ABSTRACT

AN INVESTIGATION OF THE OXYGRAPHIC RESPONSE OF STRESSED BOVINE BLOOD

by

Cristin McKenna

Oxygraphic profiles of stressed bovine blood in a temperature controlled 2 ml reactor vessel provide the basis for an investigation of blood's response to various conditions. This response is broken down into an initial response (within 3 seconds), the response over 1 hour and the response over 24 hours. Fast-assay runs which investigate the initial response display a dose-response relationship when chemically stressed by phenol. Phenol concentrations in the 1,000 ppm range indicate a decrease in dissolved oxygen (DO) and higher phenol concentrations of over 20,000 an increase in DO. In addition to phenol concentration, other factors investigated with respect to the initial response are the effects of temperature, blood amount, DO before blood is added, blood age, blood aeration, addition of 1-butanol, and handling stress.

Long term response profiles for blood exposed to phenol or other stresses (such as decreased osmotic pressure or increased temperature); indicate the existence of oxygraphic activity in the form of molecular oxygen peaks (MOPs). A technique is presented which summarizes the activity of these long term response profiles conveniently with a single number in micrograms O_2/ml packed cells. Also, attempts to predict the appearance of MOPs based on oxygraphic profiles, and, separately, on pH, are presented.

The effect on the DO consumption rate, during the first hour of the run, of osmotic stress, blood amount, phenol concentration is also explored. The oxygraph, traditionally used by enzymologists to investigate enzyme kinetics through observation of DO, has potential applications in developing toxicity assays for xenobiotics.

Key words: bovine blood, oxygen concentration, xenobiotic, molecular oxygen peak, phenol, MOP

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by
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v

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TABLE OF CONTENTS

Chapter **Page**

TABLE OF CONTENTS
(Continued)

Chapter **Page**

TABLE OF CONTENTS
(Continued)

Chapter Page

TABLE OF CONTENTS
(Continued)

Chapter Page

TABLE OF CONTENTS

(Continued)

LIST OF TABLES

LIST OF FIGURES

LIST OF FIGURES
(Continued)

Figure **Page**

LIST OF FIGURES
(Continued)

Figure Page

LIST OF FIGURES
(Continued)

Figure Page

CHAPTER 1

INTRODUCTION

1.1 Objective

Elucidating the interaction between the immune system and its environment is an exciting and challenging area of environmental science. This research delves into that portion of this interaction which involves oxygen, blood, and phenol. The objectives of this work center around the use of an oxygraph to observe bovine blood *in vitro* under various conditions. The primary focus of this work is on blood stressed by a selected toxicant, phenol. Other stressful conditions that were observed include temperature and osmotic stress. These oxygraph observations were conducted in two time frames: fast assay runs (under 5 seconds), and long term runs (5 hours and up). The primary work utilizing the oxygraph was buttressed by additional work including observation of pH in the reactor concomitantly with oxygraph measurements.

1.2 A Brief Overview of Oxygen's Role in Mammals

Because Environmental Science brings many disciplines to bear on the areas it encompasses, a brief introduction to pertinent biological aspects of this problem is presented. This is intended to allow scientists without a strong biological background to comprehend the nuances of the work presented herein.

The sometimes extreme specialization required to become adept in any scientific discipline can cause researchers to become cut off from all but a small circle of like-

minded individuals. In the general interest of scientific sharing and this researcher's personal goal to always eschew obfuscation, a "quick and dirty" dash through the halls of biology is presented.

As the reader will see, oxygen's essential role in vastly different capacities of mammalian life make it a molecule of variable service. In mammals, blood is pumped by the heart to the capillaries via arteries (large blood vessels) and returns from the capillaries via veins (which are also large blood vessels). In the capillaries, which are a complex network of very thin walled blood vessels, gas exchange takes place. The primary purpose of this blood circulation is to supply all the cells of the body with oxygen and to remove carbon dioxide and other waste products from the cells. Besides pumping blood to all areas of the body, the heart also pumps blood separately to the lungs where carbon dioxide is released to the environment and oxygen is taken into the body through the alveoli.

The hearts of mammals are separated into two atria and two ventricles. The heart's exquisite structure ensures that blood coming from the lungs which is oxygenated (has relatively large amounts of oxygen and small amounts of carbon dioxide) is sent to the rest of the body without mixing with the deoxygenated blood which is sent to the lungs to get rid of the relatively large amounts of carbon dioxide it carries and replenish its oxygen supply. As the reader may already have suspected, the practical upshot of all this is that mammals breathe in oxygen and breathe out carbon dioxide. This process on a much smaller, namely single cell, level is referred to as cellular respiration.

1.2.1 Cellular Respiration

Hamlet: Your worm is your only emperor for diet: we fat all creatures else to fat us, and we fat ourselves for maggots: your fat king and your lean beggar is but variable service, two dishes, but to one table: that's the end.

King: Alas, alas!

Hamlet: A man may fish with the worm that hath eat of a king, and eat of the fish that hath fed of that worm.

(Shakespeare, Act IV, Sc. II, 20-30)

And what is that "one table"? A worm's gut. Just as food cycles through various creatures, so does oxygen. One could consider the "one table" to be oxidation. It is no use to eat food for energy without a way to release the energy contained in the chemical bonds in food. "The stepwise release of energy from food molecules, accompanied by storage of the energy in short-lived energy intermediates (Arms and Camp 1987, 1116)," is cellular respiration. Because of oxygen's essential role in aerobic cellular respiration, supplying all cells of the body with oxygen is requisite for mammalian life.

1.2.2 Hemoglobin

The oxygen needed for cellular respiration is carried by hemoglobin. Hemoglobin is contained in the blood cells known as erythrocytes (the cells formerly known as red blood cells). Hemoglobin is a protein molecule composed of four polypeptide chain subunits which can each bind one molecule of oxygen.

Hemoglobin's four subunits undergo cooperative binding of oxygen. This means that once one molecule is bound by one subunit, it becomes easier for the next oxygen

molecule to bind. This cooperative binding affects all four subunits and leads the hemoglobin's characteristic sigmoidal shape of the oxygen binding curve seen in Figure 1.1 in Appendix A. (Campbell 1991, 187). The physiological implication of this sigmoidal curve is that hemoglobin becomes nearly fully saturated when the oxygen concentration in the surrounding medium is high (such as in the lungs) but releases oxygen easily when the oxygen concentration in the surrounding medium is low (such as the capillaries) (Matthews and van Holde 1990, p223).

One of hemoglobin's many fascinating facets which warrants exposition in this work is the Bohr effect. Hemoglobin's cooperative binding behavior help it perform its essential role in supplying the body with oxygen while removing carbon dioxide. When the body is expending a lot of energy in heavy exercise, the muscles can undergo fermentation to supply more energy than would otherwise be available. Under these conditions of heavy exercise, lactic acid, a byproduct of fermentation, can build up in the muscles. This lactic acid lowers the pH in the surrounding area. This lower pH decreases hemoglobin's affinity for oxygen and thus increases the amount of oxygen released to this area. This phenomenon is known as the Bohr effect and can be see in Figure 1.1 in Appendix 1. Note that the sigmoidal curve is intact at all pH levels. (Matthews and van Holde 1991, 231).

1.2.3 Immune Response

All animals have defense mechanisms that protect them from disease. Immune responses have evolved to help protect animals from pathogenic agents such as bacteria, viruses, eukaryotic (non-bacterial) invaders, and exogenous chemicals. Defenses against the last pathogenic agent, exogenous chemicals, help the body cope, for example, with chemicals introduced via food, such as chemicals found in plants.

An inevitable byproduct of civilized life is anthropomorphic production of substantial quantities of chemicals. Some of these chemicals occur naturally while others are entirely synthetic. These chemicals can and do enter the biosphere intentionally, such as chlorine in water or medicine prescribed by a doctor, or unintentionally, such as industrial and domestic releases in leaks and spills. Both types of chemicals can elicit an immune response in organisms. Exogenous substances enter organisms through three primary routes: inhalation, ingestion, and dermal absorption (absorption through the skin). As a result, the immune system honed by countless generations of evolution is facing challenges that it may or may not be able to overcome.

The primary portion of the immune response which involves oxygen is production, from dissolved oxygen, of radical oxygen species which are used to destroy pathogenic substances. There are oxygen-dependent killing mechanisms in blood which utilize oxidizers such as hydrogen peroxide to attack invading bacteria. These bacteria are attacked and ingested by certain leukocytes (formerly known as white blood cells), particularly macrophages and granulocytes in a process called phagocytosis. Once inside the leukocyte, the bacteria are destroyed. This phenomenon is covered in greater detail in Section 2.1.

CHAPTER 2

LITERATURE REVIEW

There are many studies which examine oxygen in blood but the vast majority of these studies focus on erythrocytes which carry oxygen to all the body's cells using hemoglobin to carry the oxygen. The interaction between oxygen and leukocytes is in comparison little studied, yet is of great interest to this research due to the possibility of an immune response influence in the oxygraphic profiles. The experiments presented in this work are completely unaddressed in the literature as they represent a truly novel approach to examining blood. This literature survey presents several areas of investigation hopefully of interest to any reader interested in this research.

2.1 Respiratory Burst

In a respiratory burst by activated macrophages, O_2 is catalyzed to superoxide anion. The superoxide anion generates other powerful oxidizing agents including hydroxyl radicals and singlet oxygen (Kuby 1994). These chemical species have potent antimicrobial properties (Rautelin and von-Bonsdorff *et al.*, 1994, 667). Excess hydrogen peroxide is destroyed within the organism by catalase or by reduced glutathione (Erslev 1985).

During phagocytosis, a respiratory burst takes place in activated macrophages (Sanguinetti 1992, 20). In a respiratory burst, molecular oxygen is consumed as superoxide and hydrogen peroxide are generated, along with other powerful oxidizing

agents including hydroxyl radicals and singlet oxygen (Kuby 1994,). Polymorphonuclear leukocytes have the ability to liberate large amounts of reactive oxygen species.

The biochemical basis for the respiratory burst is the activation of an enzyme which catalyzes the one electron reduction of oxygen to O_2^- oxidizing NADPH (nicotinamide-adenine dinucleotide phosphate) to $NADP^+$ (nicotinamide adenine dinucleotide phosphate). This accounts for the consumption of oxygen that occurs during the respiratory burst. Also during the respiratory burst, one molecule of oxygen and one molecule of hydrogen peroxide are produced by the reaction of O_2^- with itself (Babior 1984, 959).

These chemical species have potent antimicrobial properties. Production of Reactive Oxygen Intermediates (ROI) by the NADPH oxidase of neutrophils is a major mechanism of bacterial killing and, in pathologic circumstances, tissue damage (Waddell *et al.* 1994, 18485). Not all ROIs are released at once. In one study which examined different methods of provoking ROI release, H_2O_2 (hydrogen peroxide) was produced without concomitant O_2^- release. Excess hydrogen peroxide is disposed of within the organism by catalase or by reduced glutathione (Erslev 1985). NADPH oxidase is the oxidative burst enzyme (Sozzani *et al.* 1994, 3895). The initiation of oxidants and maximal rate of their generation can initiate without protein kinase C and phospholipase D but these substances are required to sustain oxidase activity (Watson *et al.* 1994, 91).

In studies of the respiratory burst, time and dose dependent stimulation of superoxide anion production was induced through addition of beta-naphthylamine (Ciarn 1992, 1841). This and other studies of respiratory burst indicate immune systems reacting to molecules with a mechanism previously shown only in response to bacteria. The

respiratory burst of phagocytes is not fully understood. Many of the body's immunological defenses respond especially well to proteinaceous invaders (Kuby 1994) but the respiratory burst can be triggered in response to many nonproteinaceous substances such as methotrexate, an anti-cancer drug (Gresader *et al.* 1994, 679). Other respiratory burst stimulators include heat-inactivated Haemophilus influenzae type b, and phorbol myristate acetate (Wolf *et al.* 1994, 235).

Neutrophils can be primed to increase the percentage of cells generating an oxidative burst in response to subsequent stimulation (Daniels *et al.* 1994, 465). Several types of cells can undergo the respiratory burst and these cells do not always respond in tandem. An illustrative example of this phenomenon is a study by Turyna *et al.* (1994) indicates that turpentine and thioglycollate cause a decrease and lipopolysaccharide an increase in respiratory burst activity of rat alveolar macrophages while these three compounds all cause an increase in respiratory burst activity in rat peritoneal macrophages. This is not to suggest that the respiratory burst never occurs in tandem amongst a cell population. In a novel study using neutrophils from pig blood, it was found that neutrophils stimulated to undergo respiratory burst can activate a second, chemically separated, but optically coupled population of neutrophils (Shen *et al.* 1994, 963).

In one study, native and synthetic collagen-like polypeptides stimulated the release of superoxide anion and hydrogen peroxide from alveolar macrophages. These effects were reported to be dose and time dependent, reaching a maximum after 72 hours (Laskin 1994, 58). However, these findings relate only to the respiratory burst which is an uptake of oxygen, as opposed to the oxygen release which is the subject of this report.

H_2O_2- forming oxidase activities include glucose, xanthine, fatty acyl, fatty-acyl CoA (Coenzyme A) oxidases, and NAD(P)H-O_2 oxidoreductase activity (Turner *et al.* 1985, 13163). As noted in a review article by Hellstrand *et al.* (1994, 4940), catalase is a scavenger of H_2O_2, SOD scavenges superoxide anion, mannitol and deferoxamine scavenge hydroxyl radicals, taurin scavenges hypochlorous acid, and NG- monomethyl-L-arginine (L-NMMA) inhibits nitric oxide synthetase.

2.1.1 Measurement of the Respiratory Burst

Zueller and Sullivan report that although luminol-CL (chemiluminescence) is a sensitive measure of phagocyte respiratory burst activity, this method is limited because the nature of oxidation products contributing to the light emission and the their site of generation remain incompletely defined. To describe more precisely the oxidative burst of monocytes and neutrophils, Zueller and Sullivan measured superoxide anion release by cytochrome c reduction. In addition they distinguished the extracellular release of hydrogen peroxide from hydrogen peroxide generation by using a phenol red oxidation. For an index of intracellular peroxide production, a flow cytometric determination of dichlorofluorescein oxidation was used. Measurement of hydrogen peroxide production by haemocytes can be quantified by using horseradish peroxidase-dependent oxidation of phenol red while superoxide anion generation can by quantified with reduction of nitroblue tetrazolium. (Pipe 1992, 111) Generation of superoxide radicals can be detected by reduction of ferricytochrome c and spin trapping (Shen *et al.*1994, 963). Oxidative burst reactions can be measured by chemiluminescence (Rautelin *et al.* 1994, 667).

2.2 Respiratory Burst and the Environment

Exogenous insult of free radicals to the respiratory tract may derive from polluting environmental agents, cigarette smoke, drugs, and toxic compounds (Sanguinetti 1992, 20). The types of exogenous substances which can trigger the respiratory burst is very wide and includes many types of bacteria, fungi, unicellular parasites and metazoa (larval stages of schistosomes). The respiratory burst can be unleashed upon both phagocytized particles (such as bacteria) and targets too large to be ingested (such as a fungal hypha). In either case, the destructive power of the radical oxygen species released during the respiratory burst can take their toll on both the target and the attacker (Babior 1984, 961).

It is known that excessive production of reactive oxygen species by alveolar macrophages in response to inhaled toxic substances is a major cause of oxidative lung injury (Harrison *et al.* 1994, 1110). It has been demonstrated by Grzybowski *et al.* (1994, 18) that patients with atopic asthma had a defect in skin granulocyte migration. This study concluded that the ability of granulocytes from skin infiltrations to migrate was inversely proportional to the degree of oxidative burst in patients with atopic asthma. The respiratory burst can be stimulated by dialysis membranes which has led to examination of reactive oxygen intermediate production by human neutrophils with different dialysis membranes as an early marker for biocompatibility (Rosencranz *et al.* 1994, 300). The neutrophil oxidative burst response as measured by the reduction of ferricytochrome-c by superoxide or the oxidation of dihydrorhodamine 123 by H_2O_2 is recommended by Lemke *et al.* (1994, 104) to study the effects of biocompatibility on neutrophil function in haemodialysis patients during extracorporeal circulation.

The microassay recorder used in this dissertation is more cost effective than either of these methods and it is hoped that the work done here will lay the foundation towards an extracorporeal immune assist device.

2.3 Inhibitors and Stimulators of the Respiratory Burst

There is much interest in dampening the respiratory burst (Gressder *et al.* 1994, 679; Courreges *et al.* 1994, 135; Rosenkranz *et al.* 1994, 300; Rabe *et al.* 1994, 166) because increased production of reactive oxygen intermediates following medication administration (Wolf *et al.* 1994, 235) can be damaging to tissues (Waddell *et al.*1994 18485). The potential for stimulated microglia to generate oxygen free radicals may have implications in several degenerative neurological diseases (Klegeris and McGeer 1994, 83). Klegeris and McGeer compared adult with newborn rat macrophages and microglia. They lysed the cells with deoxycholate or disrupted them with sonication then measured oxygen consumption in the microglia. This implies that the stress of the stir bar in the microassay reactor may not prevent the reactions we are trying to study .

Apoxynin (4-hydroxy-3-methoxy-acetophenone) acts as an inhibitor of the respiratory burst and has been administered orally to rats as an anti-inflammatory agent ('t Hart and Simons 19992, 119). Administration of native antioxidant enzymes such as catalase and superoxide dismutase have been suggested to protect the lungs from oxidative injury (Harrison *et al.* 1994, 1110). Immunoglobulin A, a respiratory burst inhibitor, acts in a dose-dependent manner (Wolf *et al.* 1994, 235).

Melia azedarach leaf extract's effect on the respiratory burst and phagocytic capability of mouse peritoneal exudate cells was examined by Courreges *et al.* (1994 135)

Inhibition of phagocytosis was observed to be time and dose dependent; this inhibition reverted 48 hours after removing the extract from the culture medium. Other compounds which dose-dependently inhibit superoxide anion generation include sulfonated shale oil fractions (Rabe *et al.* 1994, 166). Supplemental zinc blocked the exercise-induced increase in reactive oxygen species in a study of five male runners (Singh *et al.* 1994, 2298) The synthetic opoid peptide methionine enkephalinamide is reported to be an extremely potent inhibitor of the respiratory burst of neutrophils (Rickinger *et al.* 1994, 118).

Azelastine hydrochloride suppresses neutrophil respiratory burst both *in vivo* and *in vitro* but neutrophil superoxide dismutase activity is negligibly suppressed by Azelastine (Osaki *et al.* 1994, 331). This is just one example of the separation of reactive oxygen species production from respiratory burst activity.

Despite the potentially damaging effects of overstimulation of the respiratory burst, the fact remains that the respiratory burst and production of reactive oxygen intermediates (ROIs) have evolved as defense mechanisms (Adema and van Deutekom-Mulder *et al.* 1993, 379; Passwell and Shor *et al.* 1994, 277; and Rautelin and von-Bonsdorff *et al.* 1994, 667) and as such, their selective stimulation may be regarded by some as beneficial (Zeller and Sullivan 1992, 449; Daniels and Elmore *et al.* 1994, 465; Turna, Bos, and Jurek 1994, 101). In this vein of investigation, researchers have observed a lower respiratory burst response in HIV (Human Immuodeficiency Virus)-infected children and adults. They imply that the decreased ability of the phagocytic cells to produce reactive oxygen intermediates may be a contributing factor in HIV-infected patients' increased susceptibility to bacterial and fungal infections (Chen and Roberts *et al.*

1993, 544). It is interesting that in patients with common variable immunodeficiency, monocytes exhibited reactive oxygen species generation (Aukrust *et al.* 1994, 232). The presence of intracellular *Leishmania amastigotes* (a parasitic organism) in human mononuclear phagocytes decreases the oxidative burst and may contribute to parasite survival (Passwell *et al.* 1994, 277). Examination of the effects of the therapeutic use of interferon-alpha revealed that interferon-alpha may protect against viral infection indirectly by promoting neutrophil respiratory burst responses (Little *et al.* 1994, 802). Human neutrophils *in vitro* were found to release large quantities of $O2^-$ in response to tumor necrosis factor (TNF) alpha. Another study investigated TNF-alpha's enhancement of the effect of radiation against human colon tumor xenografts. This study suggests that themechanisms of action may be related to increased oxygen radical production (Gridley *et al.* 1994, 1107).

Investigation into the chemiluminescent process used inhibitors of oxygen radicals and enzymes. Catechol-like phenols suggested the involvement of NADPH-oxidase and peroxidase in oxidative metabolism of mussel hemocytes. This study suggested a variable immunocapacity for individuals and separated hemocyte subpopulations. (Noel *et al.*1993. 483) A study involving macrophage-like defense cells (hemocytes) showed similarities to the production of reactive oxygen intermediates (ROIs) by mammalian leukocytes during respiratory burst. Coincubation but not preincubation with five different catechol-like phenols inhibited oxidative activities of zymosan-stimulated hemocytes. According to this study's authors, Adema *et al.*, their findings imply

similarities in composition and regulation of the ROI-generating mechanisms of both mammalian and snail defense cells.

The oxygen solubility in water exposed to water-saturated air at atmospheric pressure can be found in Table 2.1 in Appendix 2.

2.4 Phenol

Benzene is a known human myelotoxin and leukemogen. Benzene is metabolized by liver cytochrome p-450 monooxygenase to phenol. Subsequent hydroxylation of phenol by cytochrome p-450 monooxygenase results in the formation of hydroquinone, (a bone seeking species, accumulating in bone marrow) (Subrahmanyam and Ross *et al.* 1991, 495). It is well established that benzene requires metabolism to phenol to induce its effects (Subrahmanyam and Ross *et al.* 1991, 495).

Phenol, as a benzene metabolite, is a well studied toxicant. For example, urinary phenol levels have long been used in occupational and public health research and practice as an indicator of benzene exposure (Schulte 1991, 435). Phenol's oxidative fate has also been explored. Some researchers suggest that peroxidative metabolism of benzene's phenolic metabolites may be responsible for the increased free radical activity and toxicity produced by benzene in bone marrow (Schulte 1991, 435). Also, bone marrow cells and their microsomal fractions isolated from rodents following benzene treatment have a higher capacity to form oxygen free radicals (Subrahmanyam and Ross *et al.* 1991, 495).

2.4.1 Respiratory Burst and Phenol

Phenol has been examined as a stimulator of the respiratory burst with subsequent damage to the body (Haynes *et al.*1993, 49; Simons *et al.*1990, 251).

Peripheral blood leukocytes contain a variety of enzymes that are capable of metabolizing xenobiotics. Especially important is the enzyme myeloperoxidase (MPO) which is a peroxidase/oxidase and generates the powerful oxidant hypochlorous acid. MPO or MPO-generated oxidants are capable of oxidizing a wide variety of compounds and a broad range of functional groups (Hofstra and Utrecht 1993, 221). Other examples of chemicals such as aspirin and salicylate are transformed by human polymorphonuclear leukocytes (PMN) as measured by Haynes *et al.*(1993, 49). In this paper, it was also reported that some phenols e.g. 5-aminosalicylate inhibited H_2O_2 production.

Diethyldithiocarbamate (DDC) is a superoxide dismutase inhibitor (Pipe 1992, 111). Phenols isolated from the traditional medicinal plant *Picrorhiza kurroa* also inhibit the release of superoxide anion (O2-) by activated human neutrophils. Interestingly, Picrorhiza leaves the neutrophils' phagocytic activity intact (Simons *et al.* 1990, 251). This study by Simons *et al.* states that catechols react directly with reactive oxygen species (ROS) from the oxidative burst. For the activation of the orthomethoxy-substituted catechols the combined activity of ROS and myeloperoxidase (MPO) is obligatory. Catechols with a dimethoxy substitution cannot be activated metabolically by neutrophil-derived ROS.

CHAPTER 3

MATERIALS AND METHODS

Bovine blood is routinely collected from the carotid arteries of freshly killed cows at a slaughterhouse. During the course of this study, blood from several cows is used. The blood is kept on ice during transportation from the slaughterhouse to the NJIT Biotechnology Laboratory. The blood is collected directly into one - gallon containers (3,785 ml) with 407 ml of pre-chilled anticoagulant. ACD anticoagulant consists of 3.95 g citric acid, 10.9 g sodium citrate, and 12.1 g dextrose, all anhydrous, diluted to 407 ml with distilled water. Fresh blood is poured to fill the containers and when full, the containers are stoppered and mixed gently. The blood is kept on ice during transportation and stored at $4^{\circ}C$ until use.

Leukocyte rich samples are obtained through centrifugation of blood in 15 ml test tubes and subsequent removal and use of the buffy coat. The buffy coat is the leukocyte rich interface between plasma and erythrocytes produced by centrifugation of blood.

3.1 Measurement of Amount of Blood Cells

Immediately before each run, the blood is gently mixed and the hematocrit measured. In this work, the term hematocrit is used interchangeably with packed cell volume (PCV). Blood is drawn into a microcapillary tube and centrifuged in a Damon/IEC Division IEC MB centrifuge - microhematocrit. The hematocrit is then measured with a Damon microcapillary reader. Hematocrit refers to the volume of packed red blood cells. For the

purposes of this work, packed cell volume (PCV) and hematocrit (HCT) are regarded as interchangeable.

In this work, all measurements of blood cells are reported as packed cells because the microhematocrit is designed to measure packed cell volume. Most of these packed cell volumes are applied to whole blood which is what the instrument was designed for. When the packed cell volume is applied to leukocyte enriched blood, then the HCTs are much lower, therefore measurement may be less accurate. To check this, the packed cell volume at low HCT was measured and calibrated using an improved Neubaur hemocytometer.

