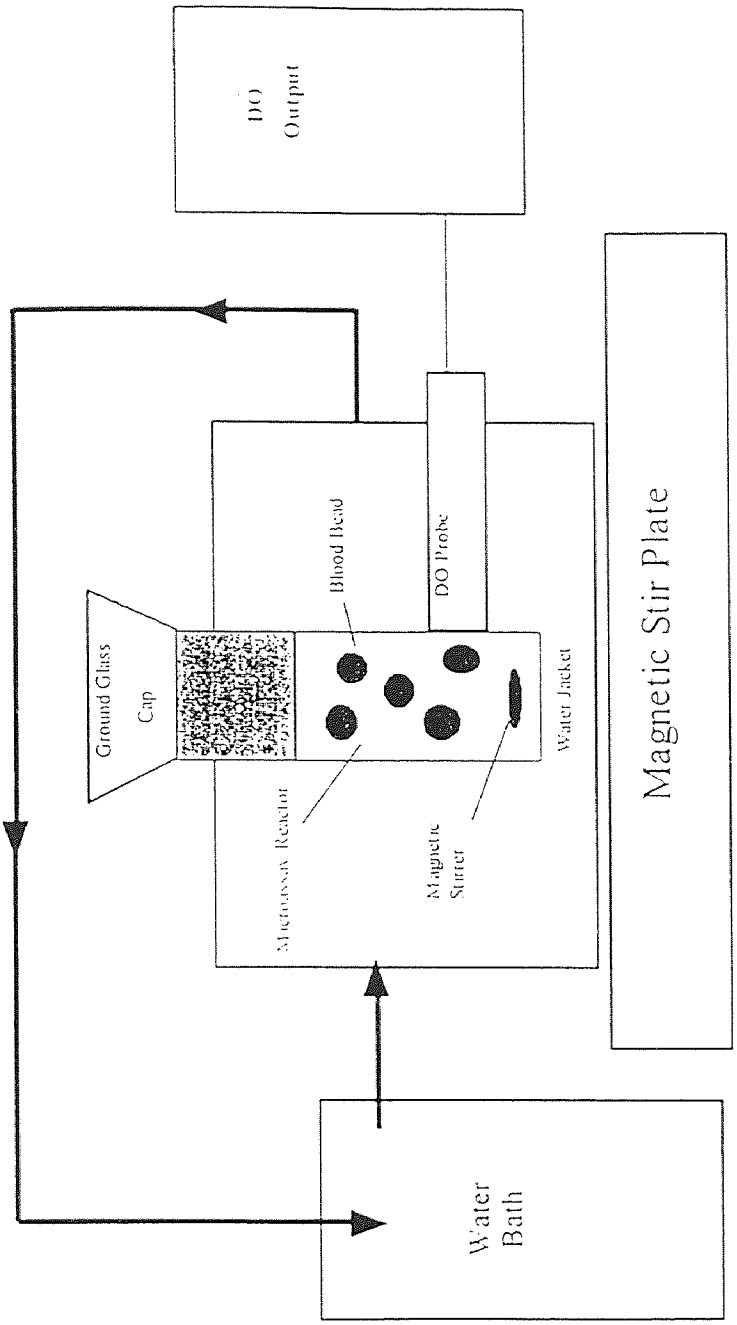

Figure 4 Microassay Reactor Configuration

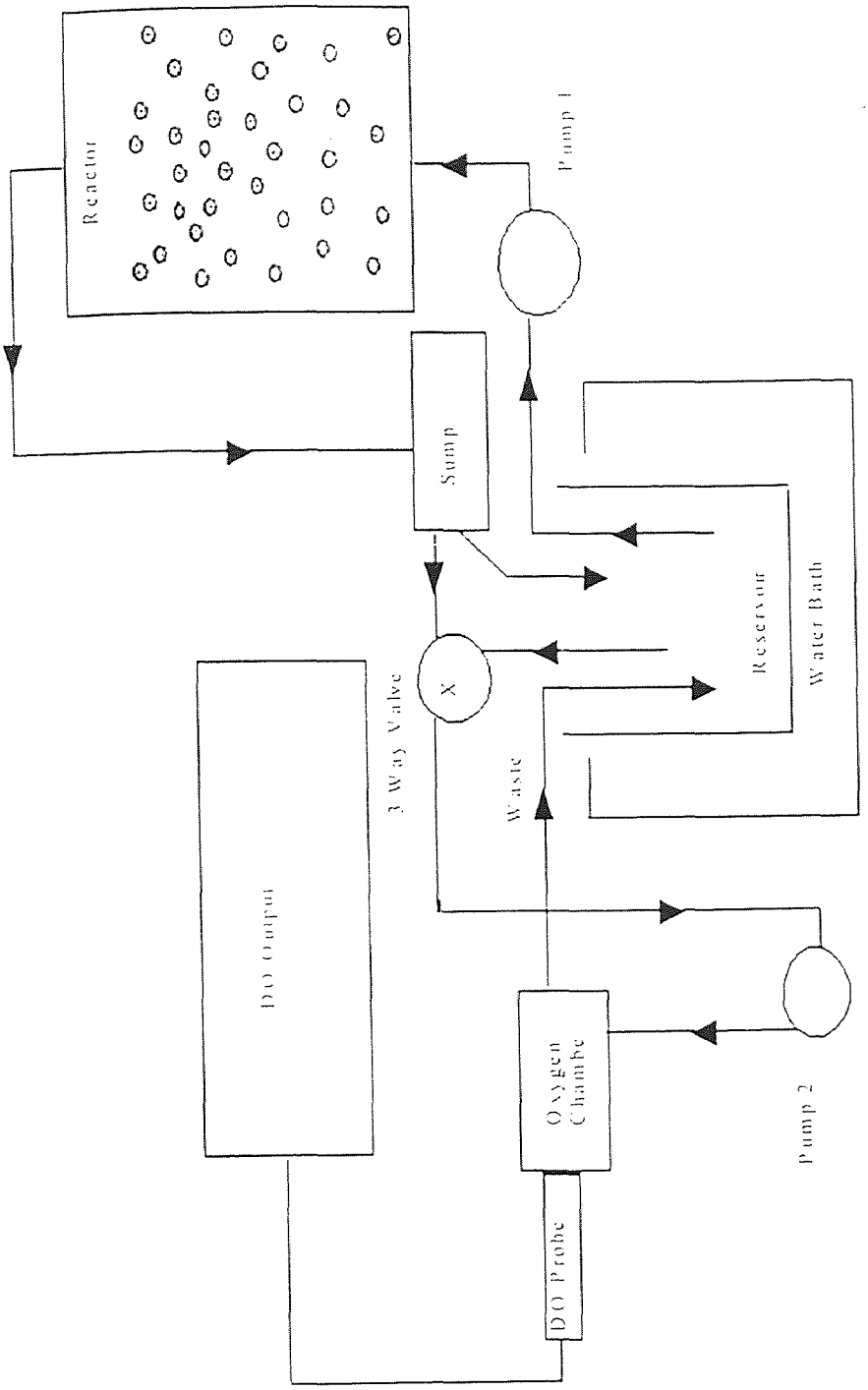

Figure 5 Recirculation Reactor Configuration

4.3.2 Recirculating Reactor

A dissolved oxygen probe and chart recorder are also used to measure dissolved oxygen concentration in the recirculating reactor. Flow across the DO probe is provided by a reactor inlet and 2) in the glass beaker or sump which collects the reactor output. A solenoid valve with a 30 minute timer switches periodically from input DO concentration to output DO concentration. The input and output measurements are constantly recorded on the chart recorder during the reaction.

4.4 Sample Calculations

The most important calculation used in this study has been the analysis of each reaction's dissolved oxygen read-out to obtain a corrected peak intensity value (Corrected Units). The following is an explanation of how each run had a Corrected Units value calculated. The basic dissolved oxygen patterns for both the microassay and recirculation reactors are discussed in the Introduction Section of this Thesis.

For both types of reactions a horizontal baseline is drawn at the point before beads are added. In the recirculation reactor the line is drawn based on the top of the "spike" of the reservoir value. In addition to the baseline, the recirculating runs also have an average oxygen concentration line drawn. This line is based on the average value of the sump and the reservoir's dissolved oxygen concentration and lies below the baseline. The actual oxygen concentration line is used in these calculations for the microassay runs. Examples of microassay and recirculation run graphs with baselines are presented in Figures 6 and 7 respectively.

A value R (Reaction Distance) is calculated at specific time intervals for both types of runs. R is the distance in centimeters from the baseline down to the average oxygen concentration line. R is calculated every hour in the recirculation runs and every half hour in the microassay runs. For each run, R is plotted vs. time. The result is