There are 5.7×10^6 erythrocytes/μl of bovine blood of packed cell volume 37.4 (Schlaim *et al.* 1975, 123). Therefore, there would be 3.0×10^5 erythrocytes/μl of bovine blood of packed cell volume 2.0. Cell counting in the hemocytometer revealed that in a sample measured by our microhematocrit instrument there are 4.4×10^5 erythrocytes/μl of bovine blood of packed cell volume 2.0. This indicates that PCV is not a precise indicator of the number of cells at low packed cell volume.

3.1.1 Differential Leukocyte Count

A differential leukocyte count was generously performed by Dr. Marguerite Hoey, D.V.M, of the Arlington Dog and Cat Hospital on three samples.

For this procedure, the packed cell volume (PCV) is measured as described above but using Lancer Critocap Micro-Hematocrit Capillary Tube Reader (Sherwood Medical, St. Louis MO 63103) and MP Readacrit Centrifuge (Clay Adams/ Becton, Dickinson & Co Parsippany NJ 07054).

The total solids content in the plasma is measured with a Goldberg refractometer #10400 (American Optical Co., Buffalo NY) which has an accuracy of +/- 0.1 g/dl.

The differential leukocyte count is a count of the different types of leukocytes within a blood sample. The red cells are lysed and the remaining white cells are counted in a hemocytometer. This gives the number of white cells per volume blood. Then, a stain of the blood is prepared using different dyes to help tell the types of leukocytes from each other. 100 cells of this stain are counted and the number of each type is the percentage of each type of leukocytes of each type in the blood sample. From these two pieces of information, the total number of each type of leukocyte is determined by multiplying the two numbers together.

The hemocytometer used is the improved Neubaur Levy Ultra Plane (Clay-Adams NY, NY). The red cells are lysed using the Unopette Microcollection *In Vitro* Diagnostic System for the Enumeration of Leukocytes in Whole Blood (Becton-Dickinson, Division of Becton, Dickinson and CO. Rutherford, NJ 07079). Each unopette contains a 1.98 ml reservoir which contains 28.6 ml glacial acetic acid, USP purified water to 1 liter. To this unopipette is added 20 µl blood. The dilution ratio is 1:100.

The differential blood stain slide is prepared using the Diffquick Stains for staining blood smears and bone marrow (Volu-Sol Stat Stain Statim Stain Buffered blood staining solutions Cas# 68 from Volu-Sol, Inc. 5095 West 2100 South Salt Lake City, Utah 84120).. This consists of three stains: Fixative (VDF-016), Solution 1 (VDE-016), and Solution 2 (VDB-016). The slide with the blood on it is dipped into each stain for 5 seconds.

3.2 Bioreactor Oxygen Measurement

All reactions are conducted in a 1.9 ml reactor (Figure 3.1 in Appendix 3) with a temperature controlled water jacket surrounding the reactor. For anaerobic runs, the reactor vessel is stoppered so that the system does not undergo gas exchange with the environment. A YSI 5331 oxygen probe measures dissolved oxygen concentration while the blood is stirred with a magnetic stir bar. A chart recorder is used to record molecular oxygen concentrations.

This extremely powerful method of oxygen concentration observation has its roots in the study of oxidative pharmaceutical kinetics (Poulsen, Ziegler, and Sofer 1976). The reactor is carefully cleaned with soap and cotton swabs between runs (chlorine bleach for the short runs). The bioreactor is calibrated by bubbling with air to determine baseline concentrations (Greenberg *et al.* 1985), and with N_2 to determine zero oxygen concentration.

3.2.1 Discussion of Potential Probe Interferences

According to the instructions for the 4004 Clark Oxygen Probe, eight gases have been tested for response. Carbon monoxide, carbon dioxide, and hydrogen show a response of only 1% as compared to oxygen. Helium and ethylene elicit no response. Hydrogen sulfide causes the probe to give a lower reading if it coats the probe. The probe is cleaned between runs to remove any buildup which might be present.

Chlorine produces 2/3 O_2 response. Nitrous oxide and nitric oxide produce 1/3 O_2 response. Chlorine may be present but the only pathway we know of for formation of

chlorine gas in blood is through myeloperoxidase activity on chlorides and halogens and is therefore also an indication of oxidation. In the case of chlorine, nitric oxide, or nitrous oxide formation, this activity is still an indication of oxidation.

If the peaks are caused by nitric oxide or nitrous oxide, there would be three times the number of moles of these gases compared with the number of moles of oxygen in the peaks if the peaks are caused by oxygen. The presence of this amount of either nitric oxide or nitrous oxide would imply an oxygen reservoir of greater magnitude than the one implied if the peaks are oxygen and our postulates regarding the MOPs (in Chapter 4) could reasonably be applied to NO and NO_2. When compared to control runs with no blood, these profiles seem to be indications of oxygraphic activity.

Temperature also affects probe reading. The probe is calibrated daily at the temperature to be used that day. Additionally, addition of ice cold saline serves as a control for the temperature effects of adding cold blood to the reactor.

3.2.2 Description of a Typical Run

The reactor is first filled with water or saline and the temperature of the reactor is recorded. The oxygen concentration is calibrated by saturating with air bubbled through the reactor liquid. Then, blood is added to the reactor producing a near immediate initial response in oxygen concentration in the reactor.

This initial response has a measurable maximum velocity. When phenol is added to the reactor either before or after the blood is added, the initial response may be an initial rise in the dissolved oxygen concentration in the reactor. In order to measure the initial

response's velocity, the chart speed is set at 10 mm/sec. These runs are very short (3-5 seconds each).

After the initial response, a more gradual oxygen consumption continues, usually until the oxygen concentration in the reactor reaches zero. This decreasing or zero oxygen concentration continues for several hours (from 1 to 48 hr., avg. = 16.1 hr) at which time the oxygen concentration in the reactor may suddenly increase and then decrease, forming a molecular oxygen peak (MOP). Many discrete MOPs may occur before the run is complete. For these long term runs, a much slower chart speed is used (1-2 mm/min). In the long term runs, the reactor may be filled with saline or water (the latter provides osmotic stress).

3.3 Sample Calculations

3.3.1 Maximum Amount of Hemoglobin (Hb) that Each Blood Sample Could Hold:

1. Erythrocytes contain 330g Hb/L packed erythrocytes (Garby and Meldon 1977). There are 588 amino acids in Hb which weigh approximately 64680 amu,
2. (330 g Hb/L packed erythrocytes)(1L/1000 ml)(6.02 x 10^{23} molecules HB/64680 g Hb in 1 mol Hb) = 3.1 x 10^{18} molecules Hb/ml packed erythrocytes,
3. (3.1 x 10^{18} molecules Hb/ml packed erythrocytes)(4 molecules O_2 /molecule Hb) = 1.2 x 10^{19} molecules O_2/ml packed erythrocytes, and

4. (1.2×10^{19} molecules O_2/ml packed erythrocytes)(32 g/mole O_2/6.02×10^{23} molecules/mole) = 6.5×10^{-4} g O_2/ml packed erythrocytes = 650 μg O_2/ml packed erythrocytes.

3.4 Cell Viability Tests

3.4.1 Vital-Nonvital Stain

The procedure for identification of vital and nonvital leukocytes is adapted from DeRenzis and Schechtman (1973). This method uses two dyes, trypan blue and neutral red, to distinguish vital from nonvital cells. The dead cells stain blue and the live cells stain red.

Add equal volume of cell suspension and 0.04% neutral red in balanced salt solution. After incubating at 37°C for 10 minutes, add an equal volume of 0.5% trypan blue in balanced salt solution. Incubate for 2-3 minutes further. Cells should now be visibly stained either ruby red or peacock blue (they're quite beautiful). If a cell contains both colors it was damaged during the staining procedure. If all cells look dark pink/purple, the staining procedure was unsuccessful and should be redone.

3.5 Leukocyte Rich Blood

Leukocyte rich blood is obtained by two methods for these experiments. The buffy coat method is by far the most frequently used method for these experiments.

3.5.1 Buffy Coat Method

In this method, several samples of about 15 ml whole blood are placed in test tubes which are then spun in a Safety Head Centrifuge (Clay Adams, Parsippany NJ) at speed "5" on

the centrifuge dial for about 10 minutes. The result of this spinning is that the blood cells sink to the bottom of the tube, the plasma is at the top, and at the interface is a buffy coat containing a leukocyte rich layer of cells which are then removed with a Pasteur pipette.

3.5.2 Erythrolysed Blood Samples

Samples are prepared by adding packed blood cells to distilled water and then adding NaCl solution to form a balanced salt solution. This procedure was designed to eliminate the erythrocytes because they are more susceptible than the leukocytes to bursting from the change in osmotic tension. The resulting solution is then spun down in the centrifuge and the cells form a pellet at the bottom of the tube and are removed. Microscopic examination of the resultant pellet indicated that many erythrocytes remained and so this method was only used a few times.

3.6 pH Measurements

The pH is measured using a 13 mm diameter pH electrode with an epoxy body and Ag/AgCl reference cell type (Cole-Palmer 1-800-323-4340). The probe is inserted into the top of the reactor and pH data are recorded on the same chart recorder as the DO (using a second pen on the same chart recorder). In all runs in which the pH is monitored, the glass stopper is not inserted into the reactor so that the pH probe can be inserted.

CHAPTER 4

OXYGRAPHIC PROFILES OF PHENOL STRESSED BOVINE BLOOD

Blood added to a saline filled, magnetically stirred reactor produces an initial decrease in dissolved oxygen (DO) in the reactor. This response occurs almost immediately upon addition of blood to the reactor. The amount (nmol O_2) and maximum velocity of the initial response (nmol/ml/sec) can be quantified. The initial response is influenced by the amount of blood injected and the temperature of the reactor. It is also profoundly influenced, in a dose-response manner, by the concentration of phenol present in the reactor. When phenol concentrations are sufficiently high (generally 20,000 ppm or more), the blood releases oxygen. The fast-assay runs take place within 3-5 seconds of blood addition to a reactor.

A second series of experiments examines the DO profile for many hours after blood addition to the reactor. Addition of phenol to bovine blood cell bioreactors affects the long-term DO concentration profiles and increases the probability that the blood will subsequently release oxygen and produce a molecular oxygen peak (MOP). Long term assays are analyzed for MOP activity, each run yielding a single number representing total activity. Activities as high as 1,300 μg O_2/ml packed cells have been observed for stressed, leukocyte enriched blood.

4.1 Introduction and Background

4.1.1 Molecular Oxygen Peak (MOP)

An MOP as defined in this paper consists of a discrete increase followed by a decrease in oxygen concentration. MOPs usually last upwards of one hour. The release of O_2 as defined in the MOP has not to the best of our knowledge heretofore been reported.

4.1.2 Choice of Phenol as a Model Chemical Stress Agent

Phenol, as a benzene metabolite, is a well studied toxicant and has been selected as the chemical stress agent for this study. Benzene is a known human myelotoxin and leukemogen. It is well established that benzene requires metabolism to phenol to induce its effects (Subrahmanyam 1991, 495). Urinary phenol levels have long been used in occupational and public health circles as an indicator of benzene exposure (Schulte 1991, 435). Oxidation of phenol has been explored. Some researchers suggest that peroxidative metabolism of benzene's phenolic metabolites may be responsible for the increased free radical activity and toxicity produced by benzene in bone marrow. Also, bone marrow cells and their microsomal fractions isolated from rodents following benzene treatment have a higher capacity to form oxygen free radicals (Subrahmanyam 1991, 495).

The ability of leukocytes to metabolize xenobiotics is well known (Hofstra and Uetrech 1993, 221). Due to its action as a toxicant, phenol does not readily lend itself to clinical application, but its study *in vitro* can reveal the general mechanics and kinetics of dose-dependent stimulation of the MOP.

4.1.3 Potential Applications of this Research

DO profiles are potentially useful in determining the ability of blood cells to defend against various stresses, especially those due to chemicals in the environment. While phenol is used as a model compound, blood cell bioreactors have potential applications as sensors for calibrating or detecting environmental pollutants, pharmaceuticals, and other xenobiotics. Blood cell bioreactors also may be used to test patient donor blood sensitivities or to activate immune properties of the cells. This work has potential applications in determining general oxygraphic capabilities of blood, and in identifying human subpopulations sensitive to chemical pollutants and other xenobiotics, including pharmaceuticals.

The experiments and results stemming from the work described in this communication may serve as a basis for inexpensive, quantitative alternatives to extant toxicological tests. Other potential applications are in determining the oxygraphic vitality of blood as well as identifying of subpopulations which are more prone to react adversely to a particular pollutant, drug, or treatment regimen.

4.2 Results and Discussion

4.2.1 Oxygraphic Recording of a Blood Age Study

Figure 4.1 demonstrates several key aspects of the work presented in this chapter. Each run is a replication of the oxygraph chart recording. In Run 1, an air saturation baseline is obtained by bubbling air through water in the temperature controlled reactor vessel. The dissolved oxygen (DO) is recorded throughout the duration of the run. The second run shows the zero oxygen level in the reactor. The readings at zero and air saturation DO

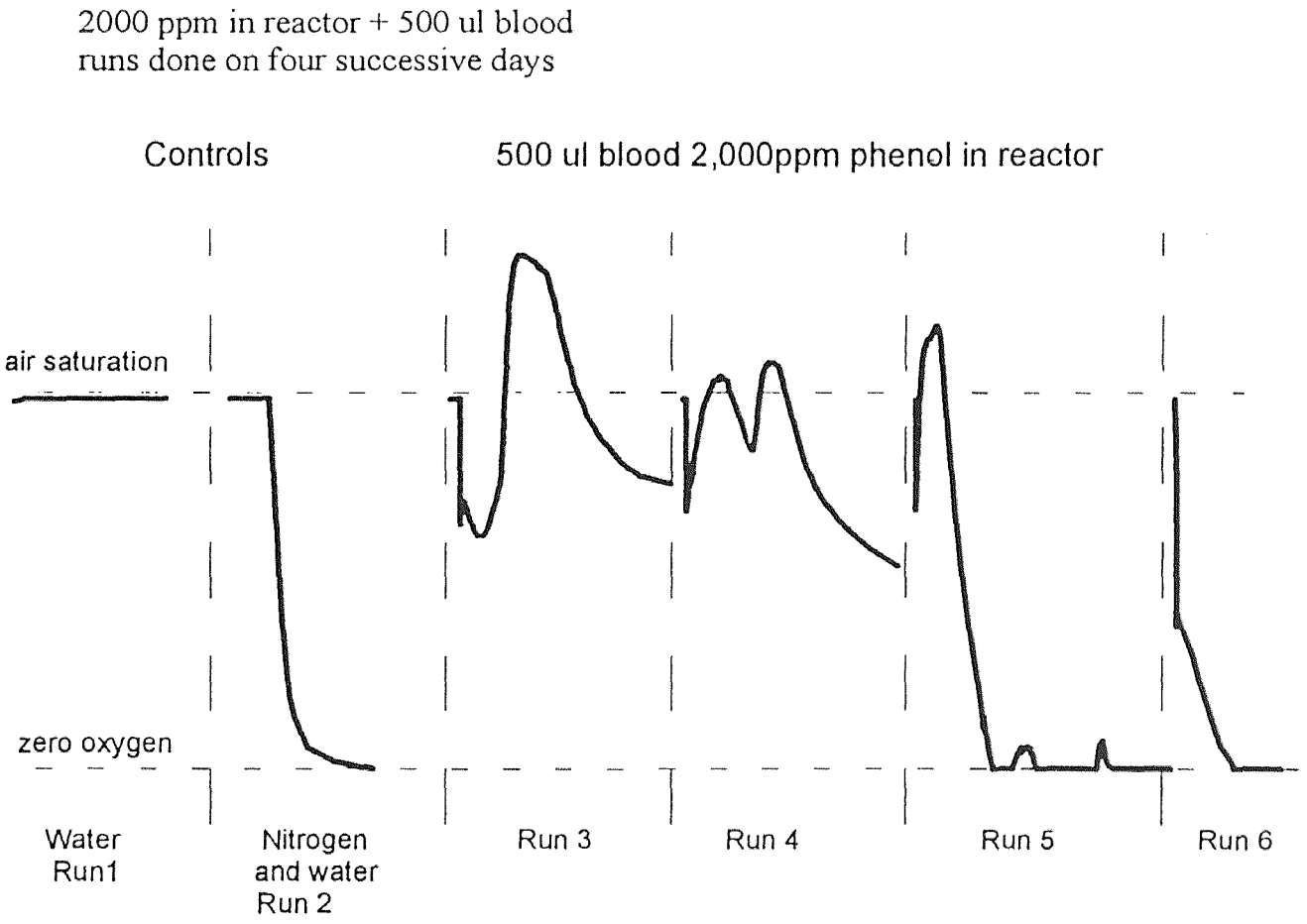


Figure 4.1 Oxygraphic recording of a blood age study.

provide us with a calibrated instrument and because the amount of oxygen required to saturate water fully at a given temperature is known, accurate quantitation of all oxygraph measurements is possible.

Runs 3-6 are 24 hour observations of the same size blood samples subjected to the same phenol stress at the same temperature. The blood for each of these runs was taken from the same *in vitro* source, thus the samples differ only in age, each sample being one day older than its predecessor. Each of these runs provides us with two types of information. First, note that immediately upon addition of blood to the reactor, there is an initial response, in this case an initial decrease in the DO in the reactor. For Runs 3-5, this initial response appears to be quite similar. Run 6 has a larger initial response than the previous runs. By significantly expanding the chart speed, to 10 mm/sec, we can measure both the amount of oxygen involved and the velocity of this initial response. Observations of the initial response of blood are dubbed "fast-assay" runs. The fast-assay runs provide very interesting observations, as will be shown in Figures 4.2 -4.4.

The second major type of observation is also shown in Figure 4.1. Long term DO profiles often provide the opportunity to observe MOPs. Note that in Run 5, the MOP commences when the DO in the reactor is zero. This is interesting indeed, as hemoglobin would not have any oxygen bound to it if the surrounding medium contained zero oxygen. Also note the variability in the profile for these samples. Although these samples differ ostensibly in age by one day, there is considerable variation in the time of MOP onset, the duration and magnitude of the MOPs, and in the number of MOPs which appear. In the final run, no MOPs are observed. The variability is typical of these experiments and has

led to the examination of MOP occurrence as a statistically probable phenomenon. This statistical examination of MOP occurrence is presented in Figure 4.5.

4.2.2 Initial Response vs. Amount of Blood Added

Fast-assay runs indicate the dose-response relationship observed in Figure 4.2 which relates the initial response to the amount of blood added and the amount of phenol present in the reactor before blood addition. The initial response may be a decrease in DO in the reactor (dubbed an "initial drop") or it may be an increase in DO in the reactor (dubbed an "initial rise"). There is a dose-response relationship for both the amount of blood added and the amount of phenol present in the reactor before blood addition.

There is a similar dose-response relationship when the velocity of the initial response is examined in the same manner as the amount of oxygen in the initial response. Figure 4.3 in Appendix 4 contains the velocity of the initial response .

4.2.3 Initial Response per Milliliter of Packed Cells

Figure 4.4 summarizes these data and provides an examination of the dose-response relationship as a function of the milliliters of packed cells (n=90, coefficient of determination or R-squared = 0.98 for the amount of oxygen in the initial response and 0.99 for the velocity of the initial response). In Figure 4.4, both the amount of oxygen present in the initial response (the slope of each line from Figure 2) and the velocity of that initial response are shown. Both indicate dose-response relationships and are useful indicators for a rapid assay.

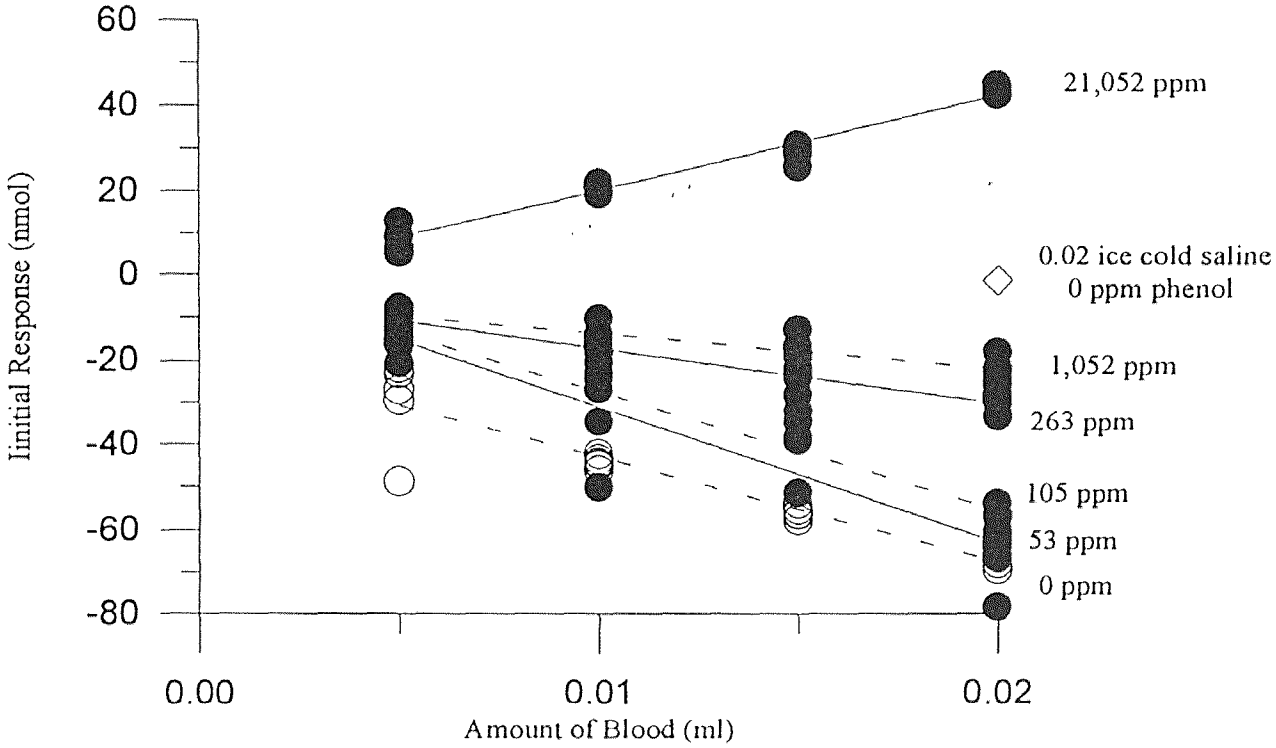


Figure 4.2 Initial response vs. amount of blood on 7/17/96. The open circles are with no phenol in the reactor; the closed circles are runs with various amounts of phenol in the reactor, the open diamond is ice cold saline injected into the reactor as a control. There is a dose-response relationship for both the amount of blood added and the amount of phenol present in the reactor before blood addition.

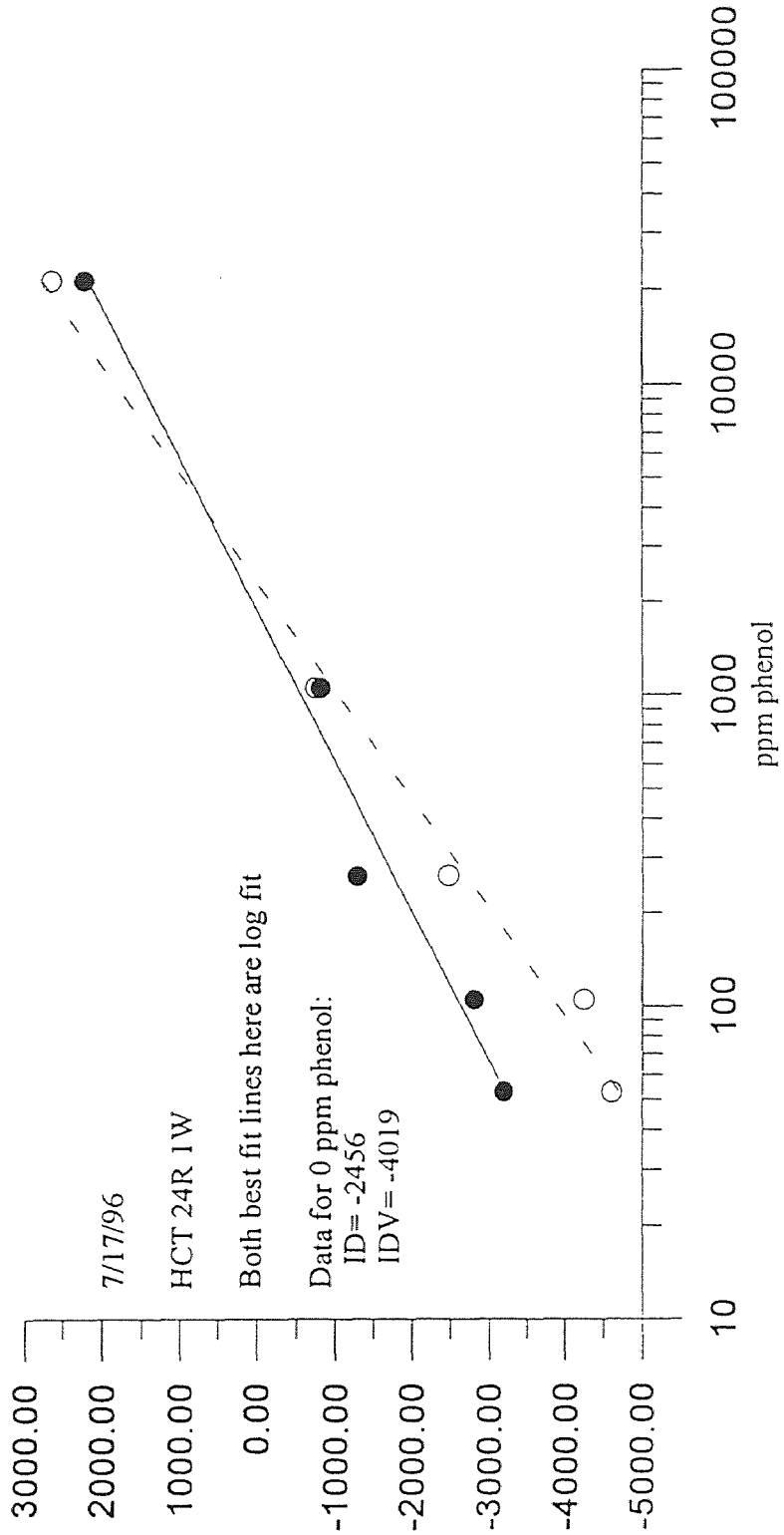


Figure 4.4 Initial response per ml of packed cells on 7/17/96. The closed circles and solid line show the amount of oxygen in the initial response per milliliter of packed cells and the open circles and dashed line show the initial response velocity per milliliter of packed cells. Both the amount of oxygen present in the initial response (the slope of each line from Figure 2) and the velocity of that initial response are shown. Both indicate dose-response relationships and are useful indicators for a rapid assay.

As examination of toxicological effects of pollutants is likely to continue and become more refined, the demand for tests such as these fast-assay runs can only increase. These fast-assay runs are technically simple and inexpensive and rapid. They could provide information about subpopulations sensitive to a particular drug or pollutant, as well as give an indication of tolerant subpopulations.

4.2.4 Statistical Examination of MOP Incidence

Figure 4.5 and Table 4.1 both refer to oxygraphs of stressed blood which were conducted for much, much longer than the runs introduced as fast-assay runs. The fast assay runs take a few seconds each while these long term runs are often 24 hour runs.

Figure 4.5 shows the statistical analysis of MOP occurrence. The effects of various types of stresses are examined. MOPs are more likely to be observed in the reactor when blood is stressed. High concentrations of phenol, at the 2,000 ppm level, yield the highest oxygraphic activity (48%, n=56 runs) when compared to lower concentrations of 200 ppm and under (31%, n=49 runs, (1-p) = 0.9222). Higher temperature, 42C, yields more MOP activity (50%, n=125 runs,) than lower temperature, 32C, (34%, n=79 runs, (1-p) = 0.9474). Leukocyte enriched blood yields more MOP activity (78%, n=18) than ordinary blood (30%, n=118, (1-p) = 0.999032). The quantity (1-p) is the probability that the response falls within the Normal curve. A decrease in osmotic pressure increases the chance that the blood will subsequently and suddenly release oxygen to form the MOP. Likelihood of MOP generation by blood in distilled water is higher (38%, n=120 runs) than in saline solution (23%, n=31 runs, (1-p) = 0.9057).

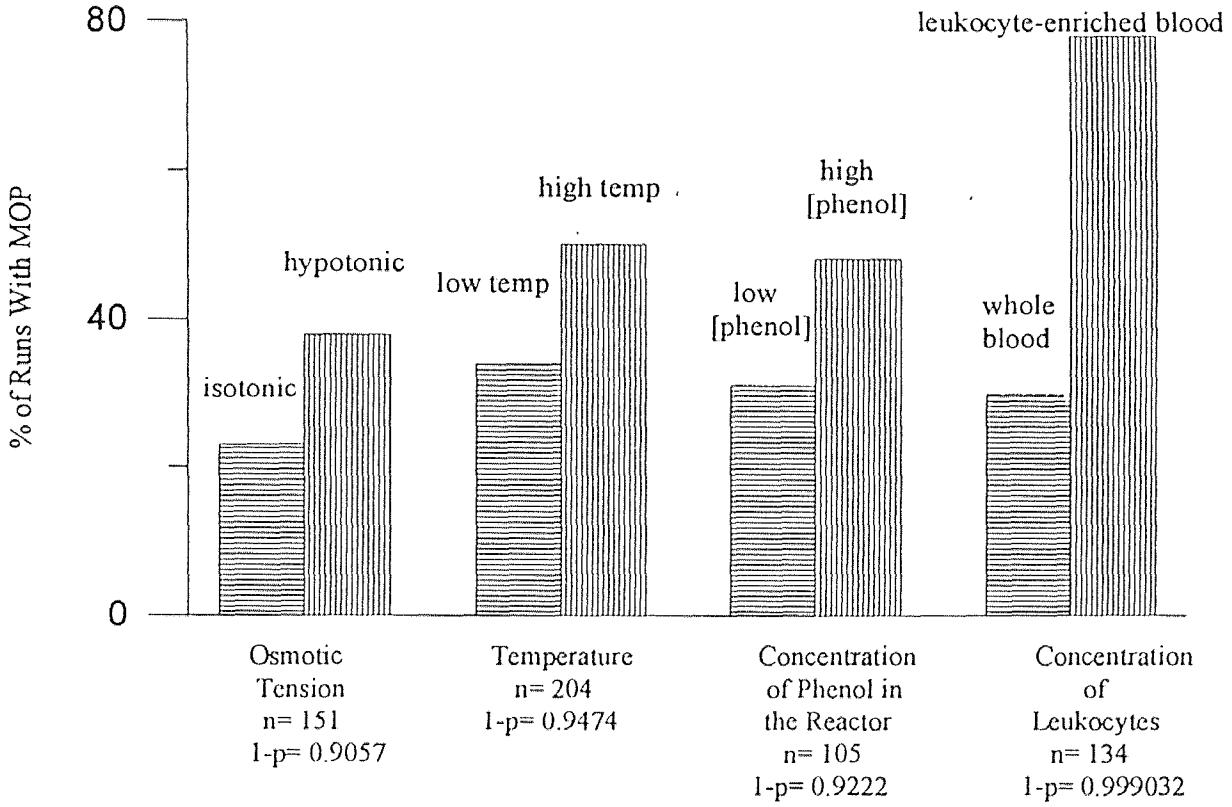


Figure 4.5 Statistical examination of MOP incidence. All runs shown are over 5 hours in duration. This graph represents approximately 6,950 hours of reactor data.

4.2.5 Quantitation of Runs with High MOP Activity

Table 4.1 is an in-depth quantitative examination of four particularly active runs which yielded MOPs. The MOPs' existence may be of interest to the scientific community because of the conclusions stemming directly from their observation under the conditions that they are observed, that is, a reactor with no external oxygen source.

Table 4.1 Quantitation of selected long-term runs with high MOP activity. Each run is described and MOP oxygen generation summed. Whether MOP oxygen is consumed or simply shifts from reservoir to reservoir, the total oxygraphic activity for long term runs may be represented by a single number, micrograms O_2 per ml of packed cells shown in the last row. Long term summaries are reduced to a single row as shown in this table.

Run #	143-1	212-2	213	216-1
amount blood (ml)	0.015	0.3	0.4	0.35
whole (wh) or leukocyte-rich (lr) blood	wh	lr	lr	lr
ppm phenol in reactor	105	158	210	0.5
temperature (°C)	42	30	32	32
MOPs, peaks per run,	4	2	6	2
Time (hr) of peaks,	3.1, 11.6, 16.6, 19.3	22.5, 25.7	32.4, 34.2, 61.6, 70.4, 77.0, 81.2	1.0, 2.9
µg O_2/peak	2.1, 2.1, 2.8, 0.7	4.4, 2.9	3.4, 3.1, 15.2, 13.7, 15.8, 13.1	2.35, 0.8
Average hours per MOP	12	22	18	22
Duration of run	50	44	110	44
O_2 found in all peaks (mg)	7.64	7.35	64.18	3.14
Maximum hemoglobin O_2 capacity in run (mg)	3.45	6.08	1.35	1.22
Erythrocyte/leukocyte content of blood (µl)	5.1/0.8	9.0/12.0	2.0/48.0	10.3/0.3
Oxygraphic activity (µg O_2 /ml packed cells)	1,310	350	1,280	260

4.2.6 Implications of MOP Occurrence

Looking at Run 5 in Figure 1, one is compelled to attempt some clarification of this puzzle: namely, what is the source of oxygen for MOPs that start at zero saturation? We have hundreds of hours of data showing MOPs arising from zero DO.

Control runs show no MOPs with water, saline, or plasma alone. No MOPs arise upon addition of phenol to water, saline, or plasma alone. MOP activity is present only when blood cells are present. Many of these discrete increases in oxygen concentration take place when the dissolved oxygen concentration is zero. At zero dissolved oxygen concentration, hemoglobin would have given up all of its oxygen so hemoglobin, as currently understood, is likely not the oxygen reservoir from which the MOPs are derived.

Three postulates are presented:

1. The MOP is not an equilibrium-dependent release of oxygen. Therefore this cannot be a conventional oxygen source such as hemoglobin, myoglobin, or oxygen dissolved in lipids or water because as currently understood, these oxygen releases are equilibrium dependent; at zero DO, they store no oxygen.

2. An increase in DO that is not equilibrium dependent may be explained by an oxygen reservoir within the blood. It is not an undetected leak from the atmosphere because there is a net positive pressure (a liquid column in the cannulus) on the reactor vessel, and leaks, even small ones, are easily visible through the clear glass of the 1.9 ml reactor vessel.

3. The existence of the MOPs indicates the existence of an oxygen reservoir within the reactor vessel, or a unique and new property of hemoglobin. That these MOPs

do not occur with water or saline or phenol alone or in any combination without blood implies that the oxygen reservoir is within the blood.

4. That the MOPs increase the DO in the reactor in a discrete, non-equilibrium manner points to a storage mechanism such as O_2 being locked physically within a macromolecule or similar trapping environment, or to chemical bonding within metals or peroxides. This implies a potential catalytic energy barrier capable of containing oxygen against a concentration gradient.

O_2 is the simplest form for the oxygen source for the MOPs. It could be stored physically as oxygen as a polymer of oxygen within a chemical or electrochemical cage or it could be stored chemically, as a peroxyacid or as a metallic oxide, or attached within a large protein molecule. The existence of a catalytic energy barrier requires some activation energy to release the O_2, the measurement of which is an area of planned research for our group.

4.3 Conclusions

The fast assay runs hold great promise for developing a rapid toxicity test or a supporting test for other toxicological evaluations. The dissolved oxygen probe during long-term runs can prove a powerfully accurate and precise measure of the overall state of a given blood sample. The existence of MOPs and their implications will hopefully lead to new and exciting discoveries about the action and nature of blood. Long term assays also are of potential use in developing a new toxicity test. The molecular and cellular level

response to xenobiotics is a dynamic research area that will benefit from the information in this dissertation.

CHAPTER 5

AERATION STUDIES

In this chapter, the effect on the dissolved oxygen (DO) consumption rate of osmotic stress, blood amount, phenol concentration, and temperature is explored. It is largely the results of these experiments which have prompted the thinking behind the fast assay runs. The initial goal of these aeration studies is to design an assay for blood which requires less than one hour to perform. The results of the aeration studies make an assay of a few seconds in duration seem feasible.

Examination of the rate of oxygen consumption in blood as a function of stress is a logical part of any work examining the oxygraphic response of stressed bovine blood. This goal is complicated by the fact that, as the reader might suspect from the previous discussion of a typical run, the vast majority of the time in typical closed reactor runs involves zero DO readings. Aeration of the reactor immediately after blood addition increases the DO concentration and thus yields more data regarding oxygen consumption rate as a function of DO.

Examining changes in the rate of DO consumption as a function of stress has proven a promising line of investigation. The initial response of blood addition to the reactor is a rapid and often large decrease in oxygen concentration in the reactor followed by a much slower rate of decrease in oxygen concentration. The two areas of DO consumption which seem likely candidates for examination with respect to stress are: the

initial response, examined in the fast assay runs using an open top reactor (presented in Chapter 6); and the subsequent slower rate of DO consumption, examined in these aeration studies. These runs are aerated and the reactor subsequently closed. They provide clues to the MOP puzzle as well as examine the effects of various stressors on DO consumption in a closed reactor.

The purpose of aeration is simply to raise the DO concentration in the reactor. This provides several interesting advantages over the non-aerated runs. First, there is a greater amount of oxygen in the reactor, therefore there is more oxygen to be consumed. More data are gleaned from these runs before they reach zero DO.

Hemoglobin's characteristic sigmoidal binding curve with respect to oxygen concentration is essential to its function but could really complicate matters if one compares two experiments in which one has not only a different stress level but also a different DO in the reactor. The initial response of blood addition to the reactor often brings the DO in the reactor well beyond hemoglobin's sigmoidal range.

One way to avoid this is through reaeration beyond hemoglobin's sigmoidal range, as in these studies. Another way, employed in the fast assay runs, is to use very small amounts of blood which won't cause such a dramatic decrease in DO in the reactor.

Thus, by raising the DO in the reactor to near the air saturation level, and by taking measurements above the saturation of hemoglobin, about 50% of air saturation, it is possible to minimize, or at least normalize, the effects of hemoglobin's action on these experiments.

5.1 Experimental Procedure for Aeration Studies

The aeration studies are similar in most respects to all the runs discussed up to this point. The only difference between the aeration studies and all other studies discussed in this dissertation is that in the aeration studies, immediately after blood addition a syringe is used to re-aerate the reactor.

To aerate the reactor, the entire reactor contents are drawn into a syringe and squirted back into the reactor several times or until the DO in the reactor, as measured by the oxygraph, reaches approximately air saturation. Then, the glass stopper is placed into the reactor and the experiment continues for 24 hours. The result, which can be observed as a chart recording throughout this procedure, is that the oxygen concentration in the reactor is higher than if no syringe aeration had taken place.

Two other methods of aeration were examined before the syringe method was selected. Aeration by bubbling air through the reactor in the same manner as is done when checking the air saturation level of water or saline in the reactor before each run was attempted several times. This method is impractical once blood is in the reactor due to the extreme foaming that results when air is bubbled through the reactor after blood has been added. Foaming is likely due to the high protein content of blood. Oxygenation by addition of hydrogen peroxide was successful, but deemed inappropriate due to the possibility that oxygen radicals are involved in the phenomenon being observed in these experiments. To add another generator of oxygen radicals seems an unnecessary complication that might interfere with the experimental results.

5.2 Quantitative Analysis for Aeration Studies

The kinetics of O_2 release from blood are analyzed in Lineweaver-Burk fashion, where the x-axis is $1/[O_2]$ and the y-axis is 1/r where r is the rate of DO consumption(change in $[O_2]$ /change in time).

These data are analyzed in Lineweaver-Burk style for several reasons. First, this method makes possible comparison between runs with different DO concentrations because it plots the inverse of the DO instead of the DO. The process of re-aeration with syringe provides a higher DO level in the reactor but it does not provide the exact same DO level each time. Therefore a technique such as this, which compares relatively different DO levels in the reactor is desirable. Also, Lineweaver-Burk style analysis is traditionally used to examine the kinetics of enzyme reactions and is familiar to many biologists and biochemists.

Points near air saturation are selected for analysis because this reduces the effects of hemoglobin oxygen storage. Further, as the DO level in the reactor nears zero, its inverse gets huge and the resulting range of values for data on oxygen concentration becomes unwieldy.

These graphs do not examine MOP peaks. MOPs are not discussed in this chapter at all. These graphs record the oxygen concentration in the blood as the oxygen concentration decreases after addition of blood and reaeration of the reactor.

5.2.1 K_R

Examination of the aeration studies points inexorably to the observation that there is a rotation when the various conditions of stress are applied. "Rotation" refers to the

observation that in blood samples varying in one of the four experimental conditions examined, one sample will have a much steeper slope of the best fit line.

K_R refers to the slope of the best fit lines which compare the rate of DO consumption to the amount of oxygen in the reactor. K_R is expressed in units of 1/hr. The greater the K_R, the lower the rate of DO consumption as DO in the reactor decreases. Because these plots are double reciprocal, a steeper slope actually means that that run had a slower rate of oxygen consumption at the lower oxygen concentrations. A lower K_R implies a higher stress.

5.2.2 Conventions for Figures in this Chapter

For all figures in this chapter, the following conventions are observed in the interest of clarity.

1. All runs in which the reactor is filled with saline have dashed best fit lines. All runs in which the reactor is filled with water have solid best fit lines.

2. All runs in which the reactor is 30-32 °C have thin best fit lines and are described as 30 °C. All runs in which the reactor is 40-43 °C have heavy best fit lines and are described as 42°C.

5.3 Results of Aeration Studies

For each experimental parameter explored, Figures (5.1-5.16) are included. One figure of each stress condition examined is in the chapter text. Additional figures for each stress are found in Appendix 5. Tables 5.1-5.4 record the K_Rs for each figure.

5.3.1 Osmotic Stress

The runs done in water are under greater osmotic stress than the run in saline. All other conditions being equal, runs in a saline filled reactor have a steeper slope, thus a greater K_R, than runs in a water filled reactor. Since a greater K_R indicates less stress, this is the result one would expect. Therefore, runs in a water filled reactor had a greater rate of DO decrease as DO in the reactor decreased than runs in a saline filled reactor. A typical pair of runs demonstrating the effects of osmotic stress is shown in Figure 5.1. Further examples (Figures 5.2-5.5) are in Appendix 5.

Table 5.1 K_R Values for Osmotic Stress (Figs 5.1-5.5). Water is more stressful than saline. Therefore a lower K_R (indicative of greater stress) in water compared with saline is expected, and is observed, in these results.

Amount of blood (ml)	Amount of phenol (ppm)	Temperature (°C)	K_R with Saline (1/hr)	K_R with Water (1/hr)
0.1	100	30	5.18	0.17
0.015	100	42	28.23	0.82
0.05	100	42	44.31, 13.94 (two runs)	1.97
0.1	100	42	1.90, 2.32 (two runs)	-1.17
0.2	0	42	34.01	-0.62

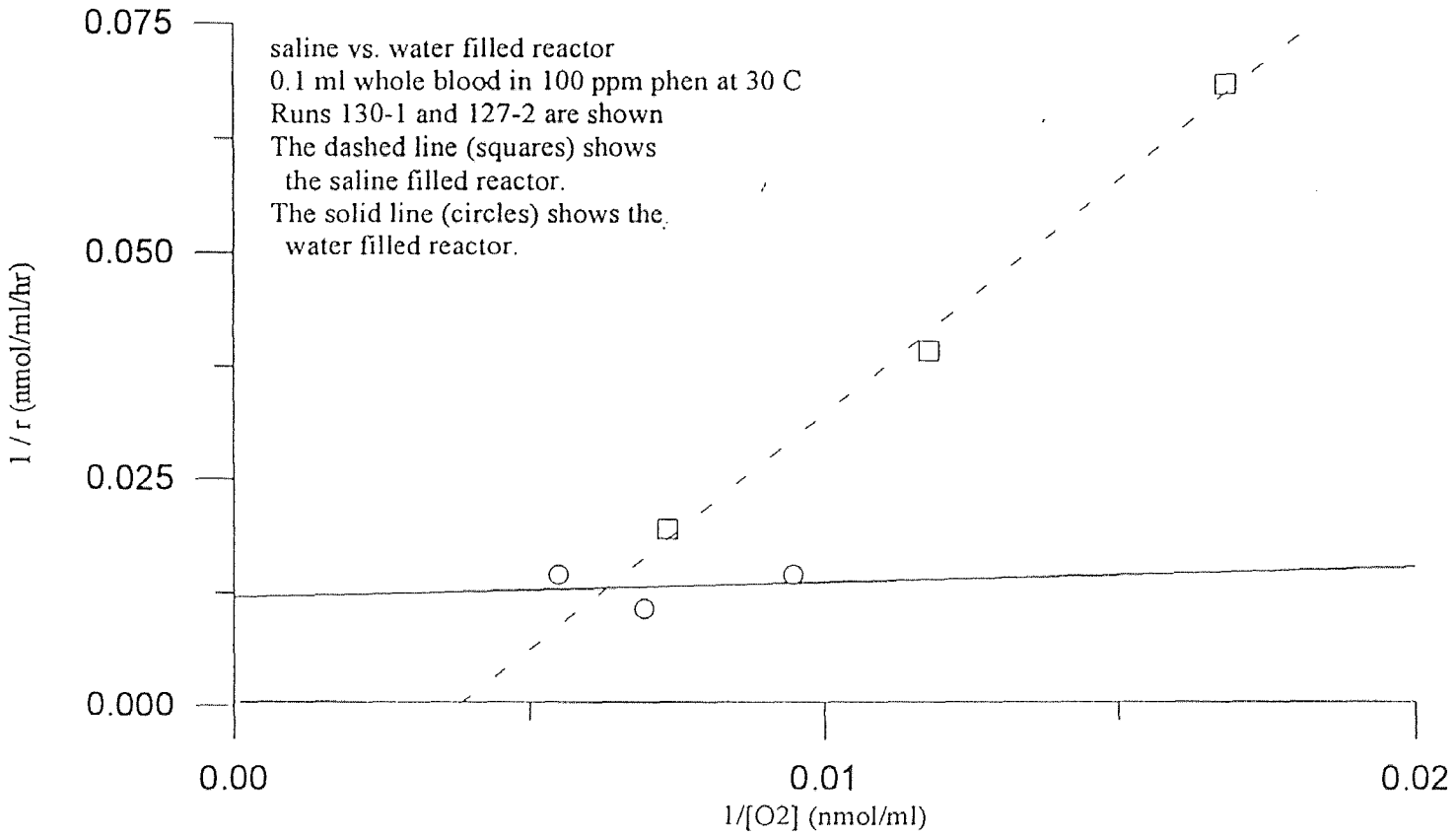


Figure 5.1 Effect of osmotic stress on DO consumption for 0.1 ml whole blood in 100 ppm phenol at 30 °C. The run done in water is under greater osmotic stress than the run in saline. The run in the saline filled reactor has a steeper slope, thus a greater K_R, than the run in the water filled reactor. Since a greater K_R indicates less stress, this is the result one would expect. Therefore, the run in a water filled reactor had a greater rate of DO decrease as DO in the reactor decreased than the run in the saline filled reactor.

5.3.2 Temperature

All other conditions being equal, runs in a reactor at 42 °C have a greater K_R than runs at 30 °C. Therefore, runs at 30°C have a greater rate of DO consumption as DO in the reactor decreases than runs at 42°C. If K_R indicates both less stressful conditions and the reaction rate, this result is counterintuitive since one would generally expect a reaction rate to increase with temperature. However, these K_R indicate more stress at hypothermic conditions. Figure 5.6 shows a comparison of K_R values at different temperatures. Further examples (Figures 5.7-5.10) are in Appendix 5.

Table 5.2 K_R Values for Temperature (Figs 5.6-5.10). The K_R at 42 °C is greater than the K_R at 30°C. Therefore, if greater K_R indicates less stressful conditions, 42 °C is less stressful to blood than 30°C.

Amount of blood (ml)	Amount of phenol (ppm)	Saline or water	K_R at 42 °C (1/hr)	K_R at 30 °C (1/hr)
0.01	525	water	3.98	-0.11
0.015	100	saline	28.23	4.91
0.05	100	saline	44.31, 13.94 (two runs)	3.64
0.1	100	water	-1.17	0.17
0.1	100	saline	1.90, 2.32 (two runs)	5.18

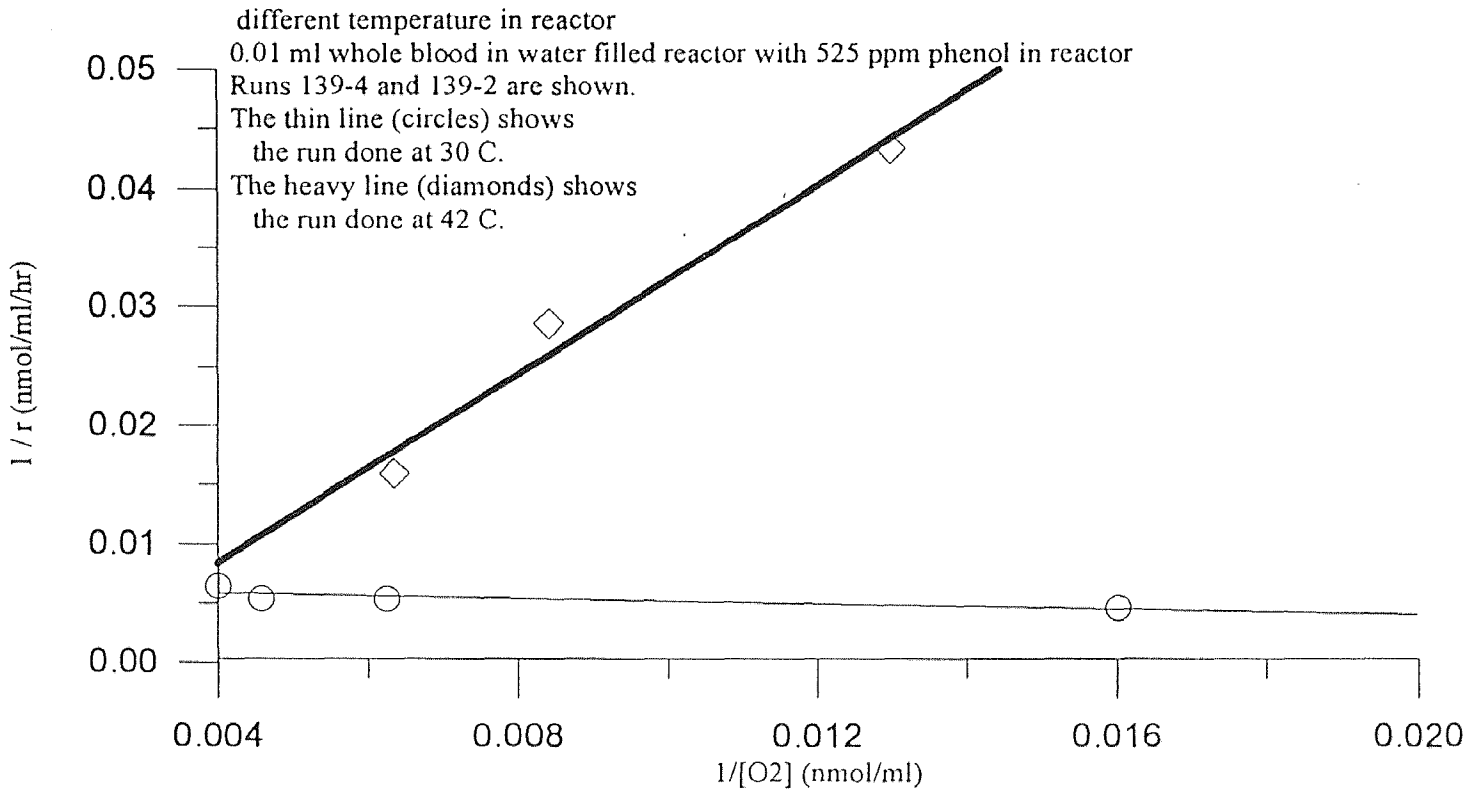


Figure 5.6 Effect of temperature on DO consumption for 0.01 ml whole blood in 525 ppm phenol in water filled reactor. The, run at 30°C has a greater K_R than the run at 42°C. If a K_R indicates both less stressful conditions and the reaction rate of DO consumption, this result is counterintuitive since one would generally expect a reaction rate to increase with temperature. However, this figure indicates more stress at lower temperatures.