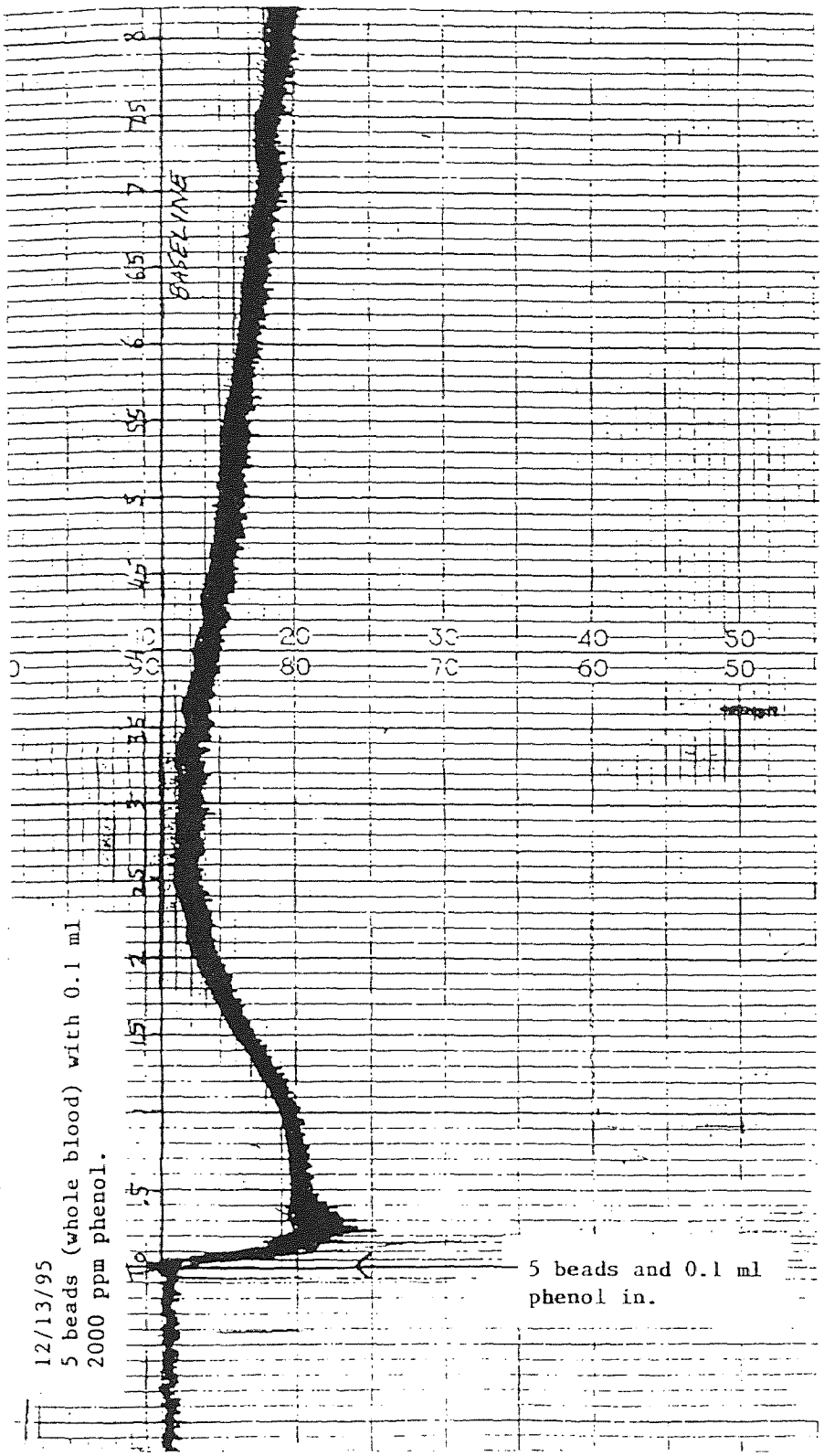

Figure 6 Example of a Microassay Reactor Run With Baselines

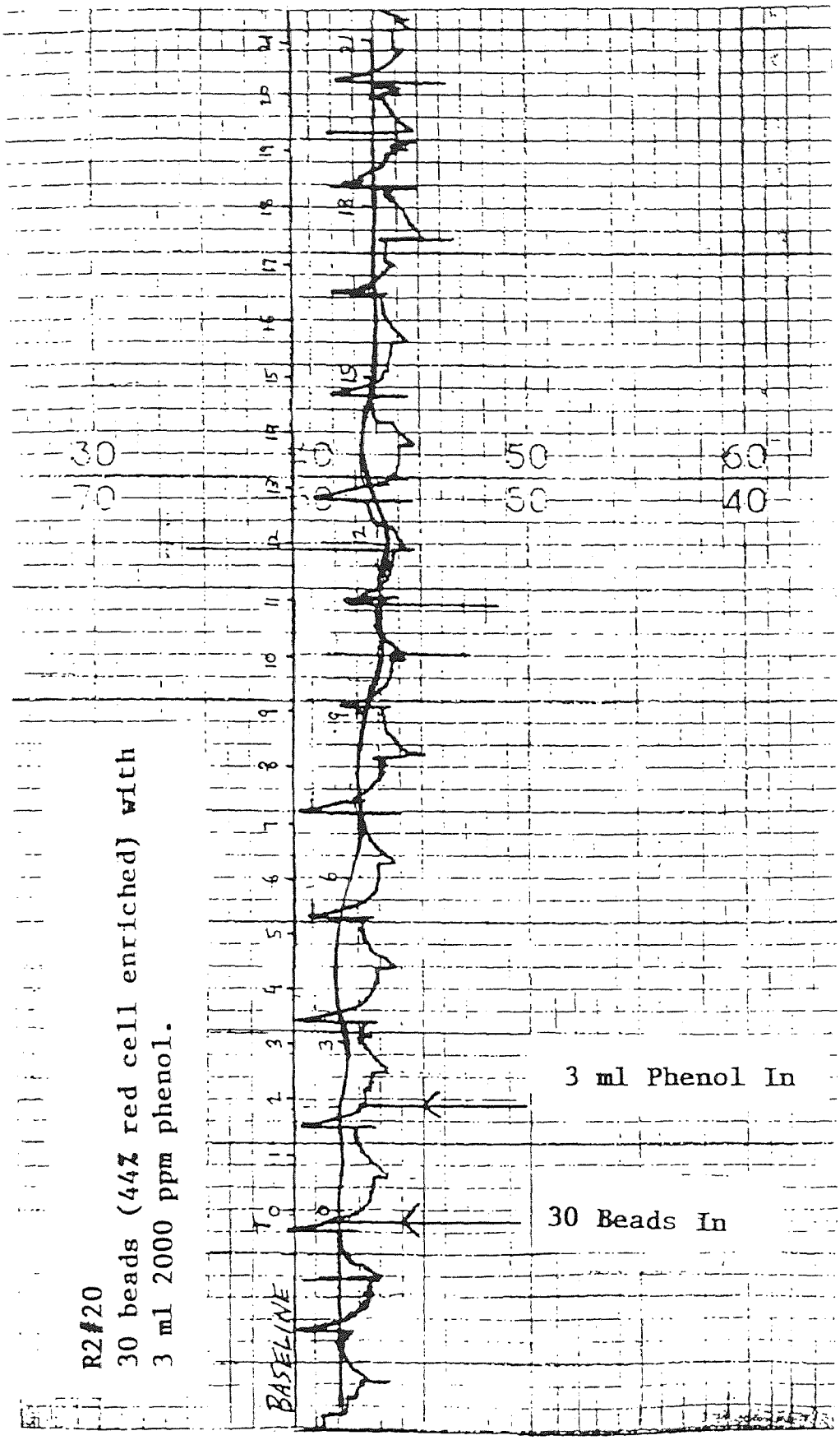

Figure 7 Example of a Recirculation Reactor Run With Baselines

linearized for both types of runs. The slope value of the regressed line is considered the average slope of the run. Examples of typical R vs. Time plots are represented in Figures 8 and 9 for microassay and recirculating reactor runs respectively.

Once the equation of the regressed line has been determined, it can be used to calculate Relative Peak Intensity Units. The slope of a line may cause certain peaks to be misrepresented due to the interference in calculating the R value. Therefore Relative Peak Intensity is used to reflect the changes in the baseline once the average slope of the line has been accounted for.

To calculate Relative Peak Intensity the equation of the linearized line is used. The time value is used as the "x" value in the equation of the line (mx + b). This quantity is then subtracted from the R value. These new values are Relative Peak Intensities (RPI). These values are multiplied by negative one and are then graphed versus time. A RPI baseline is drawn parallel to the X axis using the point of lowest oxygen concentration. Oxygen releases are represented by increases above this baseline. The highest point of each peak is used to measure the change in RPI Units. Examples of analyzed RPI graphs for microassay and recirculating runs are represented in Figures 10 and 11 respectively. Average RPI values for the recirculation runs are presented in Figure 12.

Once Relative Peak Intensity Units are calculated, the final means of measuring the intensity of the reaction can be determined. Corrected Units are the ultimate numbers used to determine the oxidative response of immobilized blood. These values are obtained by multiplying the Relative Peak Intensity Units by the time of occurrence of the highest point of the peak, and by the slope of the linear regressed line. Corrected Units reflect that the slope of the reaction is indicative of its activity. The time value is used to add weight to peaks that come later in the reaction, when the R value is highest. This enables easier qualitative comparison between peaks from different time periods.