5.3.3 Amount of Blood

All other conditions being equal, the less blood injected into the reactor, the greater the K_R. Therefore, the more blood injected, the greater the rate of DO decrease in the reactor as DO in the reactor decreased. This is what one would expect. Since blood is responsible for the DO consumption , there will be more of whatever is consuming oxygen if more blood is added. There are more data points on the figures showing the blood amount, such as Figure 11, than on the other graphs in this chapter.

Table 5.3 K_R Values for 100 ppm phenol and different amounts of blood in the reactor (Figs 5.11-5.13) As amount of blood increases, the DO consumption rate increases. This is what one would expect. Since blood is responsible for the DO consumption, there will be more of whatever is consuming oxygen if more blood is added.

Temp. (°C)	**saline or water**	**K_R with 0.01 ml blood (1/hr)**	**K_R with 0.015 ml blood (1/hr)**	**K_R with 0.05 ml blood (1/hr)**	**K_R with 0.1 ml blood (1/hr)**	**K_R with 0.2 ml blood (1/hour)**
30	saline	3.64, 3.45 (two runs)	4.91	3.37	na	na
42	saline	na	13.87	44.31, 5.57 (two runs)	2.15, 1.05 (two runs)	0.26
42	water	na	9.37	0.58, -0.05 (two runs)	-0.20	na

na (not available): no runs were done with these conditions

Different amounts of blood at 42 C in 100 ppm phenol. Reactor was filled with saline. Runs 142-1, 143-2, 144-1, 144-2, 145-1, 201-1 are shown. Each line is labeled with the volume of whole blood injected. All runs were aerated after blood addition.

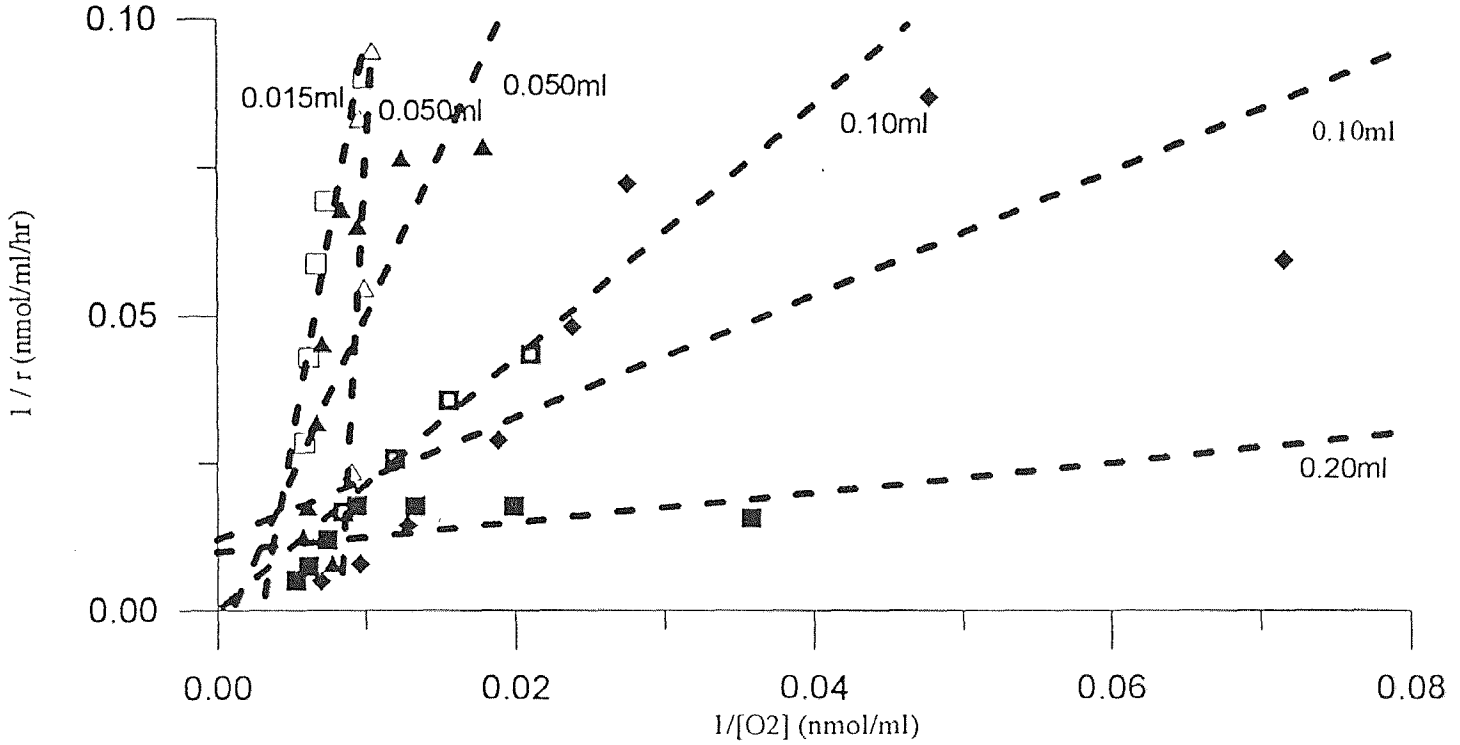


Figure 5.11 Effect of blood amount on DO consumption in 100 ppm phenol in saline filled reactor at 42 °C. Therefore, the more blood injected, the greater the rate of DO decrease in the reactor as DO in the reactor decreased. This is what one would expect. Since blood is responsible for the DO consumption , there will be more of whatever is consuming oxygen if more blood is added.

5.3.4 Concentration of Phenol in the Reactor

The trends observed in phenol stressed blood are initially puzzling since phenol at low concentrations appears to have an acceleratory effect on DO consumption while phenol at high concentrations has an inhibitory effect on DO consumption. This is consistent with observations of phenol and DO consumption rate by Lakhwala. (1991). According to Fayaz, at low concentrations, phenol is oxidized by bacteria, causing an increase in DO consumption. At high phenol concentrations, phenol's toxic effects on bacteria begin to have an inhibitory effect on oxidation and the result is lower DO consumption rate.

The non linear effects of phenol on blood are observed by both these aeration studies and the fast assay runs discussed in Chapter 6.

Table 5.4 K_R Values for different phenol concentrations in the reactor; variations from 100 ppm (Figs 5.14-5.16). The non-linear effect of phenol on blood's DO consumption observed in this table is explored further in Chapter 6 in phenol's dose-response curve.

Amount of blood (ml)	Temp. (°C)	saline or water	K_R with 0 ppm phenol (1/hr)	K_R with 100 ppm phenol (1/hr)	K_R with 475 ppm phenol (1/hr)	K_R with 525 ppm phenol (1/hr)
0.1	30	saline	na	8.32	11.24	na
0.01	42	water	na	0.49	na	0.66
0.2	42	saline	34.01	3.16	na	na

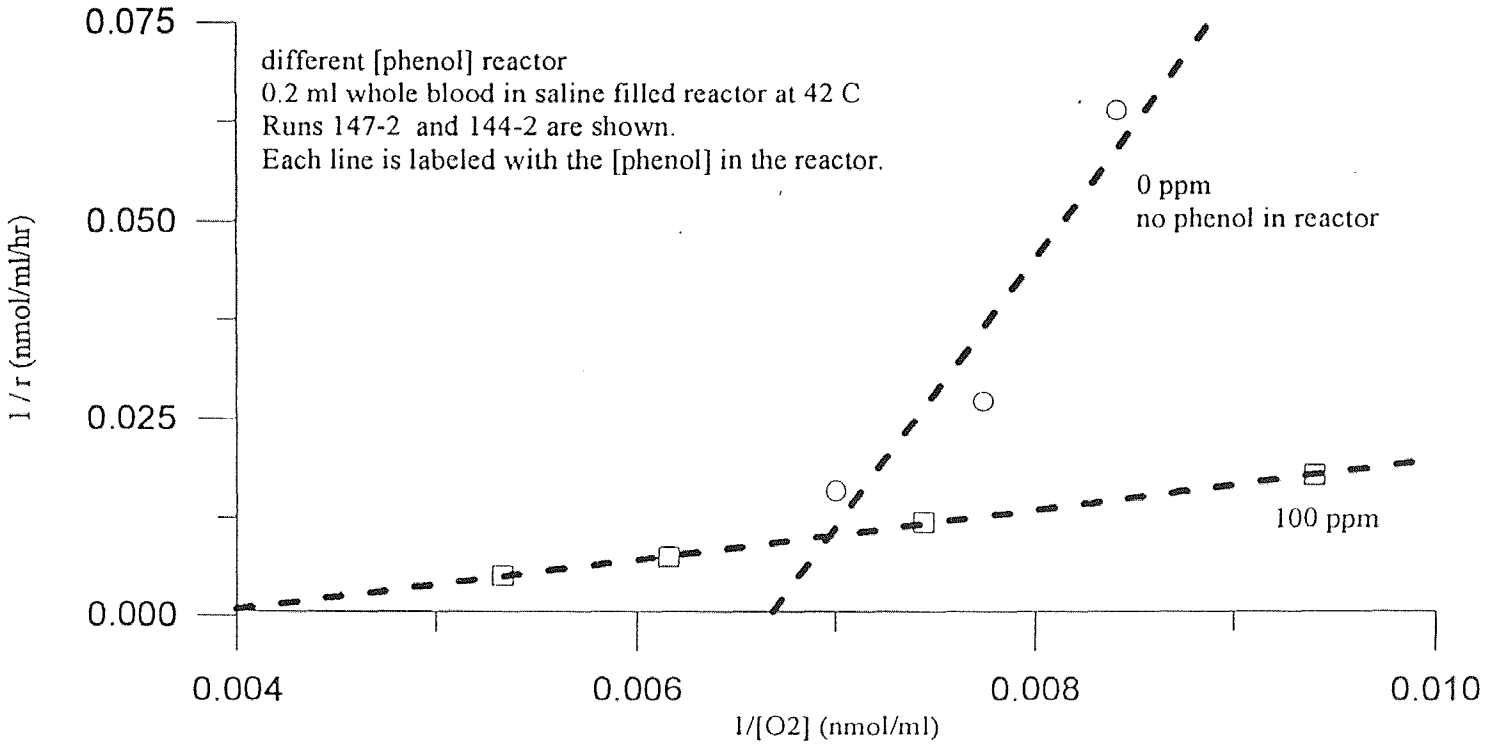


Figure 5.14 Effect of phenol concentration in the reactor on DO consumption for 0.2 ml blood in saline filled reactor at 42 °C. Since phenol is considered to be stressful to blood, one would expect the run with phenol to have a greater K_R. However, this is not observed. This non linear effect of phenol on blood's DO consumption is consistent with toxicant oxidation causing an increased DO consumption.

5.4 Advantages and Drawbacks of Aeration Studies

Unlike many immunological and toxicological methods, this method does not require expensive, hard to handle chemicals or protocols such as those using monoclonal antibodies, or rigorously aseptic techniques. We are not trying to study a systemic immune response, rather we are looking only at the cellular response. The results are straightforward in that the only reaction parameter we track is oxygen and, as with all reaction kinetics tracking, each run is data-rich.

The observation of rotation of runs is encouraging in its implication that we had achieved an indication of the stress conditions of a blood sample. The rotation is a very qualitative indication of stress and this method could stand improvement in terms of quantitation. The fast assay runs and the in-depth quantitation of MOPs are both more quantitative than the aeration runs.

Although the double-reciprocal examination of these phenomena has several advantages (discussed in Section 5.2 Quantitative Analysis for Aeration Studies), this method of analysis is labor intensive. Computerized techniques are recommended in case large numbers of runs are anticipated.

The extremely reproducible trend in rotation in these runs is encouraging. It is a consistent observation that runs which had been aerated consumed oxygen more slowly than runs which had not been aerated. This observation is so obvious to the naked eye observing the oxygraph chart recorder that it was decided to compare aeration runs and non-aeration runs separately. A characteristic example of this difference in oxygen consumption between aeration and non-aeration runs is shown in Figure 5.17 in Appendix

5. This phenomenon is not a drawback as such but it is considered a drawback because it presents a complication to the widespread use of this assay as a blood stress indicator.

These aeration runs stem from the previous 24 hour runs and the realization that valid observations may be made about the stress state of a blood bioreactor system within one hour.

CHAPTER 6

FAST ASSAY RUNS

The experiments and results stemming from the fast assay experiments described in this chapter may serve as an introduction to an inexpensive, intensely quantitative alternative to extant toxicological tests.

As introduced earlier in this work, blood added to a saline filled reactor produces an initial response in the DO in the reactor. In the absence of high concentrations of phenol, this initial response is usually a decrease in dissolved oxygen (DO) in the reactor. The initial response is influenced by the amount of blood injected and the temperature of the reactor. The initial response is also profoundly influenced, in a dose-response manner, by the concentration of phenol present in the reactor. When phenol concentrations are sufficiently high (generally 20,000 ppm or more), the initial response may be an increase in DO in the reactor.

6.1 Additional Methods for Fast Assay Runs

Blood is injected into a 2 ml saline filled reactor. This reactor is surrounded by a temperature-controlled water jacket. In the experiments described in this chapter, phenol may be added to the reactor before the blood has been added.

When blood is added to the reactor, a near immediate initial response in DO in the reactor occurs. When the initial response is a decrease in DO in the reactor, it is called an "initial drop" (ID). The ID's amount (nmol O_2) and its velocity (IDV)(nmol/ml/sec) can

be quantified. When the initial response is an increase in DO in the reactor, it is called an "initial rise" (IR). This initial rise and its velocity (IRV) can be quantified in the same manner as the initial drop.

Labeled oxygraph recordings showing the ID, IDV, IR and IRV are shown in Figure 6.1 in Appendix 6.

Each run is about 3-5 seconds long. The results from these experiments are very reproducible within any given blood sample. Each run is repeated 3-4 times. The initial response and its velocity is measured as described in Chapter 4. For all tables in this chapter, the amount of oxygen in the initial response and its velocity is reported as the average of 3-5 runs. When the initial response is shown on a graph, each run is shown separately unless noted otherwise.

The probe used to measure dissolved oxygen (DO) concentrations is temperature sensitive. Ice cold saline is used as a control to show temperature effects on the DO reading of adding cold liquid to the reactor

6.1.1 Conventions for Tables in this Chapter

For the purposes of these tables, examination of the initial response is split into two components, the initial drop (ID) which is a decrease in DO in the reactor and the initial rise (IR) which is an increase in DO in the reactor.

6.2 Initial Response as a Function of Phenol Concentration and Amount of Blood Added

Figure 6.2 compares the initial response with amount of blood added. This is a repeat of

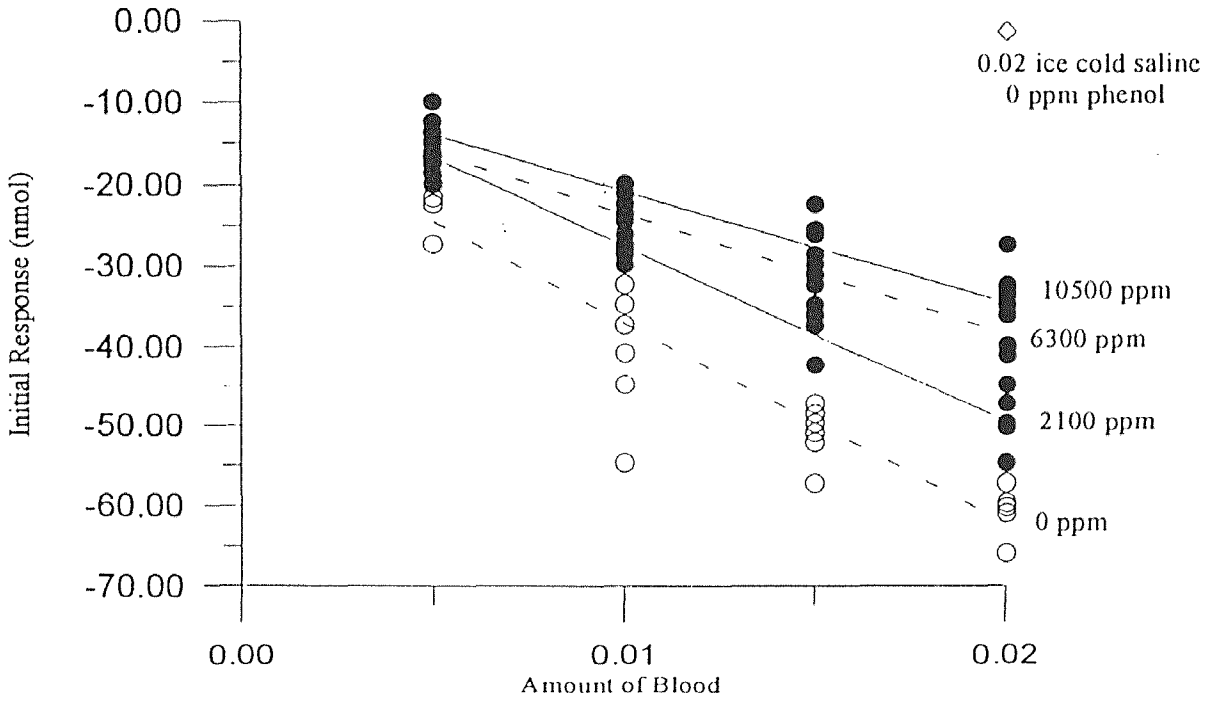


Figure 6.2 Initial response vs. amount of blood on 7/19/96. The open circles are with no phenol in the reactor; the closed circles are with various amounts of phenol in the reactor; the open diamond is ice cold saline injected into the reactor as a control. This figure demonstrates that the amount of oxygen in the initial response is directly related to the amount of blood injected into the reactor in a dose-dependent manner.

the experiment shown in Figure 4.1, done on 7/17/96. The runs shown in Figure 6.2 were done two days after the experiment shown in Figure 4.1. As in Figure 4.1, there is a dose response relationship between the initial response and both the amount of blood added and the concentration of phenol in the reactor. These figures demonstrate that the amount of oxygen in the initial response is directly related to the amount of blood injected into the reactor.

The initial response velocity compared to the amount of blood added and concentration of phenol in the reactor is shown in Figure 6.3 in Appendix 6. Figure 6.3 demonstrates that the initial response's velocity is directly related to the amount of blood injected into the reactor.

6.3 Initial Response per Milliliter of Packed Cells

The slope of each line in Figure 6.2 is plotted against the concentration of phenol in the reactor. As a result, Figure 6.4 shows the initial response per milliliter of packed cells This is based on the runs shown in Figure 6.2 and indicates that the initial response is a function of blood amount.

Figure 6.4 and Figure 4.4 are the same type of experiment done two days apart using the same blood sample. The trends in both are in the same direction but the intensity of the initial response has changed. The initial response examined by nmol O_2 per ml of packed cells has decreased by 40 % in two days when comparing the experiment done on 7/19/96 with the experiment done on 7/17/96. The effects of blood age on the initial response are explored further in Section 6.6.4.

6.4 Dose Response Curve of the Initial Response with a Wide Range of Phenol Concentrations

Figure 6.5 shows the dose-response curve of one blood amount stressed with a wide range of phenol concentrations on 7/15/97. As with many dose-response curves, this one is not linear. A closer look at the low range of phenol concentration appears in Figure 6.6. In Appendix 6 is the companion examination of this experiment's velocity (both the whole range of phenol concentrations, in Figure 6.7, and the closer examination of the lower concentrations of phenol, in Figure 6.8.)

This dose-response curve is measured on two different occasions, on 7/15/96 and on 7/23/96. The data for the latter, which exhibit basically the same dose-response curve as 7/15/96, appear in Figures 6.9-6.12 Appendix 6.

In both curves the response at lower phenol concentrations is an initial decrease in the DO in the reactor. This initial decrease in the DO in the reactor decreases in magnitude as phenol concentration increases until a plateau is reached after about 1,000 ppm phenol which continues until about 20,000 ppm phenol. During this plateau, the initial response remains the same despite increasing phenol concentrations. At about 20,000 ppm phenol, the initial response becomes an initial rise in the DO in the reactor which may or may not still be accompanied by the initial drop. Sometimes there is an ID but not an IR and vice versa. Sometimes there is both an ID and an IR. When the ID and IR occur in the same run, the ID always occurs before the IR.

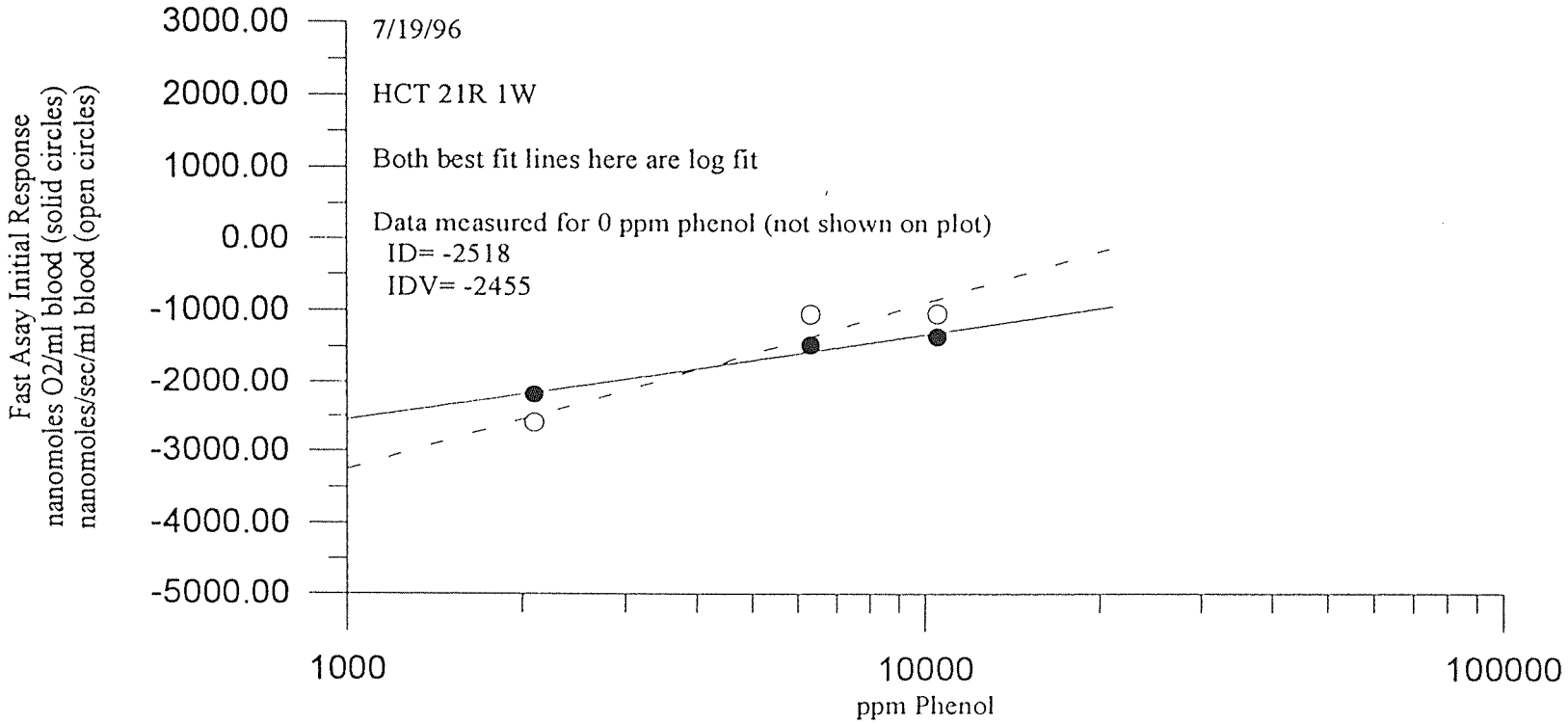


Figure 6.4 Initial response per ml of packed cells on 7/19/96. The closed circles and solid line show the amount of oxygen in the initial response per milliliter of packed cells and the open circles and dashed line show the initial response velocity per milliliter of packed cells. This figure is based on the runs shown in Figure 6.2 and indicates that the initial response is a function of blood amount.