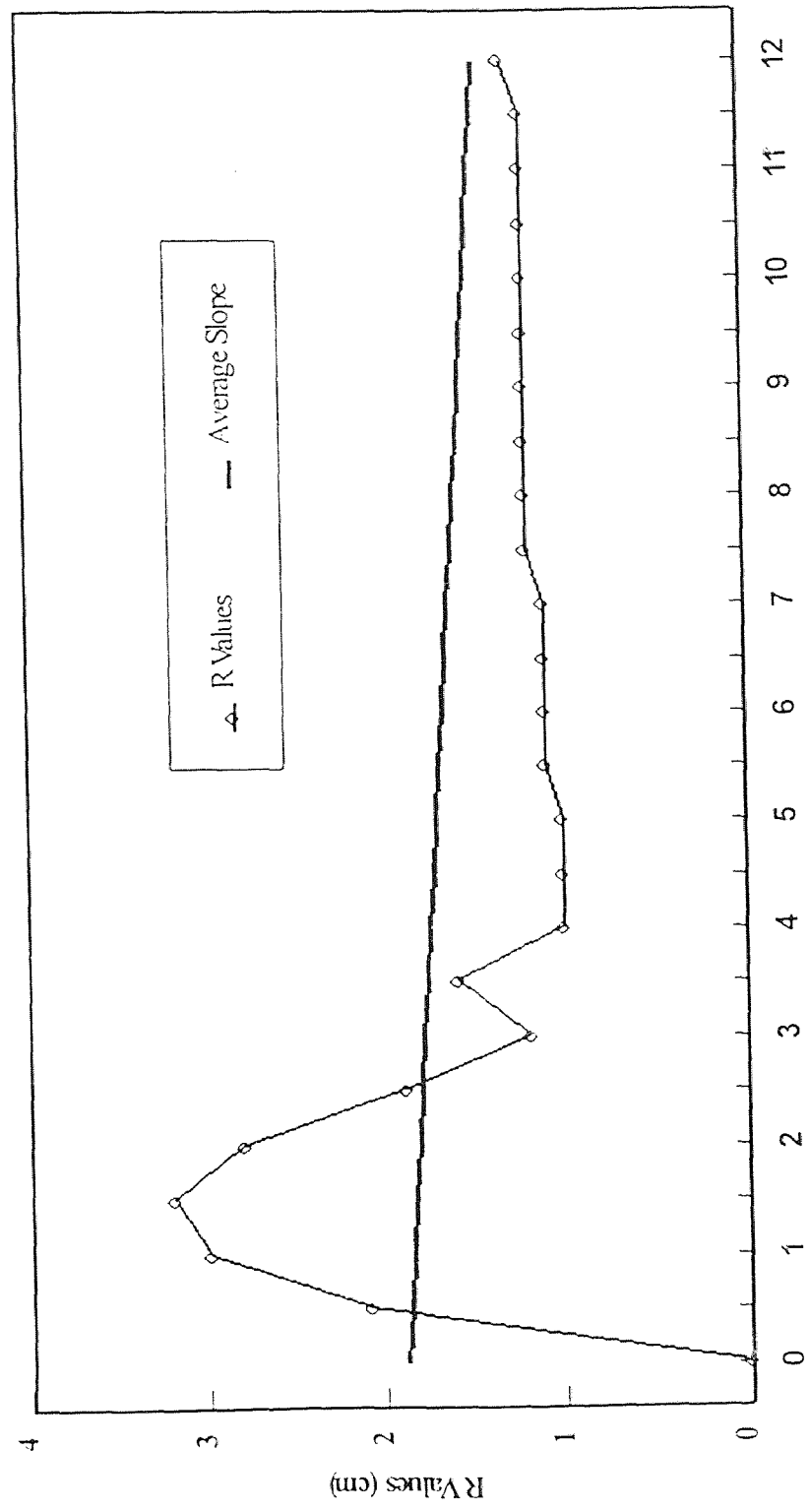

Figure 8 Example of a R vs. Time Plot for a Microassay Run

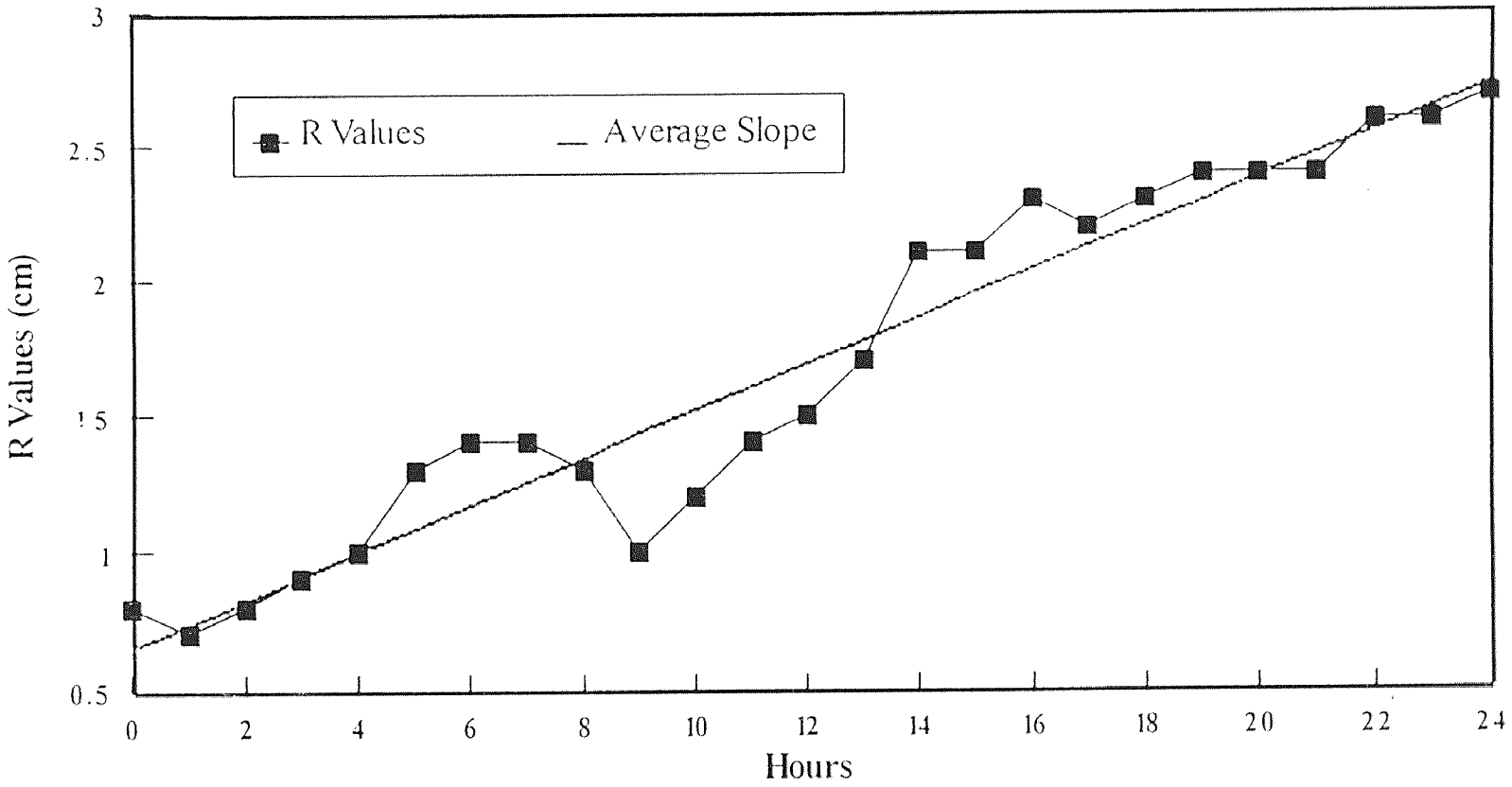

Figure 9 Example of a R vs. Time Plot for a Recirculation Run

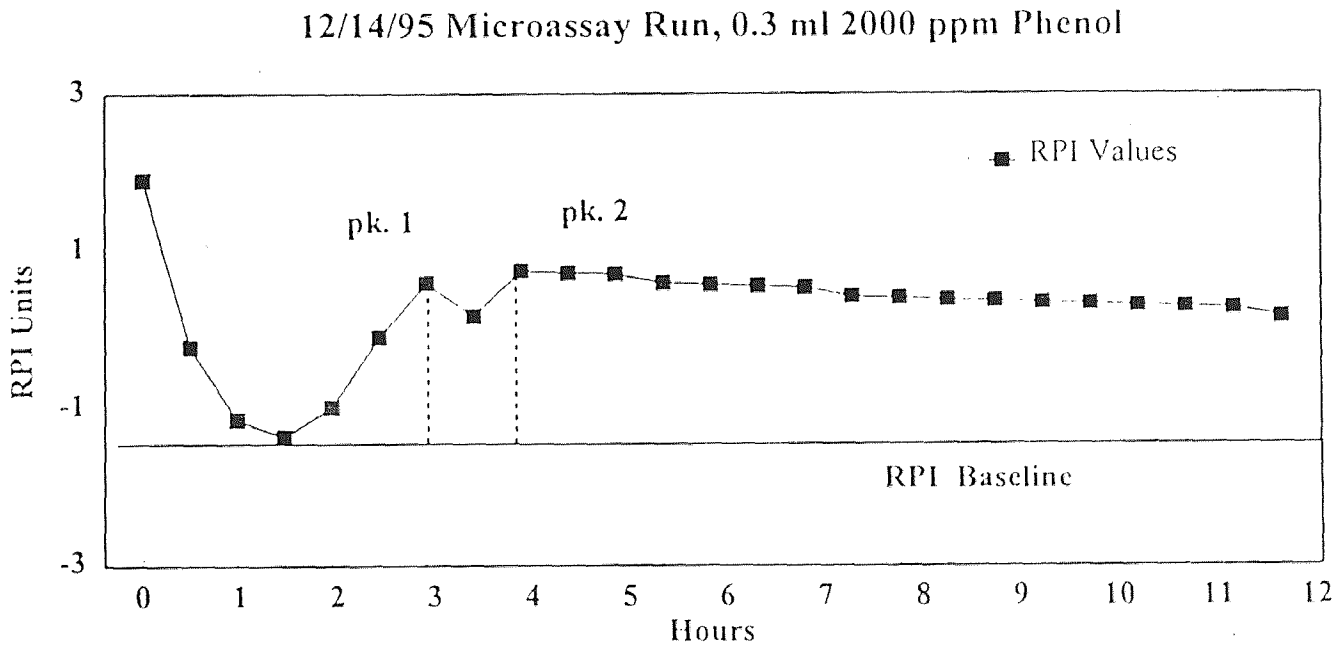

Figure 10 Example of How RPI Units Are Measured In a Microassay Reactor

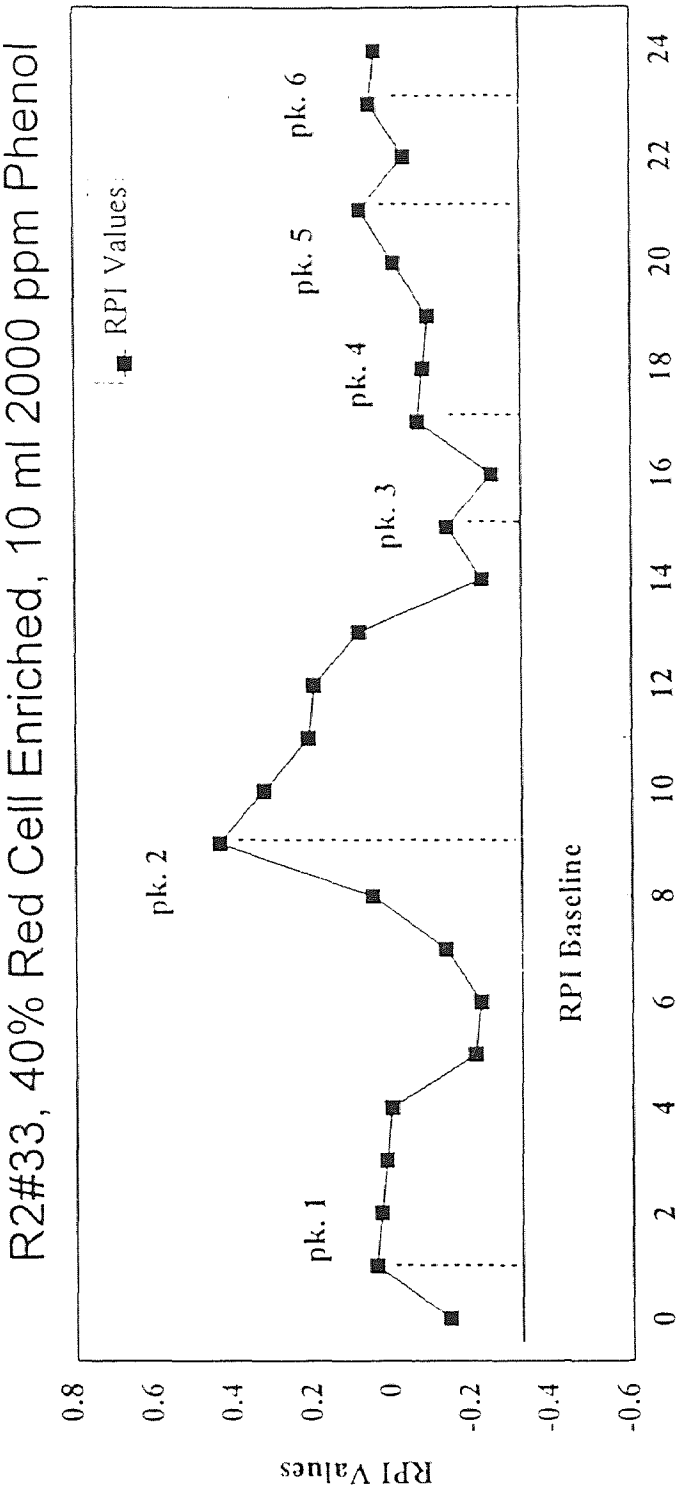

Figure 11 Example of How RPI Units Are Measured in a Recirculation Reactor