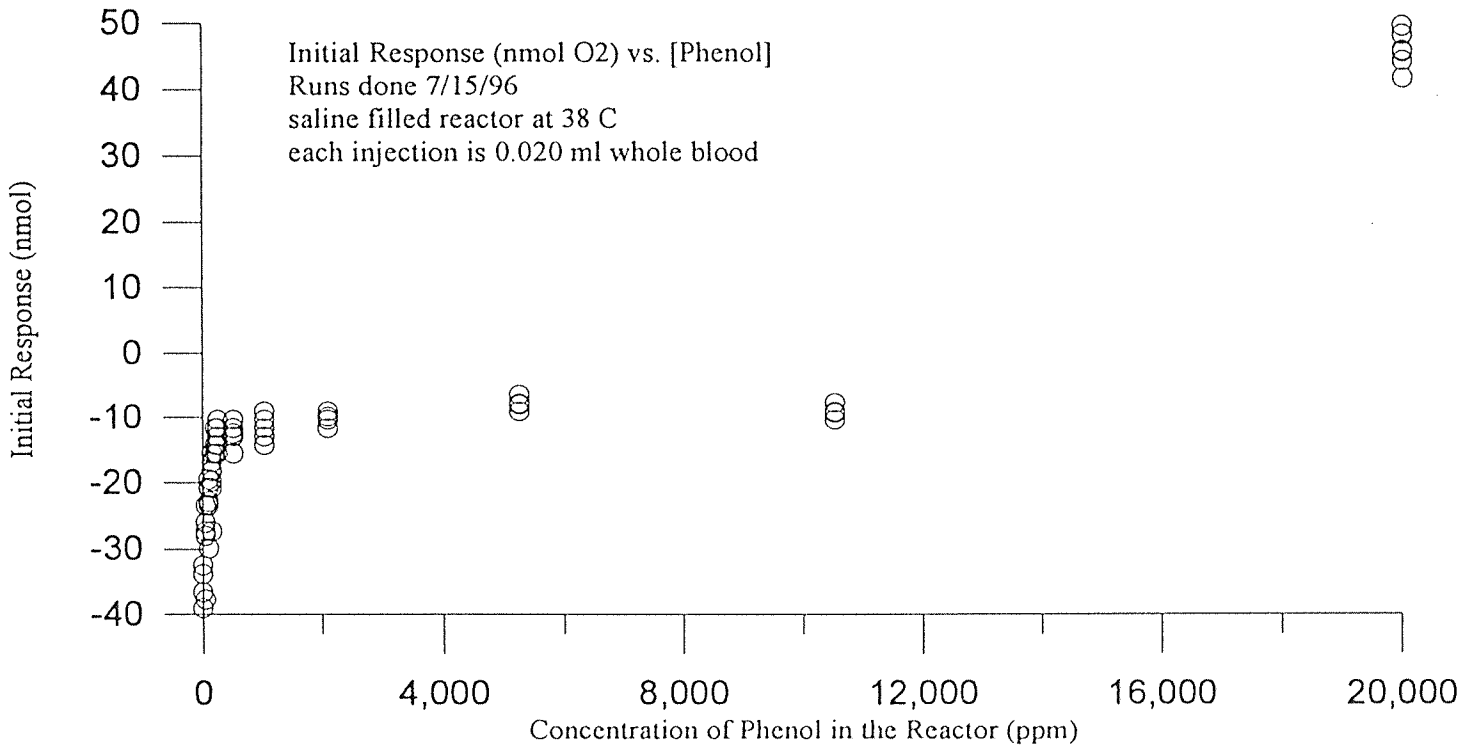


Figure 6.5 The dose response curve of the initial response of 20µl aliquots of blood stressed with a wide range of phenol. The response is not linear throughout the range of phenol. A basic description of this dose response curve of phenol consists of three phases: the low, middle, and high ranges of phenol concentration. In the low range (0-1,000 ppm phenol), the initial response is a decrease in DO in the reactor. The initial response in the low range is inversely proportional to the concentration of phenol in the reactor. In the middle range (1,000-20,000) the initial response is a decrease in DO in the reactor. The initial response remains constant during this "plateau" range. In the high range (20,000 and over), the initial response includes an initial rise of DO in the reactor. Note that the values given for low, middle and high fluctuate somewhat between different blood samples.

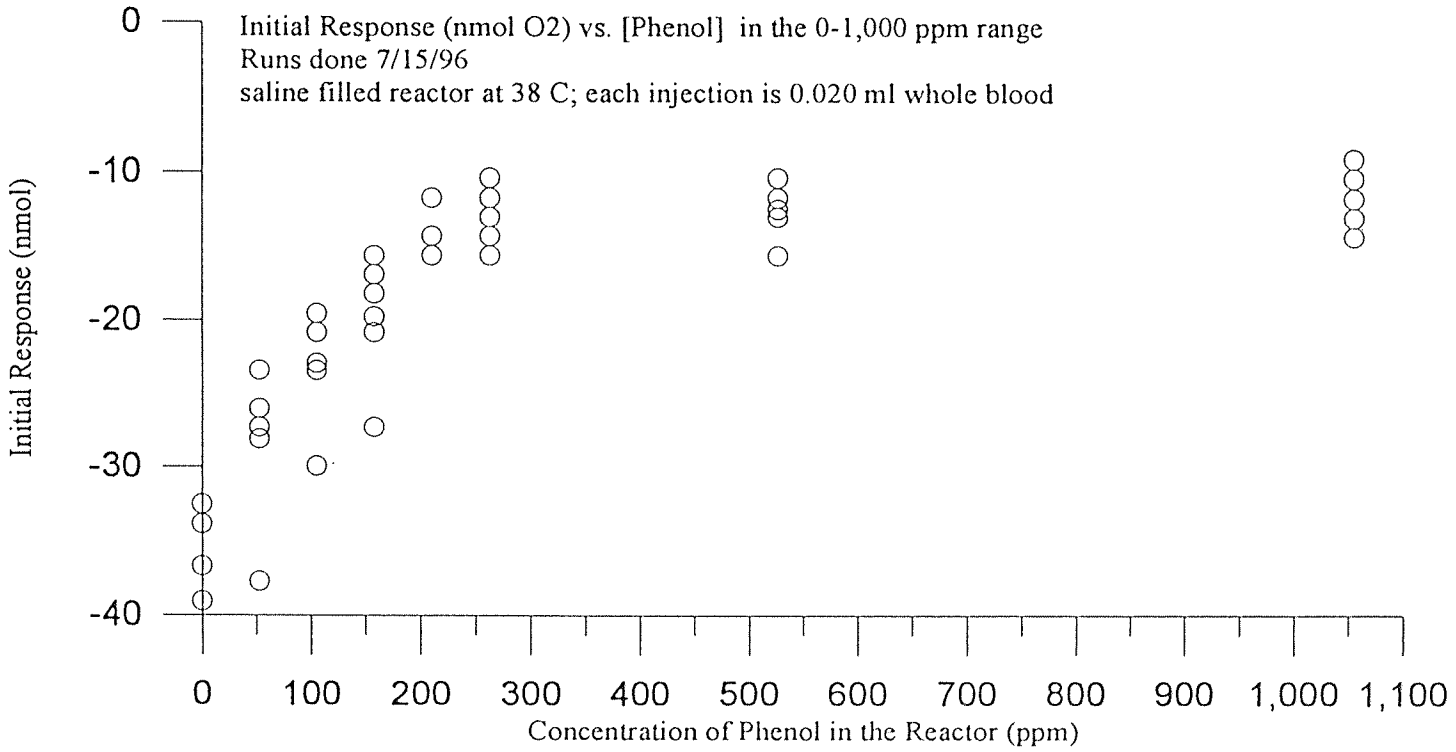


Figure 6.6 The 0-1,100 ppm phenol range of the dose response curve shown in Figure 6.5.

6.4.1 The Basic Dose-Response Curve for Blood and Phenol

A basic description of this dose response curve of phenol, seen in Figures 6.5-6.12, consists of three phases of the dose response curve: the low, middle, and high ranges of phenol concentration. In the low range (0-1,000 ppm phenol), the initial response is a decrease in DO in the reactor. The initial response in the low range is inversely proportional to the concentration of phenol in the reactor. In the middle range (1,000-20,000) the initial response is a decrease in DO in the reactor. The initial response remains constant during this "plateau" range. In the high range (20,000 and over), the initial response includes an initial rise of DO in the reactor. Note that the values given for low, middle and high are approximate. These values fluctuate somewhat between different blood samples.

The dose-response curve of phenol and blood is largely consistent with previous work done in the NJIT Biotechnology Lab. As mentioned in Chapter 5, Section 5.3.4, addition of phenol at low concentrations to bacterial bioreactor systems causes increased DO consumption as the phenol is oxidized by the bacteria. Then, as phenol concentration increases, the DO consumption is inhibited as the bacteria's oxidation mechanism undergoes substrate inhibition by phenol. Thus, at very high concentrations, phenol's toxic effects overwhelm the bacteria's ability to detoxify the phenol. It has not been established what substance within the blood is responsible for the initial response described in this dissertation. However it is not unreasonable to speculate that some similar enzymes which would oxidize phenol and would have similar DO consumption responses to phenol are present in both bacteria and blood.

Unlike the bacteria dose-response relationship, the blood fast assay runs don't indicate increased DO consumption at low phenol concentrations. However they do evidence a change in the magnitude and direction of the response with increasing phenol concentrations, since the initial response eventually becomes an initial rise.

6.5 Serial Addition of Blood into High Concentration of Phenol

In this experiment, 20 μl aliquots of blood were added sequentially to a saline filled reactor with 31600 ppm phenol. This allows examination of the following query: Does DO in the reactor above air saturation have an effect on the initial response? It appears, from Figure 6.13, that the initial response is independent of DO increases above air saturation to about 420 nmols in the reactor (about 220 nmols/ml saline). The fluctuation in initial response above 220 nmols/ml could be due to the effects of increased DO or a change in the blood during the initial rise affects the sensitivity of the DO probe.

6.6 Examination of the Effects of Temperature, Size of Sample, and Phenol Concentration on the Initial Response

Table 6.1 shows the effects of blood age, temperature and size of sample on fast assay runs. Note that the larger sample always gives a larger initial response and a faster IDV or IRV. The higher the temperature, the smaller the ID and IDV but the greater the IRV. The effect of one day's aging for all three temperatures appears to be minimal. Each value is the average of 3-5 runs. It is clear from this table that the phenomenon which causes the initial response is temperature sensitive.

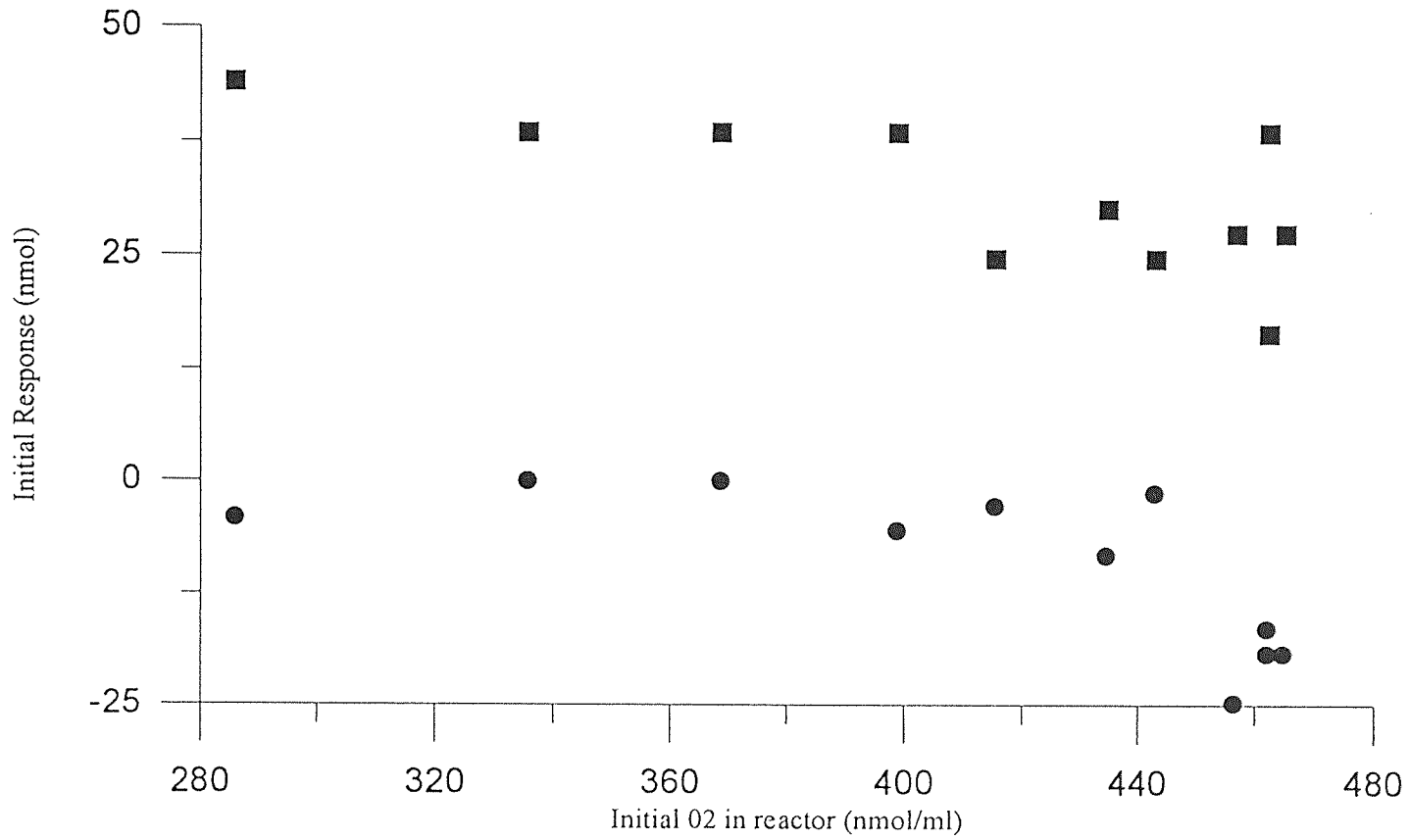


Figure 6.13 Serial addition of 20 µl aliquots of blood into high phenol concentration. The initial response in each run contains both an initial rise (squares) and an initial drop (circles). This figure examines whether or not the DO in the reactor above air saturation has an effect on the initial response. It appears, from this figure that the initial response is independent of DO increases above air saturation to about 420 nmols in the reactor (about 220 nmols/ml saline). The decrease in initial response above 220 nmols/ml could be due to the effects of increased DO or it could be that the protein in the blood affects the sensitivity of the DO probe.

Table 6.1 The effects of blood age, temperature and size of sample on fast assay style runs. Note that the larger sample always gives a larger initial response and a faster initial drop velocity (IDV) or initial rise velocity (IRV). The higher the temperature, the smaller the initial drop (ID) and IDV but the greater the IRV. Each value is the average of 3-5 runs. It is clear from this table that the phenomenon which causes the initial response is temperature sensitive. It is also clear that the initial rise (IR) is a part of the initial response only at the higher phenol concentration examined here.

ppm phenol	25°C ID O_2 nmol	ID max vel nmol/sec	IR O_2 nmol	IR max vel nmol/sec	*35°C ID O_2 nmol*	*ID max vel nmol/sec*	*IR O_2 nmol*	*IR max vel nmol/sec*	45°C ID O_2 nmol	ID max vel nmol/sec	IR O_2 nmol	IR max vel nmol/sec
runs done 11/12/96												
10 µl whole blood												
0	-47.4	-49.1	0	0	*-26.3*	*-36.2*	*0*	*0*	-15.5	-23.5	0	0
3160	-32.7	-40.9	0	0	*-23.7*	*-24.4*	*0*	*0*	-15.5	-20.0	0	0
31600	-16.4	-35.4	0	0	*-11.6*	*-20.4*	*22.1*	*14.1*	-7.8	-17.2	16.1	15.3
20 µl ice cold saline												
	0	0	0	0	*-4.21*	*-6.7*	*0*	*0*	-5.2	-10.6	0	0
20 µl whole blood												
0	-68.7	-68.0	0	0	*-47.4*	*-69.5*	*0*	*0*	-30.4	-36.2	0	0
3160	-65.4	-70.6	0	0	*-42.1*	*-49.3*	*0*	*0*	-23.3	-36.2	0	0
31600	-47.0	-49.5	0	0	*-24.2*	*-43.6*	*50.5*	*30.3*	-3.2	-8.9	38.8	44.9
runs done 11/13/96												
20 µl whole blood												
0	-65.8	-76.4	0	0	*-50.7*	*-55.8*	*0*	*0*	-29.6	-41.1	4.8	3.8
3160	-62.2	-70.7	0	0	*-42.3*	*-50.1*	*0*	*0*	-25.4	-41.9	5.5	4.1
31600	-44.2	-55.8	36.1	18.5	*-25.43*	*-35.9*	*31.7*	*23.7*	-1.0*	-4.3	44.0	69.7
20 µl ice cold saline	0	0	0	0	*0*	*0*	*0*	*0*	-6.9	-8.8	6.87	7.7
20 µl ice cold saline in 31600 ppm phenol					*0*	*0*	*0*	*0*				

* indicates that at least one run in this series had an initial response of 0

6.7 Examination of the Initial Response of an Additional Toxicant (1-butanol) and Comparison with Phenol

According to the Merck index, in rats, the LD_{50} of phenol is 0.530 g/kg while the of 1-butanol is 4.36 g/kg. This means that phenol is roughly eight times more toxic than 1-butanol. Of course, the LD_{50} is an organismal effect while the fast assay measures molecular and cellular responses. Despite these impediments to extrapolation, it is still reasonable to expect that phenol should be much more toxic than 1-butanol. If, as is our contention, inhibition of initial drop, and stimulation of initial rise are to be considered responses to stress then butanol would be considered far less toxic than phenol by our assay.

All injections in the study presented in Table 6.2 are 0.2 ml blood of HCT 2.25. The blood was prepared by mixing 2 ml of blood into 20 ml saline. An injection of 20 ml of the original sample which had HCT 30% erythrocytes and 1% leukocytes, the initial response is an initial drop of 102.7 nmol O_2.

Butanol is not totally miscible with the blood so the maximum saturation of butanol in water, 80,300 ppm is used as the maximum concentration of butanol in the reactor.

An interesting comparison would be of the respective concentrations of phenol and butanol necessary to give the same initial rise. It is possible to compare the concentration of butanol that produces an initial rise of 13.8 nmol O_2 (which is 80,300 ppm butanol) with the concentration of phenol that would be necessary to produce an initial rise of 13.8 nmol O_2. This is done by interpolating, from the initial rise at 17,700 and 35,300 ppm phenol,

what the phenol concentration would be if the initial rise were 13.8 nmol O_2. This concentration of phenol is 23,000 ppm phenol. (80,300/23,000) is about 3.5 therefore according to our test, phenol is about 3.5 times more toxic than butanol. According to the Merck Index, phenol is about 8.2 times more toxic than butanol.

Table 6.2 Comparison of the effect of butanol and phenol on the initial response of 20 µl whole blood. If, as is our contention, inhibition of initial drop, and stimulation of initial rise are to be considered responses to stress then butanol would be considered far less toxic than phenol by our assay. According to the Merck index, in rats, the LD_{50} of phenol is 0.530 g/kg while the of 1-butanol is 4.36 g/kg, thus phenol is about 8.2 times more toxic than butanol.

Runs done 3/3/97 38.5 °C	**ID O_2 (nmol)**	**IR O_2 (nmol)**
Reactor filled with saline (no phenol or butanol)	-11.1	0
17,700 ppm phenol	-13.9	6.9
35,300 ppm phenol	-2.1	52.6
52,900 ppm phenol	-2.8	60.3
80,300 ppm 1-butanol	-5.5	13.8

6.8 Exploration of Possible Influences on the Initial Response

If these fast assay runs are to serve as the basis for a toxicity test, then interfering influences must be kept at a minimum.

6.8.1 Effects of Handling Stress

The effects of both the removal of plasma and the stress of spinning in the centrifuge are examined in this table. Three samples of blood from a single 100 ml aliquot are examined. The first sample is whole blood, not spun in the centrifuge. The second sample is whole blood spun down in the centrifuge and resuspended. This third is whole blood spun down in the centrifuge, the plasma removed, and a volume of saline equal to the amount of plasma removed added to the remaining packed cells which were then resuspended in saline.

The whole blood not spun gives a much less intense response than the samples that subjected to centrifugation. This indicates that the stress of handling and spinning in the centrifuge does not dampen the response, instead this treatment makes the cells more likely to react. There is minimal difference between the samples which were both spun. The removal of plasma does not affect the response so it is unlikely that plasma is involved in the initial response.

Leukocyte rich blood for most of these experiments is obtained by spinning blood in the centrifuge and removing the leukocyte rich buffy coat. Since it has been observed and noted that leukocyte rich blood is more likely to produce a MOP than is whole blood, these centrifuge stress studies provide a crucial control.

In light of these results, what appears to be a leukocyte rich blood response is in some of our experiments indistinguishable from a spinning stress response. The fractionated blood 3 minute assays presented later are all obtained either without spinning or with spun and resuspended whole blood as a control.

Table 6.3 Effect of Handling Stress on the Initial Response. The whole blood not spun gives a much less intense response than the samples subjected to centrifugation. This indicates that the stress of handling and spinning in the centrifuge does not dampen the response, instead this treatment makes the cells more likely to react. There is minimal difference between the samples which were both spun. The removal of plasma does not affect the response so it is unlikely that plasma is involved in the response.

Runs done 11/18/96 all injections are 20 µl of whole blood	ppm phenol	*38 C* *ID O_2* *nmol*	*ID max vel* *nmol/sec*	*IR O_2* *nmol*	*IR max vel* *nmol/sec*
whole blood not spun					
	0	*-45.9*	*-48.2*	*0*	*0*
	31600	*-13.4*	*-20.1*	*9.6*	*5.2*
whole blood spun and resuspended					
	0	*-22.9*	*-22.9*	*0*	*0*
	31600	*0*	*0*	*21.0*	*19.5*
whole blood spun, plasma decanted, and resuspended in saline					
	0	*-19.1*	*-21.1*	*0*	*0*
	31600	*0*	*0*	*22.9*	*19.1*

6.8.2 Comparison of the Effects of Phenol on Whole blood and Leukocyte Rich Blood

The examination of whole blood in comparison with leukocyte rich blood at several temperatures provides an interesting effect. If leukocytes were responsible for the observed initial response, one would expect the leukocyte rich samples to give the most intense response. However, as is clear from Table 6.4, this is not the case. Therefore the results of this particular experiment do not support the hypothesis that leukocytes are responsible for the initial response.

Table 6.4 Whole Blood vs. Leukocyte Rich Blood. The examination of whole blood in comparison with leukocyte rich blood at several temperatures provides an interesting effect. If leukocytes were responsible for the observed initial response, one would expect the leukocyte rich samples to give the most intense response. However, as demonstrated in this table this is not the case. Therefore the results of this particular experiment do not support the hypothesis that leukocytes are responsible for the initial response.

	26 C				*36 C*				46 C			
ppm phenol	ID O_2 nmol	ID max vel nmol /sec	IR O_2 nmol	IR max vel nmol /sec	*ID O_2 nmol*	*ID max vel nmol /sec*	*IR O_2 nmol*	*IR max vel nmol /sec*	ID O_2 nmol	ID max vel nmol /sec	IR O_2 nmol	IR max vel nmol /sec
runs done 11/13/96												
20 µl whole blood												
0	-65.8	-76.4	0	0	*-50.7*	*-55.8*	*0*	*0*	-29.6	-41.1	4.8	3.8
3160	-62.2	-70.7	0	0	*-42.3*	*-50.1*	*0*	*0*	-25.4	-41.9	5.5	4.1
31600	-44.2	-55.8	36.1	18.5	*-25.4*	*-35.9*	*31.7*	*23.7*	-1.0*	-4.3	44.0	69.7
20 µl ice cold saline												
	0	0	0	0	*0*	*0*	*0*	*0*	-6.9	-8.8	6.87	7.7
20 µl ice cold saline in 31600 ppm phenol									-6.9	-15.4	8.3	8.4
20 µl leukocyte rich blood												
0	0	0	0	0	*-6.3*	*-12.7*	*4.9*	*15.7*	-11.0	-14.2	11.7	17.8
3160	0	0	0	0	*-1.4**	*-3.7*	*0*	*0*	-10.4	-17.2	9.4	13
31600	0	0	0	0	*-0.53*	*-1.9*	*16.9*	*14.2*	-0.69*	-1.7	19.9	21.8

* indicates that at least one run in this series had an initial response of 0

6.8.3 Effect on the Initial Response of the DO level in the Reactor Before Blood is Added

As is demonstrated in Figure 6.14, the DO level in the reactor has an effect on the initial response. As the DO in the reactor before blood addition increases, the initial drop increases. Whether the DO in the reactor is depleted by blood addition or by N_2, the change in the initial drop is the same amount, therefore this change is DO dependent not blood dependent.