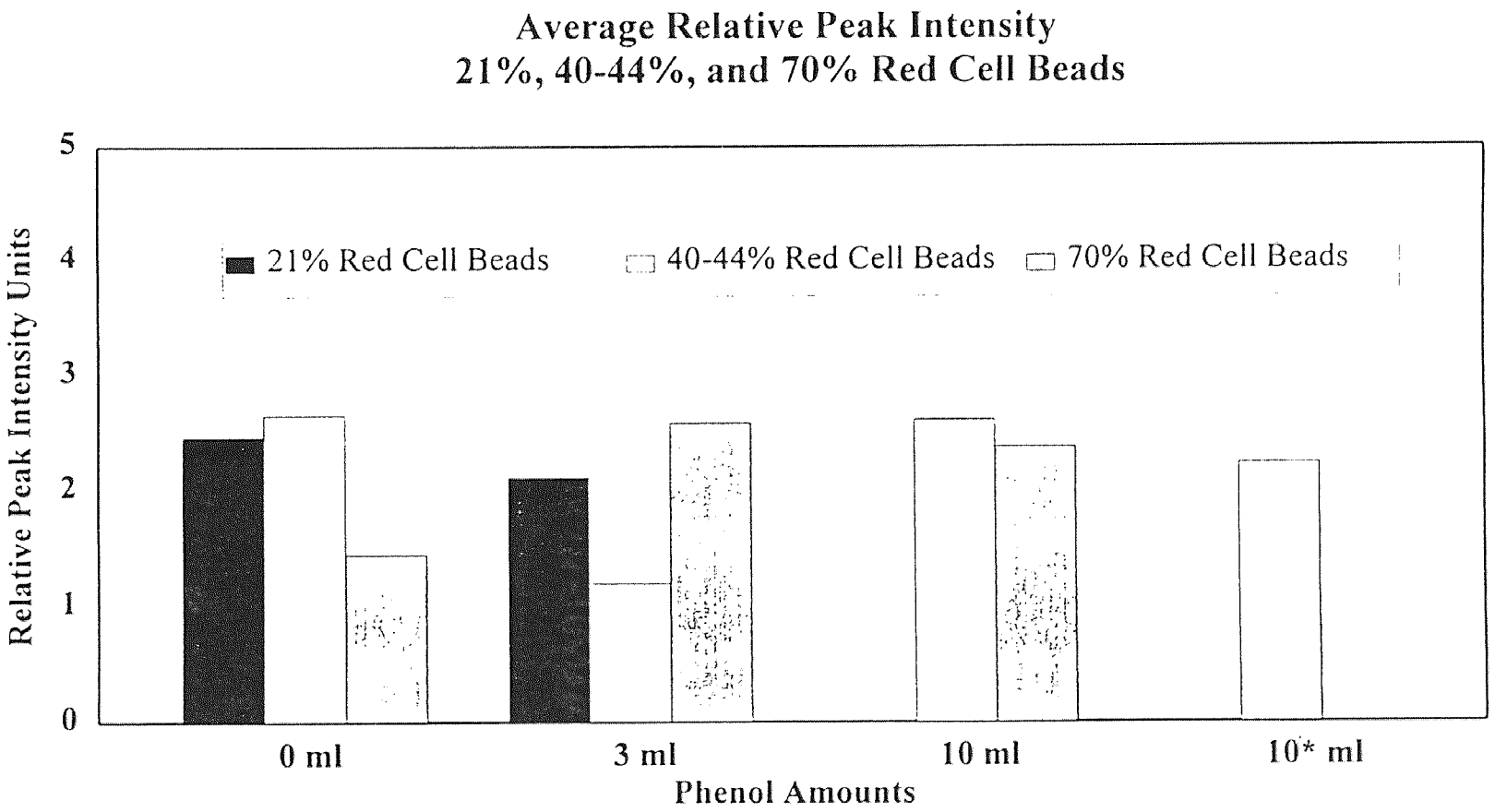

Figure 12 Average Relative Peak Intensity Units for the Recirculation Reactor

CHAPTER 5

RESULTS AND DISCUSSION

5.1 Most Active Fraction

For this study, three red cell fractions were tested for their ability to release oxygen. These fractions were 21%, 40-44%, and 70% red cells. The 21% red cell runs utilized whole blood. The 40-44% and 70% runs utilized washed cells that were centrifuged to obtain the desired red cell content. Of the three fractions the 21% red cell runs, or the whole blood runs, were most active. The second most active was the 40-44% followed by the 70%.

Figure 13 demonstrates the average activity of these runs measured in Corrected Intensity Units. Although no runs were successfully completed utilizing 10 ml phenol for the 21% red cell runs for reasons that will be discussed later in this chapter, the determination was based strictly on the control and 3 ml runs.