All runs done in this chapter have been done with the DO in the reactor at air saturation before the blood is added to avoid dealing with the effects of DO in the reactor at the beginning of the run. This avoids dealing with hemoglobin (as explained in the aeration chapter) and any other unknown influences which contribute to the effect of DO level before blood is added.

6.8.4 Effects of Blood Age on the Initial Response

The effect of blood age at different phenol concentrations is shown in Table 6.5. All runs are 20 μl blood and 36-38 C. The response is not unidirectional and is noted here as an indication of the fairly large range of response that is considered normal. It should be noted that in biological systems, the range of normal response is often large. Each value is the average of 3-5 runs.

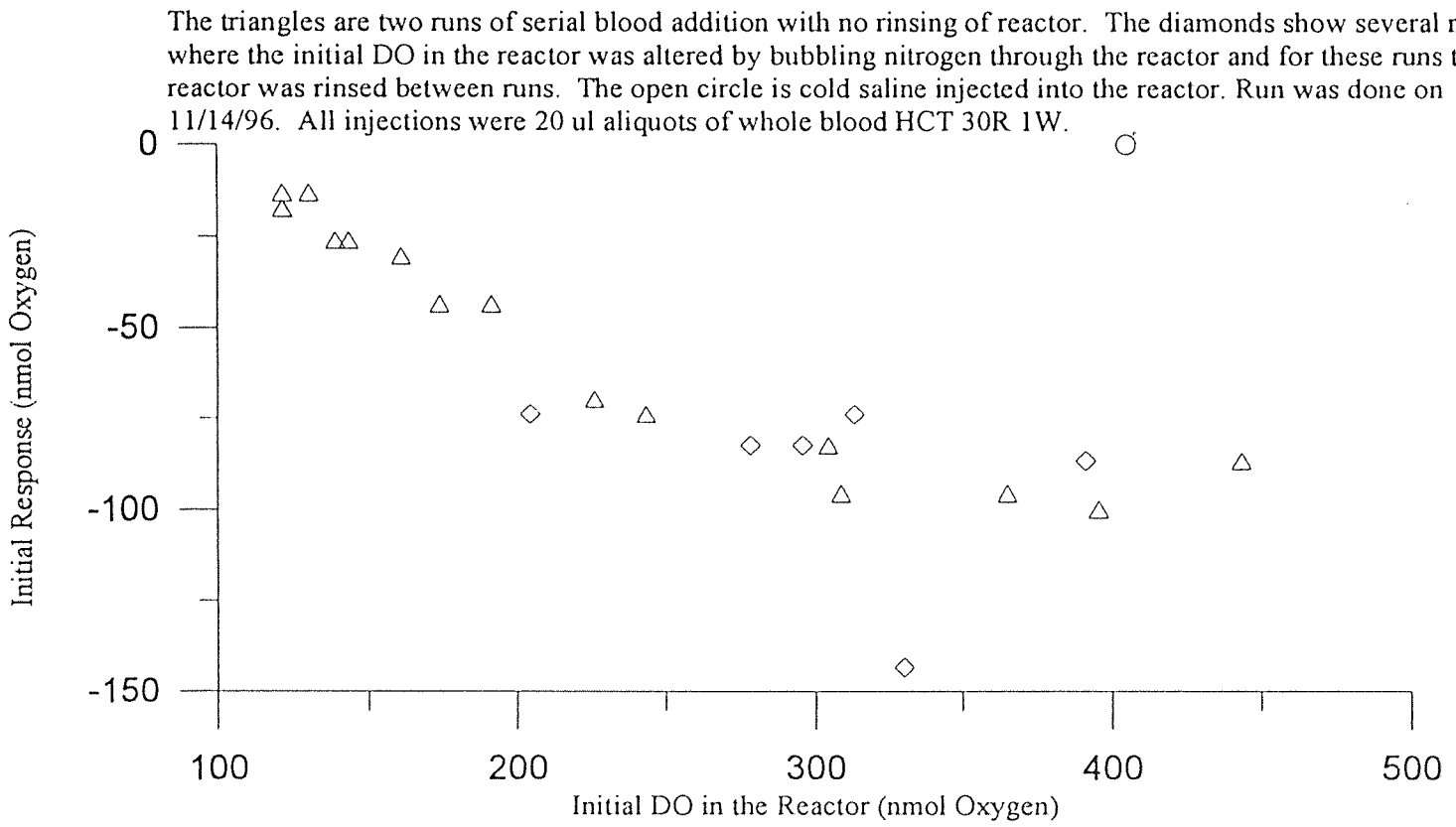


Figure 6.14 Effect on the initial response of the DO level in the reactor before blood is added. This figure demonstrates that the DO level in the reactor has an effect on the initial response. As the DO in the reactor before blood addition increases, the initial drop increases. Whether the DO in the reactor is depleted by blood addition or by N_2, the change in the initial drop is the same amount, therefore this change is DO dependent not blood dependent.

The overall trend of the dose response relationship is consistent; increased phenol causes decreased ID and high concentrations of phenol cause an IR. The magnitude of the initial response fluctuates day to day.

Table 6.5 Effect of Blood Age on the Initial Response of 20 µl of whole blood.

DATE	ppm phenol	*36 C ID O_2 nmol*	*ID max vel nmol/sec*	*IR O_2 nmol*	*IR max vel nmol/sec*
11/12/96	0	*-47.4*	*-69.5*	*0*	*0*
	3160	*-42.1*	*-49.3*	*0*	*0*
	31600	*-24.2*	*-43.6*	*50.5*	*30.3*
11/13/96	0	*-50.7*	*-55.8*	*0*	*0*
	3160	*-42.3*	*-50.1*	*0*	*0*
	31600	*-25.4*	*-35.9*	*31.7*	*23.7*
11/14/96	0	*-45.6*	*-54.7*	*0*	*0*
	3160	*-52.1*	*-52.7*	*0*	*0*
	31600	*-21.7*	*-35.6*	*35.8*	*23.7*
11/18/96	0	*-45.9*	*-48.2*	*0*	*0*
	31600	*-13.4*	*-20.1*	*9.6*	*5.2*
11/27/96	0	*-76.3*	*-85.3*	*0*	*0*
	31600	*-29.9*	*-42.3*	*18.0*	*9.4*

6.8.5 Effect of Blood Aeration on the Initial Response

The initial response of aerated whole blood is compared with non-aerated whole blood. The aerated blood is prepared by drawing about 1 ml of blood into a syringe and squirting it back out into a beaker. This is repeated several times. The aerated blood has a larger

initial rise but a smaller initial drop than the non aerated blood. This is what one would expect if aeration serves to restock hemoglobin or some other reservoir within blood. Because of the method of syringe aeration (described in Section 5.1), aerated blood is handled more than non-aerated blood. The effects of handling stress on blood, as examined in Section 6.8.1, could be responsible for the observed difference in the aerated and non-aerated samples.

Table 6.6 The Effect of Aeration on Blood's Initial Response; 20 µl Whole Blood in Each Injection. The aerated blood has a greater initial rise but a lesser initial drop than the non aerated blood. This is what one would expect if aeration serves to restock hemoglobin or some other reservoir within blood.

Runs done 11/27/96	*38 C*			
0 ppm phenol in each run	*ID O_2*	*ID max vel*	*IR O_2*	*IR max vel*
	nmol	*nmol/sec*	*nmol*	*nmol/sec*
aerated whole blood	*-18.5*	*-18.8*	*2.2*	*5.9*
non-aerated whole blood	*-76.3*	*-85.3*	*0*	*0*

6.8.6 Teasing Apart the Initial Response's Two Components, the Initial Rise and the Initial Drop

In some initial responses, there is both an initial drop and initial rise. What is the connection, if any, between the two phenomena?

Generally, when both an ID and IR are part of the initial response, both components of the initial response are smaller than if the initial response is either an initial drop or an initial rise. The initial rise generally takes place at higher concentrations of phenol and the dose-response curve of initial response as a function of phenol concentration is not linear (even in the regions without an initial rise). These factors make teasing apart the initial drop from the initial rise a tricky proposition.

Might the initial rise and initial drop always occur together and are only observed as separate entities if they're separated by time? In short, are they masking each other? It is also possible that phenol inhibits the mechanism which causes the initial drop and this is why the initial drop is smaller when both initial drop and initial rise are observed together.

To delve into this question, blood is added to a reactor that contains one milliliter of saline. The resulting initial response is measured. Then, as soon as the initial response is over, one milliliter of saline saturated with phenol is added to the reactor and the initial response is recorded. The small initial drop after the phenol addition is likely a temperature effect of adding 1 ml of liquid (about half of what the reactor can hold). The results, shown in Table 6.7, indicate that there is some masking, or inhibition, of the initial rise by the initial drop and vice versa. It is possible that the initial drop and initial rise are controlled by the same mechanism.

Table 6.7 Separation of Initial Rise from Initial Drop; 20 µl Whole Blood in Each Run. The initial rise is much greater when phenol is added separately. This indicates that the initial drop likely masks (or inhibits) the initial rise and vice versa.

Runs done 11/27/96	*38 C*			
ppm phenol	*ID O_2*	*ID max vel*	*IR O_2*	*IR max vel*
	nmol	*nmol/sec*	*nmol*	*nmol/sec*
0	*-76.3*	*-85.3*	*0*	*0*
31600	*-29.9*	*-42.3*	*18.0*	*9.4*
blood into 1 ml saline				
0	*-107.2*	*-74.8*	*0*	*0*
.....Then add 1 ml 60,000 ppm phenol				
31600	*-2.7*	*-9.2*	*74.5*	*45.6*
1 ml saline + 1 ml phenol (no blood)				
31600	*-24.7*	*-33.7*	*0*	*0*

6.9 Potential Mechanisms for the Observed Initial Response:

Following are several possible causes for the observed increase and subsequent decrease in DO. The catalysis back to O_2 of free radicals generated by the immune system may be a source for the increase in DO observed in the MOPs. It is possible that the initial drop and initial rise are controlled by the same mechanism. The ID and IR have an effect on each other as seen in Table 6.7. Also, the initial rise per ml of packed cells falls on the same best fit line as the initial drop (Fig 4.4 and Fig 6.4). It is unclear from these data whether this relationship is mechanistic or simply because they are temporally related and thus influence each other.

6.9.1 DO Decreases

Besides the most familiar mechanism for oxygen consumption in a biological system (namely cellular respiration), there are several other potential mechanisms for oxygen consumption in a blood system. The generation of oxygen free radicals from O_2 by the immune system may be responsible for the observed decrease in DO. Enzymes producing superoxide, a charged free radical, or nitric oxide (NO) will consume O_2, as will enzymes attacking xenobiotics and pollutant chemicals, such as the cytochrome P-450 and FMO-oxidase systems. A potential mechanism for oxygen consumption is the respiratory burst.

6.9.2 DO Increases: Potential Sources of Oxygen for Initial Responses that Include an Initial Rise

The oxygen source for the IR includes, but is not limited to, the possible oxygen sources for the MOP. However, hemoglobin is also a potential oxygen source for the IR (yet hemoglobin is not a potential oxygen source for the MOP.)

6.10 Conclusions and Recommendations

As examination of toxicological effects of pollutants is likely to continue and become more refined, the demand for tests such as these fast-assay runs can only increase. These fast-assay runs are technically simple and inexpensive and rapid. They could provide information about subpopulations sensitive to a particular drug or pollutant, as well as give an indication of tolerant subpopulations.

CHAPTER 7

MOP ACTIVITY

What would compel researchers to tie up their instruments for what eventually reached thousands of reactor hours? Besides the comfort provided by the knowledge that the reactor is busily collecting data while the researchers are sleeping or eating, these long term runs have provided the most intriguing, tantalizing, frustrating results.

7.1 Prediction of MOP Activity

Can the reliability of the aeration studies in their relationship to stress be used towards strengthening the evidence about MOPs? The trends observed in aeration encourage us to attempt to predict, by monitoring the oxygen consumption within the first hour of an experiment, the likelihood of MOP activity several hours later in the run. The ability to predict MOPs would lend tremendous credibility to our assertions regarding MOPs.

The first step is to get a preliminary determination whether this line of inquiry would be fruitful. This is done by compiling pairs of runs done under the same conditions (temperature, osmotic stress, amount of phenol and blood in the reactor, and aeration by syringe or not). The runs are then plotted against each other in the now familiar double reciprocal plot. These plots are shown in Figure 7.1 and Figures 7.2. The latter is in Appendix 7. Table 7.1 shows the K_R values for the runs compared in this initial investigation.

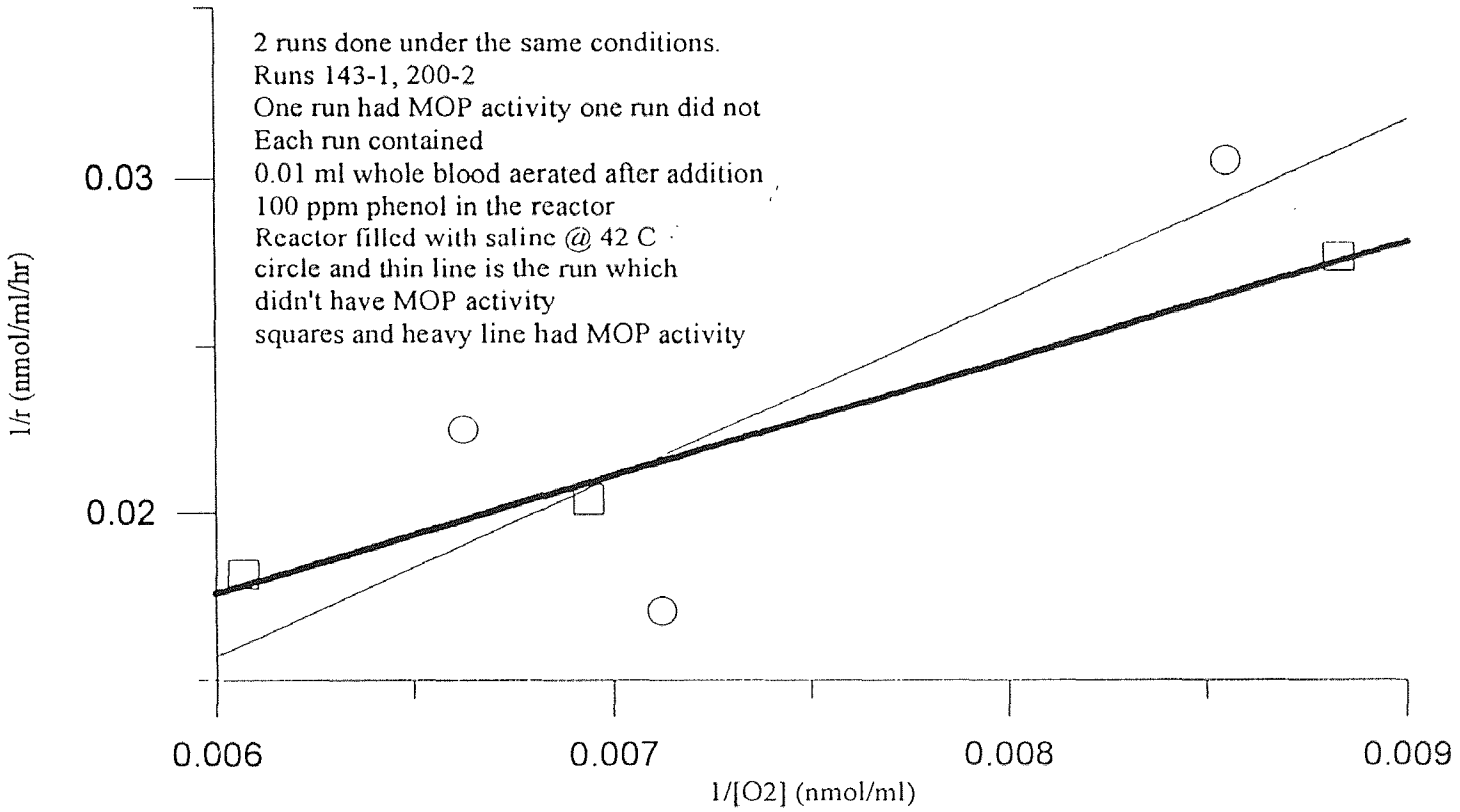


Figure 7.1 Two runs with the same reactor conditions. One run has MOP activity, one does not. The run which later had MOP activity has a markedly different rate of DO consumption than the run which did not have MOP activity. This finding implies that perhaps we can predict within an hour of blood addition whether there will be MOP activity in a run.

Table 7.1 K_R Values for runs with and without MOP activity (Figs 7.1-7.3). In one of the two groups of runs, the runs which had MOP activity have a lower K_R value than the runs which didn't have MOP activity.

Amount of blood (ml)	Amount of phenol (ppm)	Temp (°C)	Saline or water	K_R for run with MOP activity (1/hr)	K_R for run w/o MOP activity (1/hr)
0.01	100	42	saline	3.52	5.34
0.05	100	42	saline	5.02	13.63, 6.07 (two runs)

Figure 7.1 is a double reciprocal plot of two pairs of runs. Each pair of runs is done under the same conditions and yet one run exhibits MOP activity and the other does not. The runs which have MOP activity are shown with heavy lines and the runs which do not have MOP activity are shown with thin lines.

In Figure 7.1, the runs which later had MOP activity have markedly different rates of DO consumption than the runs which did not have MOP activity. This finding implies that perhaps we can predict within an hour of blood addition whether there will be MOP activity in a run.

Possibly the difference in rates between runs that produce a MOP and runs that do not implies that some mechanism has been set into motion -- a mechanism which evidences itself in one or more MOPs within several hours. Oxygen is released, and this release may to be coordinated amongst the cells because the oxygen is released in discrete and unique patterns.

Because runs done under identical conditions do not always produce the same amount (or lack thereof) of MOP activity, the propensity to generate MOP activity may be a function of the intrinsic state of a sample. Additional stress may or may not generate molecular oxygen. This is demonstrated by Figure 7.1 in which one run in each pair gave a MOP while the other run did not. Kinetics of the initial oxygen consumption suggested that a higher rate of decline tends to predict future MOP activity. This finding, made within an hour or two, may be of potential utility in developing a test to determine the sensitivity of blood to stress.

7.1.1 Experimental Procedure for Comparison of MOP vs. no MOP Studies

After comparing existing data on runs with and without MOP activity, a series of experiments which compared a split sample of blood under identical conditions seemed the best way to examine runs which differ only in the presence of MOP activity. Two reactors which shared the same temperature bath are used. The reactors contained the same osmotic and phenol conditions and blood samples are split and injected into the reactor within 1 minute of each other. These experiments are 24 hours long in duration. This experiment is repeated each day for five days.

At the completion of the pair of runs which ended on 6/14/96 (about 24 hours), each reactor is reaerated and phenol is added in three aliquots of 0.1 ml of 20,000 ppm phenol. The interval between each addition is about 10 minutes.

7.1.2 Conventions for Figures in this Chapter

The runs which have MOP activity are shown with heavy lines and the runs which do not have MOP activity are shown with thin lines.

Dashed lines show the best fit of data taken phenol addition and solid lines show the best fit of data after phenol addition. Heavy lines show data for runs which had MOP activity while thin lines show data which didn't have MOP activity.

7.1.3 Quantitative Analysis for Comparison of MOP vs. No MOP Studies

These runs are analyzed in the same modified Lineweaver-Burk method as the aeration studies described in section 5.2. The kinetics of oxygen consumption before and after phenol is added are analyzed separately. In the pair of runs (6/14/96) in which three additional aliquots of phenol are added, the rates of oxygen consumption are analyzed after each addition.

Figures 7.3-7.8 contain the information for the experiments shown in Table 7.1.

7.1.4 Results and Discussion of MOP vs. No MOP Studies

Each experiment is repeated five times. All runs are aerated after the blood is added. As seen in Table 7.2, on 6/10, 6/14 and 7/14/1996, one reactor's contents shows MOP activity and one did not. These are the types of comparisons we are seeking. These runs are shown in Figures 7.4 and 7.6. The results of the other pairs are plotted and appear Figures 7.3, 7.5 and 7.8 in Appendix 7. The K_R value for each run in shown in Table 7.3 in Appendix 7.

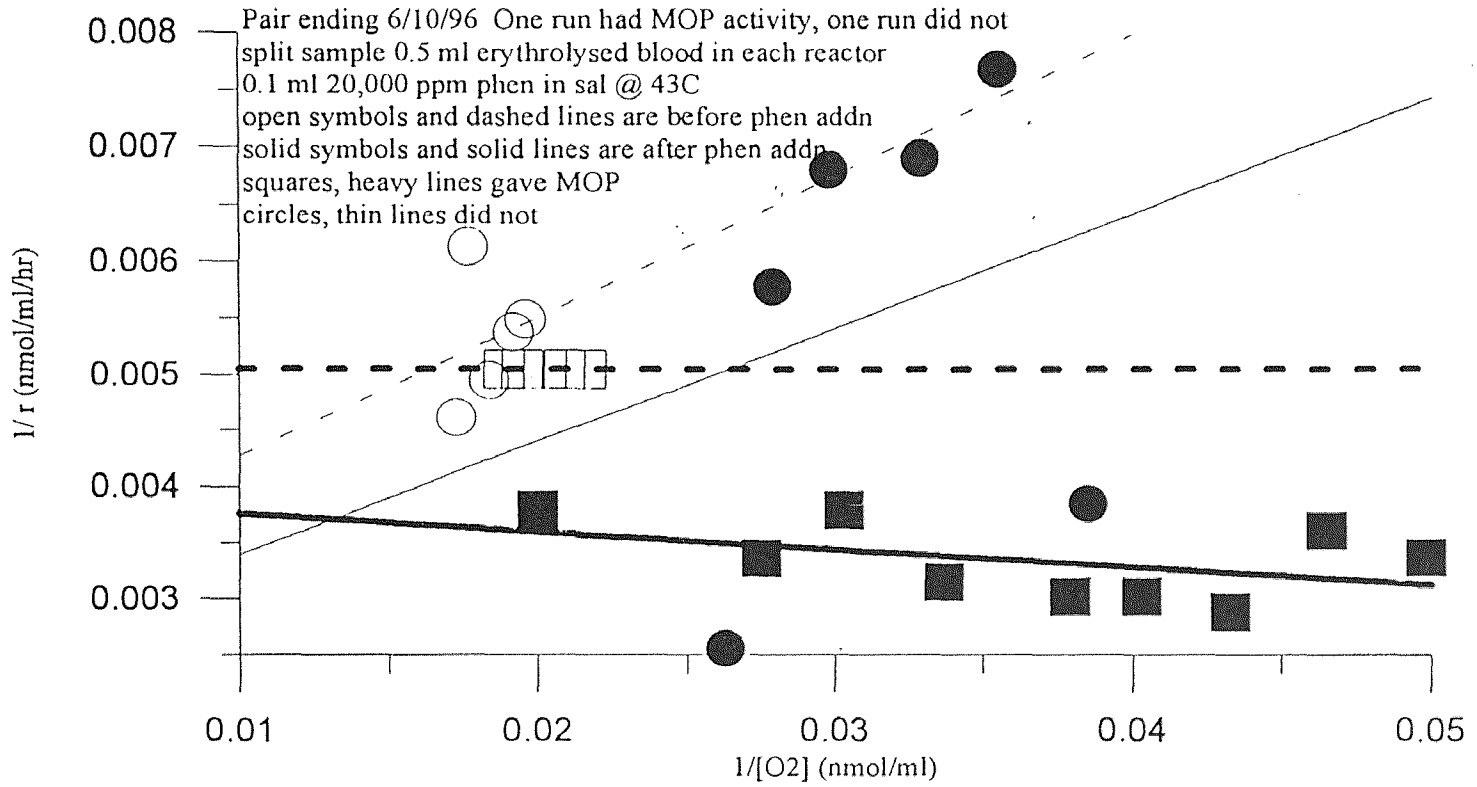


Figure 7.4 Two runs done with a split sample on 6/10/96 in two reactors sharing the same temperature controlled water bath. One run has MOP activity, one does not. To explore the influence of phenol on MOP generation, the kinetics of oxygen consumption before and after phenol addition is added is analyzed separately. This allows investigation of the effect on oxygen consumption of phenol addition. In addition, this may help determine which better predicts the likelihood of MOP generation, the kinetics of oxygen consumption in blood before, or after, phenol addition. This may help determine to what extent MOP generation is influenced by phenol stress.