5.2 Effects of Phenol on Oxygen Release

5.2.1 Microassay Reactor

The runs that were injected with 0.1 ml 2000 ppm phenol, followed by those injected with 0.3 ml phenol were more active than the control runs. However the control and 0.3 ml runs had very similar corrected unit values. The 0.5 ml ranked fourth based on oxygen release. The average values of each of these types of runs is presented in Table I

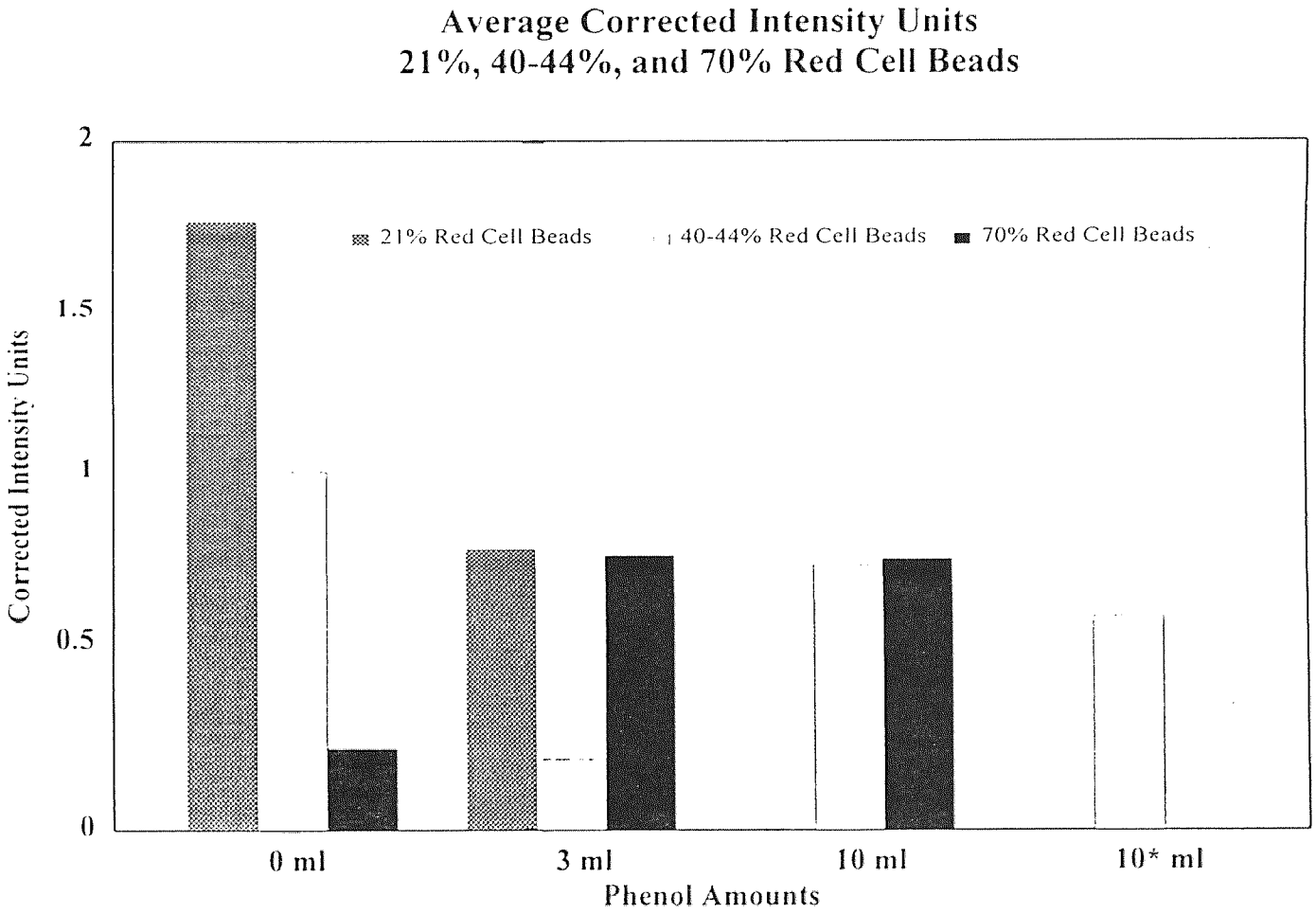

Figure 13 Average Corrected Intensity Units for Recirculation Runs

5.2.2 Recirculation Reactor

As evidenced in Figure 13 the highest amounts of oxygen release were recorded for the control runs for 21% and 40-44% respectively. Increasing phenol amounts lead to a decreasing in the amount of oxygen released in the 21% red cell runs. The opposite was noted for the 70% red cell enriched runs. Increased phenol lead to an increase in the amount of oxygen released. However, the amount of oxygen released for the 70% runs plateaued.

The 40-44% red cell enriched runs had a pattern of oxygen release different from either the 21% or 70% runs. As mentioned earlier the control runs were the highest responding fraction. The 3 ml faction however showed a sharp decrease in the response (approximately -80%). The 10 ml phenol runs showed an increase compared to the 3 ml runs. An additional phenol concentration (20,000 ppm) in the amount of 10 ml was utilized for testing the 40-44% fraction. This had the effect of reducing the amount of oxygen released compared to the 10 ml 2000 ppm runs. This may indicate that increased concentrations of phenol inhibit the amount of oxygen released.

5.3 Comparison Of Slope and Oxygen Release

5.3.1 Microassay Reactor

The microassay runs were put into groups based on the amount of phenol placed in each reactor. The average initial slopes of these groups were compared with the corresponding average corrected oxygen release numbers. Based on this information it appears that the greater the initial slope, the larger the amount of oxygen released. The initial slopes and Corrected Units are presented in Table I.

Table I Comparison of Average Initial Slope and Corrected Units

Phenol Amount	Initial Slope	Corrected Units
0 ml	6.55	1.12
0.1 ml	11.4	3.50
0.3 ml	8.94	1.17
0.5 ml	4.23	0.47

5.3.2 Recirculation Reactor

Utilizing the slope from the linear regressed line for each run, shows that there is a correlation between slope, Relative Peak Intensity, and Corrected Units as shown in Table II. The greater the slope the larger the value of the Corrected Units. This relationship however is biased by the fact that slope value is used in calculating the corrected units. However, when slope is compared to Relative Peak Intensity which is not derived using slope a similar relationship exists.

Table II Comparison of Ave. Slope, Relative Peak Intensity, and Corrected Units.