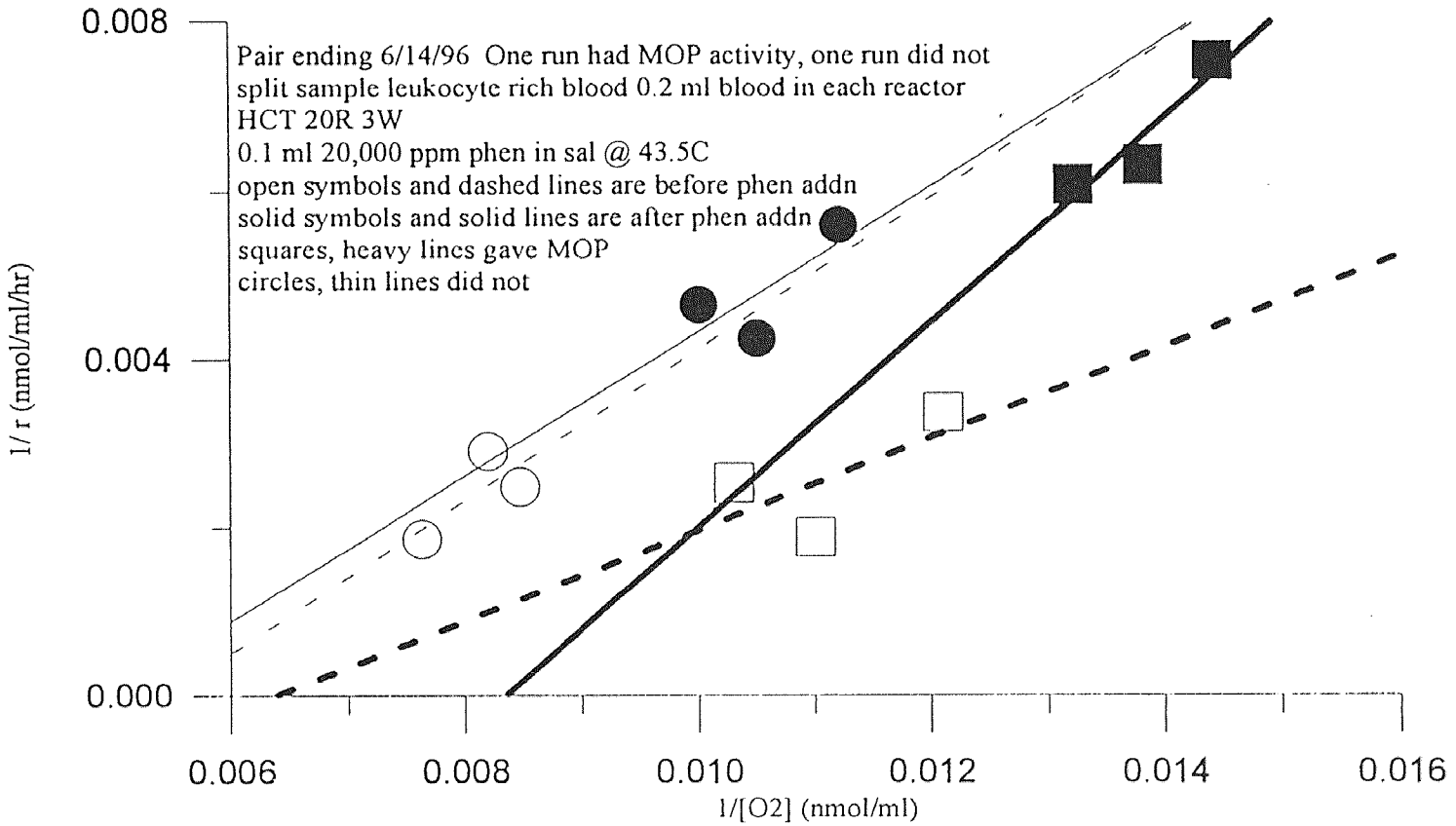


Figure 7.6 Two runs done on 6/14/96 with a split sample in two reactors of the same reactor conditions. One run has MOP activity, one does not.

Table 7.2 MOP activity in split sample runs done under the same conditions. All runs are done in saline with 1000 ppm phenol in the reactor. On 6/10 and 6/14, one reactor's contents showed MOP activity and one did not. These are the types of comparisons we are seeking for the possible prediction of MOP activity within the first hour of the run.

Date runs ended (1996)	Temperature °C	volume of blood used (ml)	type of blood sample	HCT of blood used	MOP activity in the reactors
6/10	43	0.75	whole blood	33.5R 1W	one reactor had MOP activity, one did not
6/13	43.5	0.75	leukocyte enriched	NA	neither reactor had MOP activity
6/14	43.5	0.2	leukocyte enriched	20R 3W	one reactor had MOP activity, one did not
6/15	43.5	0.2	whole blood	38R 0.5W	both reactors had MOP activity
7/14	37.5	0.4	leukocyte enriched	NA	one reactor had MOP activity, one did not

7.1.5 Influence of Phenol on MOP Generation

To explore the influence of phenol on MOP generation, the kinetics of oxygen consumption before and after phenol addition is added is analyzed separately. This allows investigation of the effect on oxygen consumption of phenol addition. In addition, this will help determine which could better predict the likelihood of MOP generation, the kinetics of oxygen consumption in blood before, or after, phenol addition. This will help determine to what extent MOP generation is influenced by phenol stress.

Further exploration of phenol's influence on runs which had MOP activity as opposed to runs which didn't have MOP activity is found in the runs which ended on 6/14/96. At the end of that pair of runs (about 24 hours), each reactor is reaerated and

phenol is added in three aliquots of 0.1 ml of 20,000 ppm phenol. The interval between each addition is about 10 minutes and the rates of oxygen consumption are analyzed after each addition. The results are shown in Figure 7.7.

Note in Figure 7.7 that before phenol is added, the DO consumption rate in each reactor is the same. After phenol addition, the DO consumption rate in the reactor increases in the reactor which has MOP activity (although the MOPs have long since finished). In contrast, the DO consumption rate decreases in the reactor which does not have MOP activity. After the second phenol addition, the DO consumption rate in both reactors decreases. This is an experiment done only once but is very interesting because increased DO consumption is consistent with oxidative activity in biological systems. Also, the decrease in DO consumption at the second and third injection of phenol is consistent with either substrate inhibition or toxic effects on biological systems.

7.2 Viability Test for Leukocytes Before and After Split Sample Runs

Viability tests were on several 24 hour runs using the neutral red trypan blue dye exclusion method described in Ch. 3. The presence of both viable and nonviable leukocytes both before and after 24 hours in the reactor indicates that the stress of being in the reactor for 24 hours did not destroy all of the leukocytes.

7.3 Conclusions Regarding MOP Prediction

This study which generated only two pairs of runs which could be compared took a week to complete. The amount of time that would be required to reach statistical significance of results was deemed prohibitive to continuing this avenue of exploration in this dissertation.

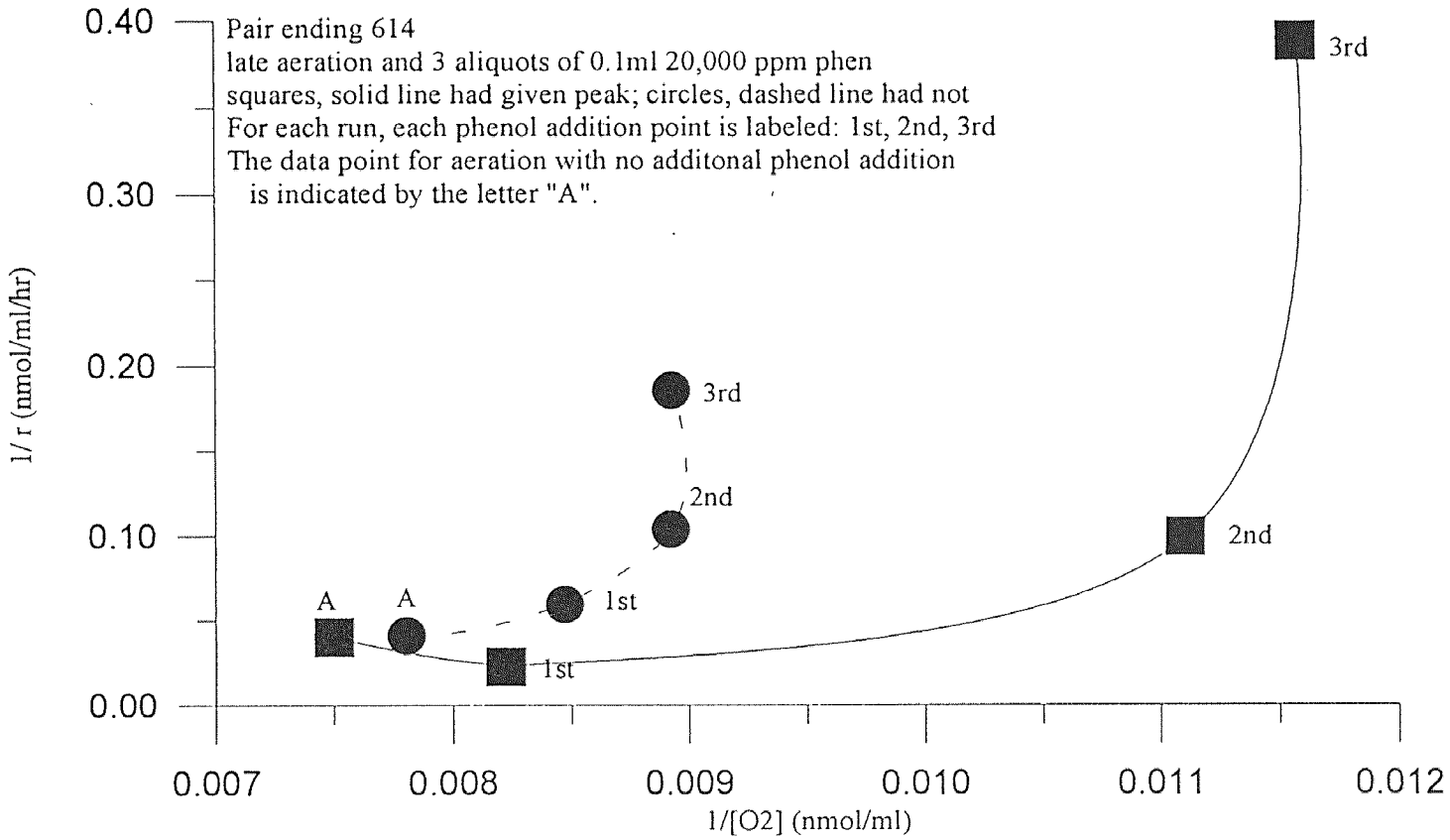


Figure 7.7 Late aeration then addition of 3 aliquots of 0.1 ml 20,000 ppm phenol to the runs which ended 6/14/96. At the end of that pair of runs (about 24 hours), each reactor is reaerated and phenol is added in three aliquots of 0.1 ml of 20,000 ppm phenol. The interval between each addition is about 10 minutes and the rates of oxygen consumption are analyzed after each addition. Note that before phenol is added, the DO consumption rate in each reactor is the same. After phenol addition, the DO consumption rate in the reactor increases in the reactor which has MOP activity (although the MOPs have long since finished). In contrast, the DO consumption rate decreases in the reactor which does not have MOP activity. After the second phenol addition, the DO consumption rate in both reactors decreases. This is an experiment done only once but is very interesting because increased DO consumption is consistent with oxidative activity in biological systems. Also, the decrease in DO consumption at the second and third injection of phenol is consistent with either substrate inhibition or toxic effects on biological systems.

However, the immense benefit of the ability to predict MOPs make following this line worthwhile.

7.3.1 Potential Sources of Oxygen for MOPs

Potential sources of molecular oxygen generation include the decomposition of hydrogen peroxide by catalase or peroxidases to generate water and O_2 and the enzymic or chemical dismutation of superoxide to hydrogen peroxide and O_2 with the consumption of $2H^+$ (Mathews and van Holde 1990).

The oxygen release reported in this paper may take place after the respiratory burst or some other, perhaps unknown mechanism. In the respiratory burst, leukocytes uptake oxygen and catalyze the oxygen into reactive oxygen species (ROS). ROS are used to destroy bacterial invaders and toxicants. The catalysis of ROS back to molecular oxygen following the respiratory burst is necessary to protect the body from the adverse effects of the ROS.

MOPs are not proposed as synonymous with the respiratory burst. Rather, the catalyzation of ROS to O_2 following the respiratory burst is merely listed as a potential source of O_2 observed in the MOP.

It should be noted that for the catalysis of ROS produced during the respiratory burst to be the oxygen source for MOPs which begin at zero DO, the existence of an oxygen reservoir of some sort is still necessary. If the respiratory burst is involved, it is unclear whether the reservoir is composed of ROS or molecular oxygen. However, the latter is more likely because the former would be highly reactive.

There are two conventional sources of oxygen introduced into the reactor: the amount of oxygen present in the blood when it is added to the reactor, and the amount of oxygen present in the reactor at the beginning of the run. The latter varies from 370 to 460 nanomoles (nmol), depending on the temperature of the 1.9 ml air saturated reactor. The former is calculated separately for each run, depending on the hematocrit and volume of blood of each run.

The stopper has a narrow injection pathway 4.5 cm long and 2 mm in diameter. Once stoppered, there is no gas exchange with the environment. The blood may uptake oxygen from the liquid inside the reactor. Oxygen can be bound by hemoglobin (Hb). However, as DO in the Hb containing medium decreases, Hb gives off oxygen. At zero DO in the medium, Hb contains zero oxygen (Mathews and van Holde 1990).

7.4 Quantitative Examination of MOPs

This section contains quantitative examination of MOPs unlike that presented in Chapter 4, Section 4.2.5.

7.4.1 Amount of Oxygen in Each MOP Compared with the Packed Cell Volume

Table 7.4 summarizes the average MOP size for 95 MOPs. The amount of oxygen in each MOP is examined in three different ways: comparison with (1) the amount of packed leukocytes, (2) the amount of packed erythrocytes, and (3) the amount of total packed cells in each sample.

A single run may yield more than one MOP. The number of MOPs observed is greater for the total amount of packed cells because there are 11 peaks recorded here that

use whole blood in which the hematocrit wasn't measured so the relative amounts of erythrocytes and leukocytes can not be determined.

Linear regression of the μg O_2 in peak/μl packed cells shows a positive correlation with both leukocytes and with the total number of cells. If erythrocytes are the causal agent for the MOP, one would expect there to be a positive correlation between the number of erythrocytes and the size of the MOP. However, the linear regression for the MOP size compared to the number of erythrocytes shows a negative slope.

As the number of leukocytes or total cells increases, the size of the MOP increases. This observation implies that leukocytes are responsible for the MOPs and that erythrocytes are not. As the number of red cells increases, the size of the MOP decreases. Runs which gave more than one MOP are represented by more than one data point because each MOP is examined individually. As indicated by the minimum and maximum y values, there is considerable variation in the size of MOPs. Because this data covers such a large data set, the overall trend is credible.

Some of the MOPs examined in Table 7.4 occurred in runs with whole blood in the reactor. Centrifugation and fractionation of other blood samples resulted in leukocyte rich samples which are never devoid of erythrocytes. The runs with more leukocytes contain a smaller fraction of erythrocytes than do the whole blood runs. This strengthens our assertion that leukocytes, not erythrocytes, are responsible for the MOPs.

Table 7.4 Comparison of μg O_2 in Each MOP with the Amount and Type of Packed Cell Volume in the Run. The amount of oxygen in each MOP is examined in three different ways: comparison with (1) the amount of packed leukocytes, (2) the amount of packed erythrocytes, and (3) the amount of total packed cells in each sample. Linear regression of the μg O_2 in peak/μl packed cells shows a positive correlation with both leukocytes and with the total number of cells. If erythrocytes are the causal agent for the MOP, one would expect there to be a positive correlation between the number of erythrocytes and the size of the MOP. However, the linear regression for the MOP size compared to the number of erythrocytes shows a negative slope. This observation implies that leukocytes are responsible for the MOPs and that erythrocytes are not. Runs which gave more than one MOP are represented by more than one data point because each MOP is examined individually.

Type of cell	packed leukocytes	packed erythrocytes	total volume of blood injected
slope of linear regression (μg O_2 /ml packed cells)	25.9	-2.6	1.3
number of peaks	86	86	95
y min (μg O_2)	0.110	0.110	0.110
y max (μg O_2)	16.7	16.7	18.3
x min (μl)	0.1	1.7	10
x max (μl)	98	555	1500

7.4.2 Oxygraphs of MOPs

Some examples of oxygraph chart recorder data are presented in Figures 7.9-7.12 which appear in Appendix 7

7.5 pH and MOP Studies

The studies presented in Table 7.5 address several issues. First, the blood is fractionated by gravity settling in saline. This method of blood fractionation, without any centrifugation, and with minimum handling allows blood fractionation with minimum handling stress which, as seen in Section 6.8.1, may affect the oxygen release from blood.

The second issue addressed in this study is the relationship between the initial response and the ability of blood to generate MOPs. The blood fractions obtained through fractionation are examined in a short run. The blood is added to saline and then phenol is added. The increase in DO which follows phenol addition is used to select the most active fraction. The fraction which produces the greatest DO increase per milliliter of packed cells is deemed the most active fraction. This most active fraction is used in a 24 hour run during which both pH and DO are monitored.

If the initial response and the ability to generate MOPs are related, then the runs which have a higher activity in the short runs should have the greatest MOP activity. As seen in Table 7.5, this does not appear to be the case.

The third area examined in these experiments is the relationship between pH and MOP activity. The maximum pH increase over the course of the run is monitored. The pH increase does not appear to be a good indication of MOP activity.

Table 7.5 pH and MOP runs. All runs are in a saline filled, open top reactor at 38°C. If the initial response and the ability to generate MOPs are related, then the runs which have a higher activity in the short runs should have the greatest MOP activity. This does not appear to be the case. The maximum pH increase over the course of the run is monitored. The pH increase does not appear to be a good indication of MOP activity.

amount of packed cells (ml)	[phenol] (ppm)	activity of fraction (μgO_2/ml packed cells)	amount of O_2 in peaks (μgO_2 /ml packed cells)	amount of O_2 in peaks (μgO_2)	pH increase
0.32	7050	742	1.1, 2.8, 1.1 (5.0 total)	0.4, 0.9, 0.4 (1.7 total)	0.023
0.028	7050	710	25.1, 18.9, 6.3, 1005.4 (1055.7 total)	0.7, 0.5, 0.2, 28.2 (29.6 total)	0.030
0.023	7050	681	23.0, 15.3, 15.3, 15.3, 1713.6 (1782.5 total)	0.5, 0.4, 0.4, 0.4, 39.4 (41.1 total)	0.033
0.11	0	250	5.9	0.6	0.00
0.06	13300	40	6.3, 7.9 (14.2 total)	0.4, 0.5 (0.9 total)	0.06
0.025	7050	541	1548.4	38.7	0.02
0.042	7050	553	75.4, 226.2, 603.3 (904.9 total)	3.2, 9.5, 25.3 (38.0 total)	0.01

The observation of different activities per volume of packed cells raises the following question: Are there different cell types in these fractions? The examination of one fractionation, shown in Table 7.6, indicates that this fractionation by addition to saline and subsequent gravity settling does not drastically concentrate different types of leukocytes. These cell counts fall within the normal ranges for bovine blood (Schailm *et al.*, 1975, 122). The types of cells examined in this procedure are: neutrophils, bands (immature neutrophils), lymphocytes, monocytes, eosinophils.

Table 7.6 Differential White Blood Cell Count of Fractionated Samples. This table indicates that fractionation by addition to saline and subsequent gravity settling does not drastically concentrate different types of leukocytes in the various fractions.

Sample	Whole Blood used in this study	Sample Taken at t=0	Bottom fraction after 1 hour
Packed Cell Volume a.k.a HCT	37	3.75	7.25
Total solids in supernatant (g/dl)	7.2	<2.5 (off scale)	<2.5 (off scale)
Total Leukocytes (#cells/mm^3)	6600	1200	1300
% Neutrophils	37	28	36
% Bands	2	0	0
% Lymphocytes	54	70	62
% Monocytes	2	0	0
% Eosinophils	5	2	4

CHAPTER 8

CONCLUSIONS AND RECOMMENDATIONS

8.1 Conclusions

1. Blood added to a saline or water filled reactor produces an initial response. This initial response is a change in the DO in the reactor. The initial response has two components: an initial decrease in DO in the reactor (initial drop) and an initial increase in DO in the reactor (initial rise) Both the initial drop and the initial rise can be quantified.

2. The initial response of blood is affected in a dose-response manner by both the amount of blood injected and the concentration of phenol present in the reactor.

3. The initial response is affected by temperature. The initial drop portion of the initial response increases with temperature over a range of 25-45 °C. The initial rise portion of the initial response is inhibited at lower temperatures.

4. Examination of the initial response with both phenol and 1-butanol indicate that the initial response may prove a reliable index of toxicity.

5. Handling stress increases the initial response.

6. The initial response can take place in blood whose plasma has been removed.

7. The initial response is affected both by the DO in the reactor before blood is added and by the age of the blood sample.

8. K_R is a reliable indicator of the osmotic pressure, temperature, and amount of blood. K_R is not a reliable predictor of MOP incidence.

9. pH change is not a reliable indicator of MOP activity

8.2 Recommendations

Future work should include:

1. Identification of the cells in each fraction which give different activity, and

2. Examination of the effect on the initial response of selected toxicants

APPENDIX 1

FIGURES FOR CHAPTER 1

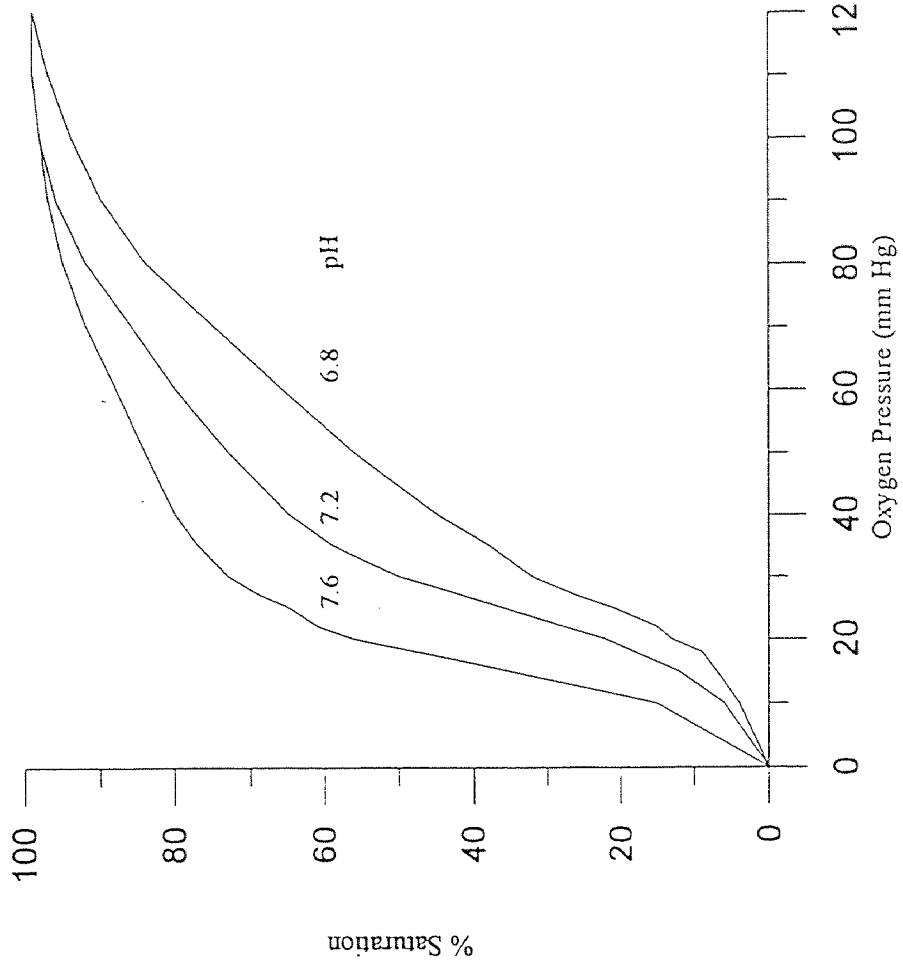


Figure 1.1 The effect of pH on the oxygen binding curve of hemoglobin. Adapted from Matthews and van Holde page 233.

APPENDIX 2

FIGURES FOR CHAPTER 2

Table 2.1 Oxygen solubility in water exposed to water-saturated air at atmospheric pressure (adapted from Greenberg *et al.*).