		21%			44%			70%	
	Slope	RPI	CU	Slope	RPI	CU	Slope	RPI	CU
0 ml	0.060	2.46	1.78	0.041	2.65	1.00	0.011	1.44	0.22
3 ml	0.037	2.11	0.76	0.012	1.19	0.19	0.021	2.58	0.74
10 ml	NA	NA	NA	0.039	2.61	0.71	0.024	2.38	0.73
10* ml	NA	NA	NA	0.030	2.24	0.57	NA	NA	NA

Note: 10 ml runs utilized 20,000 ppm phenol.*

5.4 Observations On The Operation of the Bioreactor

This section is intended to provide insight on the operations of a bioreactor utilizing blood for biomass. Unlike standard biomass reactors which utilize microorganisms, cleanliness is essential in the operation of a blood based bioreactor. For this study it was found to be necessary to clean the reactor for a minimum of at least one hour with a chlorine bleach/water (30:70 volume mixture) solution after each run. Additionally it was necessary to clean the reactor for at least three hours with the same mixture at least once a week. This method helped to prevent anaerobic bacteria from taking over the reactor.

Working with whole blood in the recirculation reactor proved difficult. Unwashed blood cells contain large amounts of protein. This protein has the ability to slowly diffuse through the alginate matrix. This causes the beads to clump together in a single large mass while in storage. This necessitates the need for beads to be made the day of each individual run. However beads are most stable after being in the $CaCl_2$ solution for at least twenty-four hours prior to immersion in the reactor. The fresh beads are more subject to the forces of shear and have a tendency to break, preventing a completion of the run. Washed cells also will exhibit this tendency, but not to the same extent.

The clumping together of the beads was not as much of a problem in the microassay reactor. The high turbidity of the water due to the magnetic stirrer, would soon break up any clumps of beads. In the recirculating reactor the turbidity is less. As a result the beads stay clumped together for a longer period of time. This prevents the maximum amount of bead surface area contact with the reactor fluid affecting the amount of oxygen uptake. Therefore washed cells were found to be necessary, and the 40-44% fraction was determined to be the primary assay in this study.

CHAPTER 6

CONCLUSIONS AND RECOMMENDATIONS

Of the three fractions 21% was the most active. The 40-44% cell enriched runs, and the 70% cell enriched runs followed second and third respectively. The most active run was the 21% control followed by the 40-44% cell enriched control runs. Average slope showed some correlation to the amount of oxygen released in the recirculation reactor.

Of the three phenol amounts tested in the microassay reactor the most active proved to be 0.1 ml of 2000 ppm. Initial slope proved to be correlated with the amount of oxygen released. Essentially the greater the initial slope, the greater the amount of oxygen released.

Further investigation utilizing the bioreactor set up for blood studies is warranted. Methods should be developed to enable easier handling of whole blood. Work should also be done to determine if phenol is being degraded by the blood. It would also be of interest to determine which portion of the blood is responsible for the oxygen releases and the degradation if any. Hemoglobin is a known source of oxygen storage but white cells may play a major role in the oxygen release. Work should be done to determine the actual maximum viability and average life-span of blood in this type of reactor.

Some additional research that may prove valuable would be to investigate a specific type of reaction. This reaction has been titled "enzyming out" in this study. Runs that enzyme out have very large oxygen demands. Oxygen is only released within the first few hours if at all.

APPENDIX A

MICROASSAY RUN DATA

Table III Selected Microassay Run Data

Date	Phenol Amount	Initial Slope	Corrected Units
10/24/95	0	4.8	2.49
10/26/96	0	8.3	2.04
11/02/95	0	4	1.27
11/07/95	0.1	11	5.98
12/13/95	0.1	11.7	8.02
11/10/95	0.3	5	8.41
11/23/95	0.3	6	2.77
11/29/95	0.3	3.7	2.07
12/04/95	0.3	13	1.36
12/05/95	0.3	8.3	1.03
12/06/95	0.3	13	1.87
12/11/95	0.3	9	0.17
12/14/95	0.3	13.5	1.02
11/08/95	0.4	14	1.04
10/26/95	0.5	4.7	2.12
10/27/95	0.5	3.2	0.54
11/06/95	0.5	4.8	0.13

APPENDIX B

RECIRCULATION RUN DATA

Table IV Selected Recirculation Run Data

Run#	%Red Cells	Phenol Amount	Phenol Concentration	RPI Units	Ave. Slope	Corrected Units
R2#1	21	0	NA	2.46	0.06	1.78
R2#2	21	3	2000	2.42	0.044	1.19
R2#3	21	3	2000	1.8	0.048	0.52
R2#5	21	3	2000	2.88	0.019	0.59
R2#8	44	0	NA	1.36	0.083	0.92
R2#9	70	10	2000	2.05	0.018	0.5
R2#15	0	0.5	2000	0.67	0.009	0.08
R2#16	44	0	NA	2.01	0.04	0.49
R2#18	44	0	NA	4.57	0.016	0.9
R2#19	44	10	2000	2.07	0.045	0.94
R2#20	44	3	2000	1.43	0.02	0.31
R2#21	44	3	2000	0.93	-0.003	0.03
R2#22	44	3	2000	4.31	0.081	4.9
R2#23	44	0	NA	2.59	0.054	0.92
R2#24	44	0	NA	2.75	0.049	1.45
R2#25	44	0	NA	1.49	0.052	0.75
R2#26	44	0	NA	3.19	0.041	1.43
R2#27	70	3	2000	2.58	0.021	0.74
R2#28	70	10	2000	2.51	0.035	1.06
R2#29	70	0	NA	1.44	0.012	0.22
R2#30	44	3	2000	1.22	0.02	0.22
R2#30A	70	10	2000	2.58	0.02	0.62
R2#31	40	10	2000	1.82	0.039	0.98
R2#32	40	10	2000	1.47	0.025	0.51
R2#33	40	10	2000	1.85	0.087	2.08
R2#34	44	12	2000	2.64	0.018	0.57
R2#36	40	10	2000	8.59	0.045	0.11

R2#37	40	0	NA	1.33	0.035	0.56
R2#38	40	12	2000	1.23	0.032	0.36
R2#39	49	12	2000	1.27	0.002	0.02
R2#44	44	10	20000	1.3	0.042	0.57
R2#45	44	10	20000	3.18	0.017	0.57

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