Temperature (°C)	mgO_2/L	nmol O_2/ml
20	9.092	284.13
25	8.263	258.22
30	7.559	236.22
32	7.305	228.28
35	6.950	217.19
37	6.727	210.22
40	6.412	200.38
42	6.213	194.16
45	5.927	185.22
50	5.477	171.16

Sample calculation: 1mol O_2 = 32,000mg O_2

At 30°C, oxygen solubility = 7.559 mg/L

= (7.559 mg/L)/(32,000 mg/mol)

= (0.000236 mol/L) * (10^9 nmol/mol)*(1 L/1000 mL)

= 236nmol/mL

APPENDIX 3

FIGURES FOR CHAPTER 3

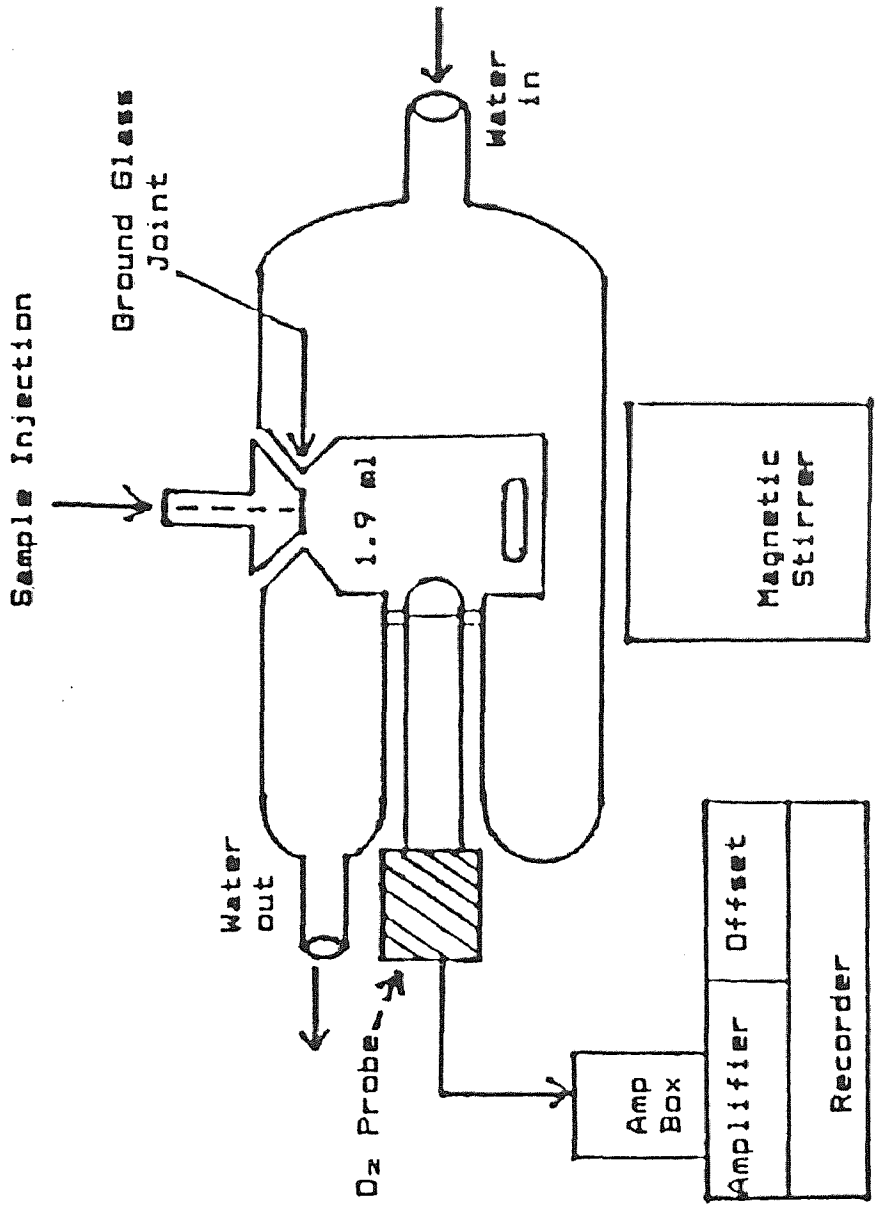


Figure 3.1 The microassay reactor used in these experiments. The Clark type oxygen probe is powered by the amp box. The signal from the probe is amplified and recorded on a strip chart. Mixing is provided by a small magnetic stirring bar.

APPENDIX 4

FIGURES FOR CHAPTER 4

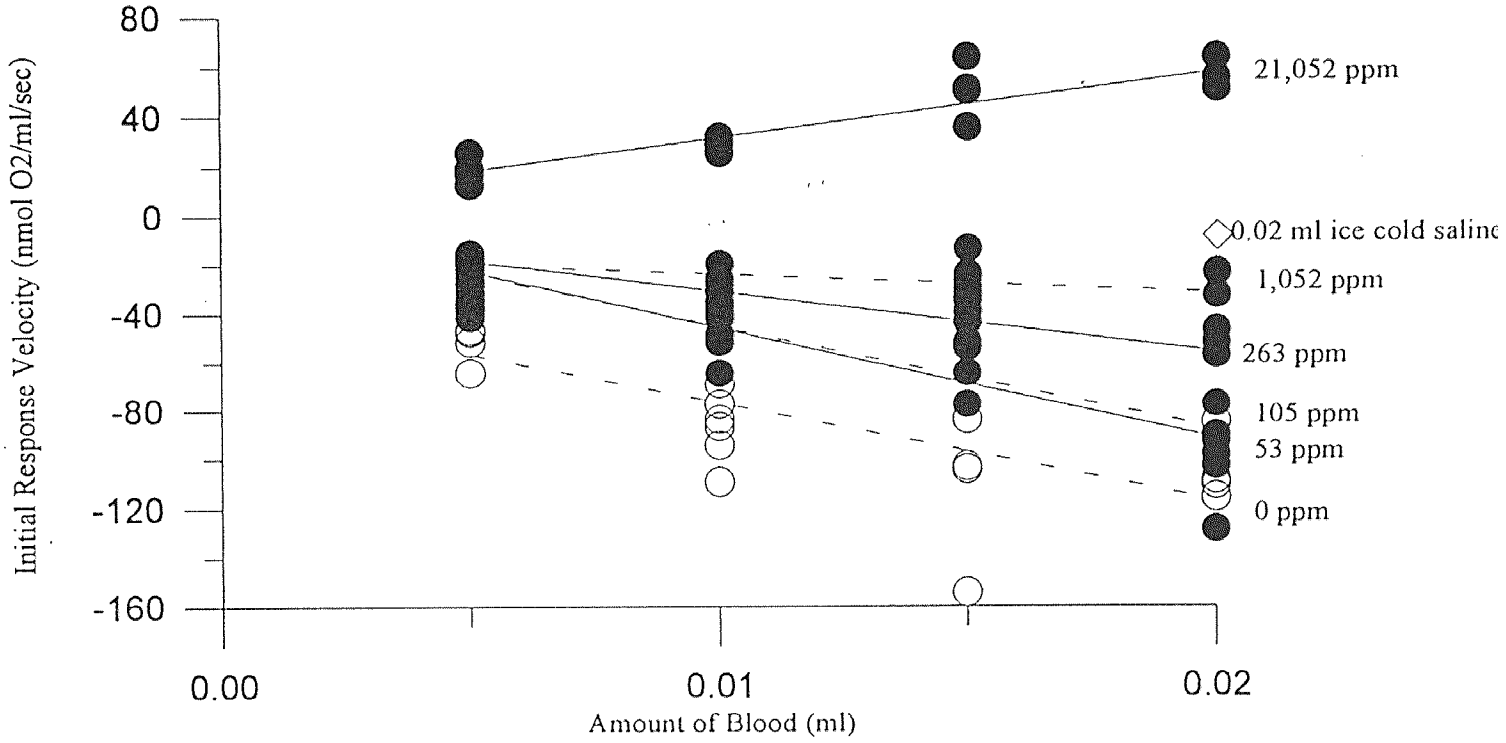


Figure 4.3 Initial response velocity vs. amount of blood added on 7/17/96. The open circles are with no phenol in the reactor; the closed circles are runs with various amounts of phenol in the reactor, the open diamond is ice cold saline injected into the reactor as a control.

APPENDIX 5

FIGURES FOR CHAPTER 5

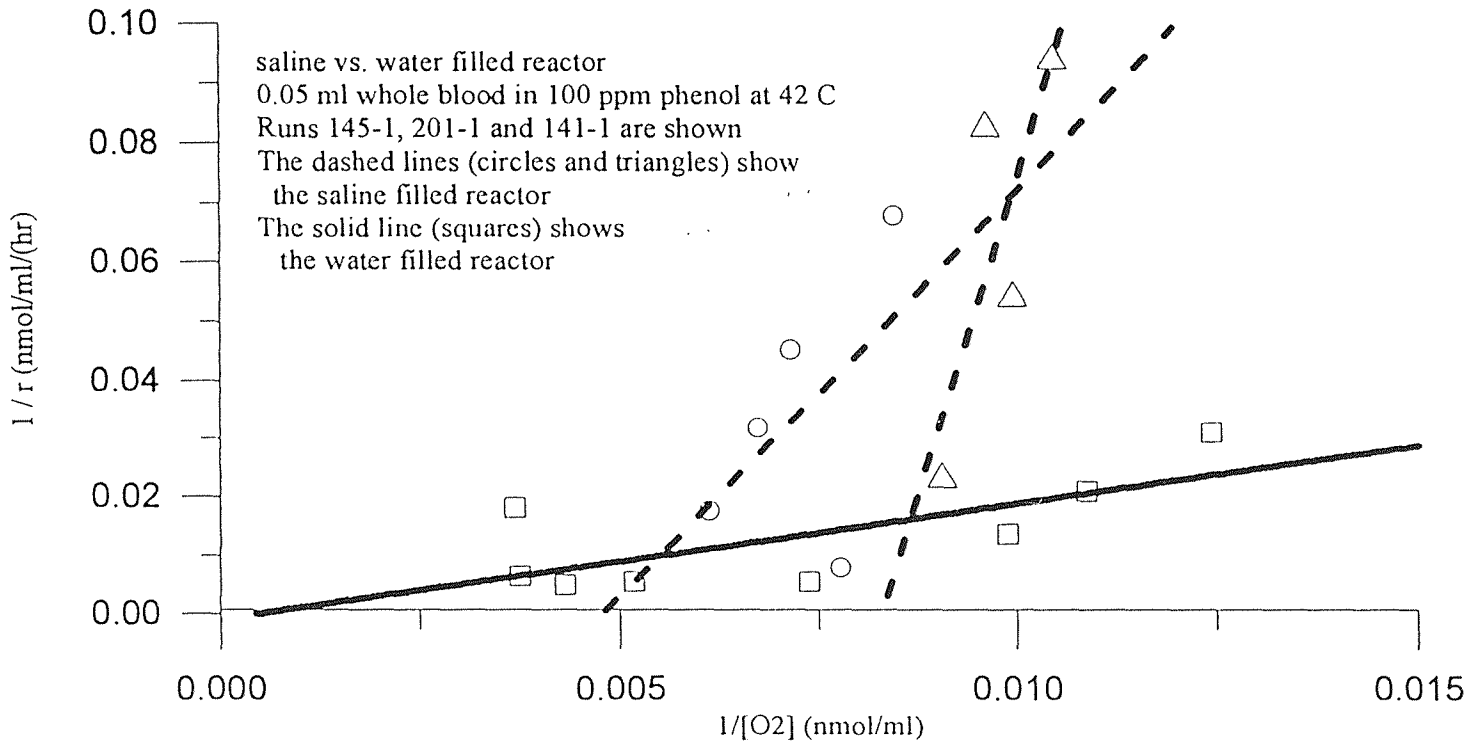


Figure 5.2 Effect of osmotic stress on DO consumption for 0.05 ml whole blood in 100 ppm phenol at 42 °C.

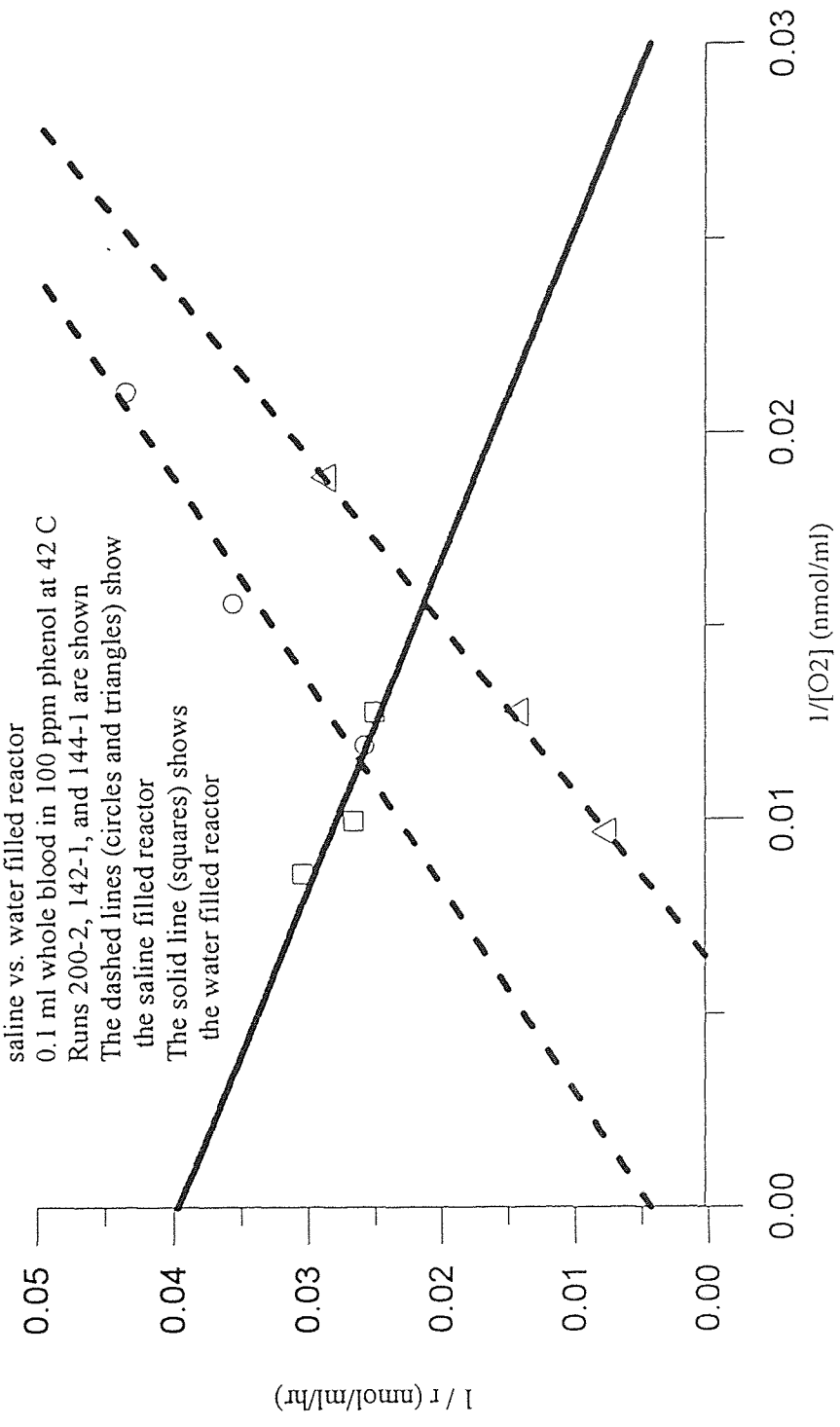


Figure 5.3 Effect of osmotic stress on DO consumption for 0.1 ml whole blood in 100 ppm phenol at 42 °C.

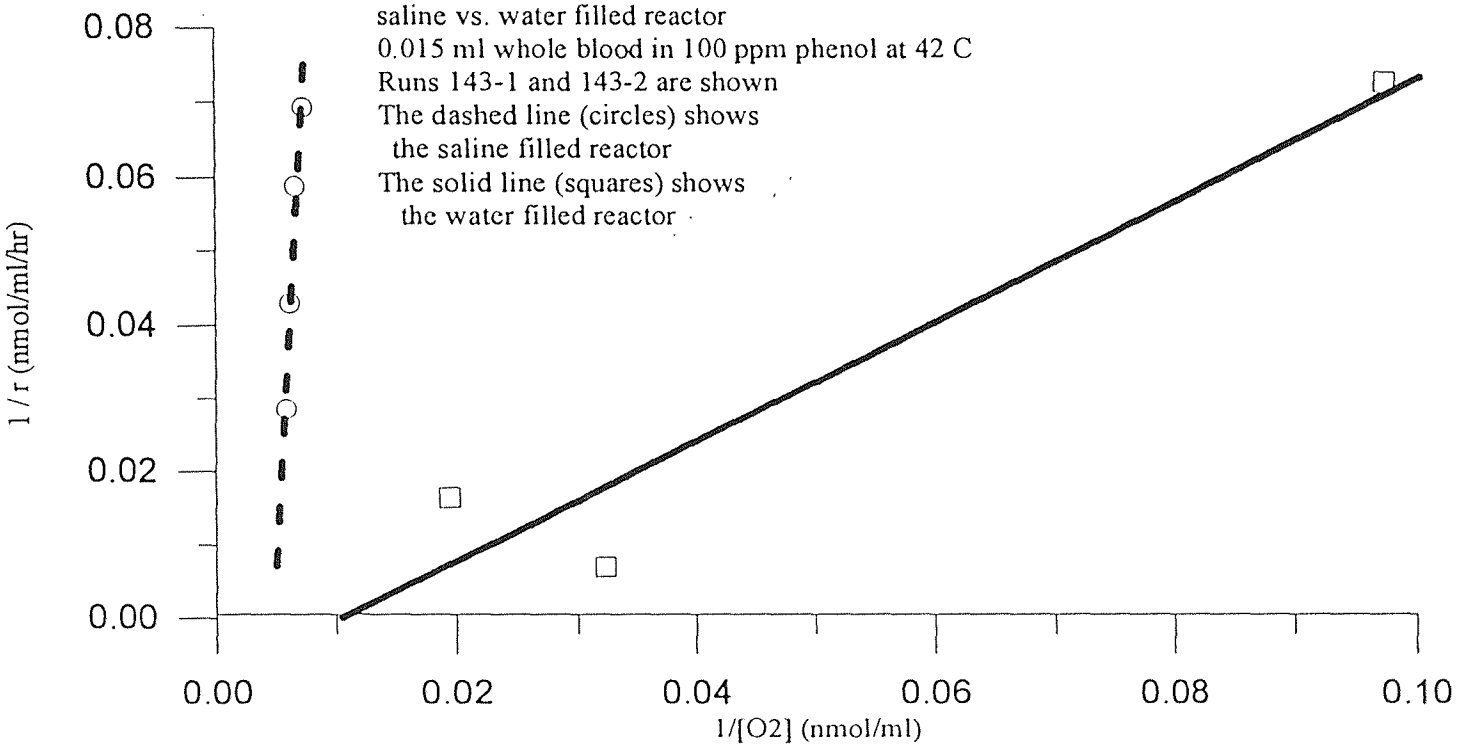


Figure 5.4 Effect of osmotic stress on DO consumption for 0.015 ml whole blood in 100 ppm phenol at 42 °C.

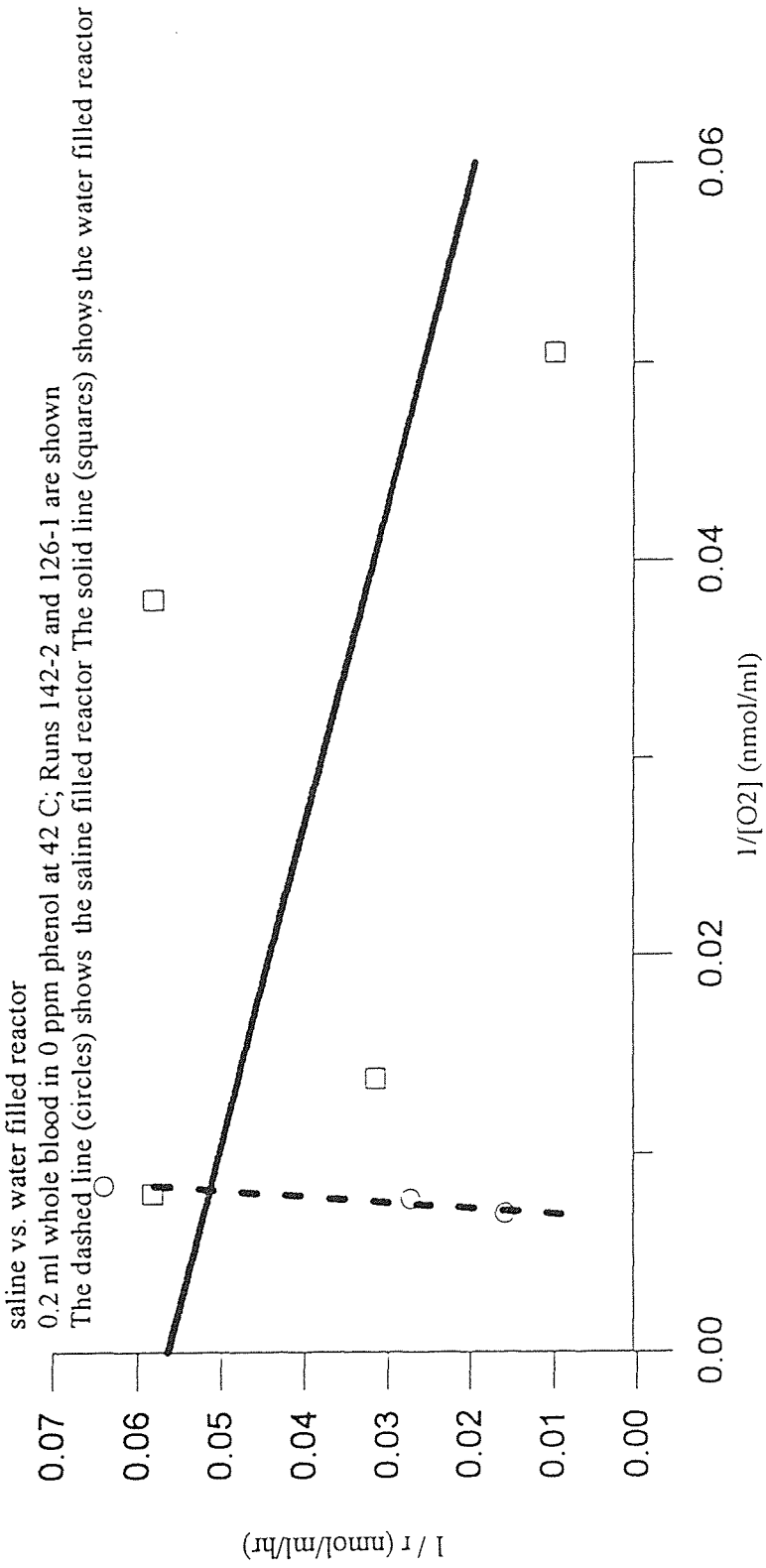


Figure 5.5 Effect of osmotic stress on DO consumption for 0.2 ml whole blood in 0 ppm phenol at 42 °C.

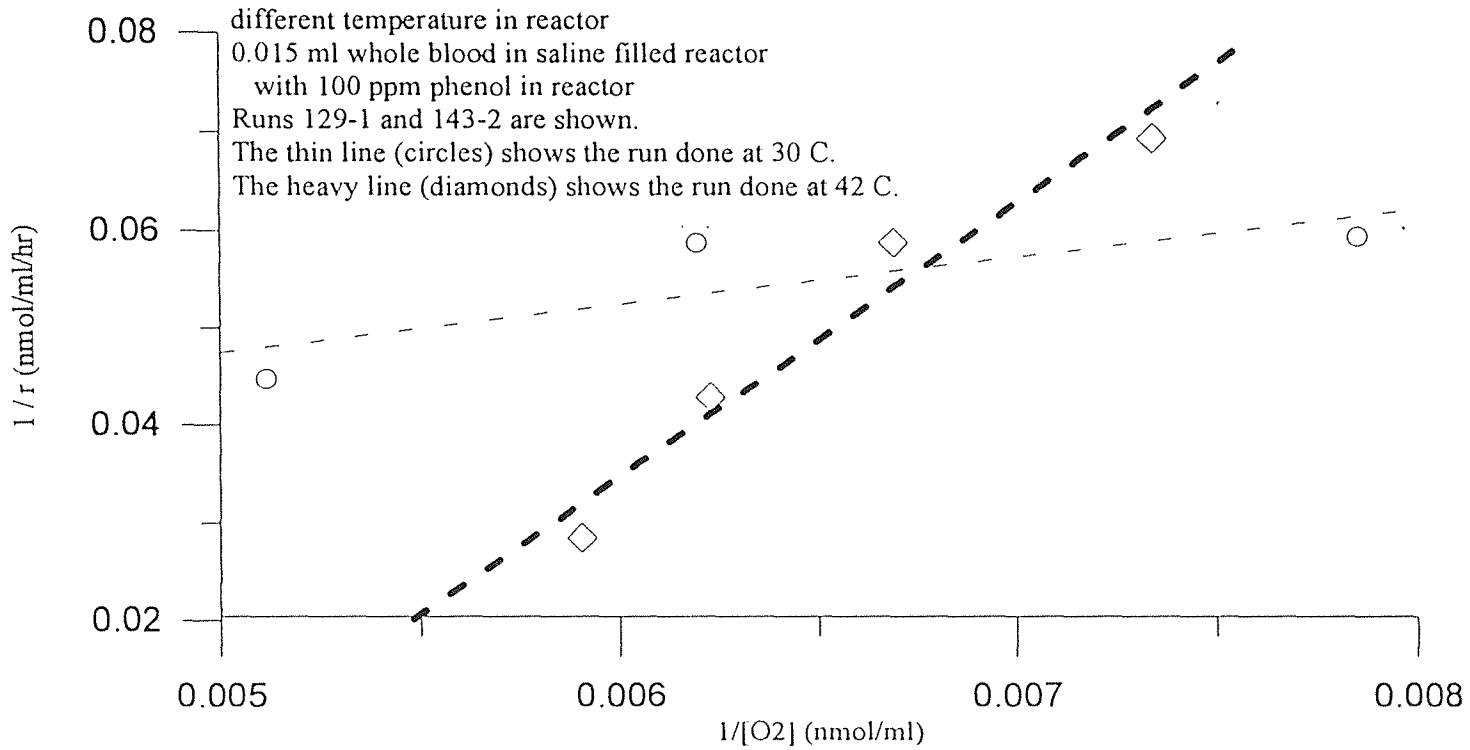


Figure 5.7 Effect of temperature on DO consumption for 0.015 ml whole blood in 100 ppm phenol in saline filled reactor.

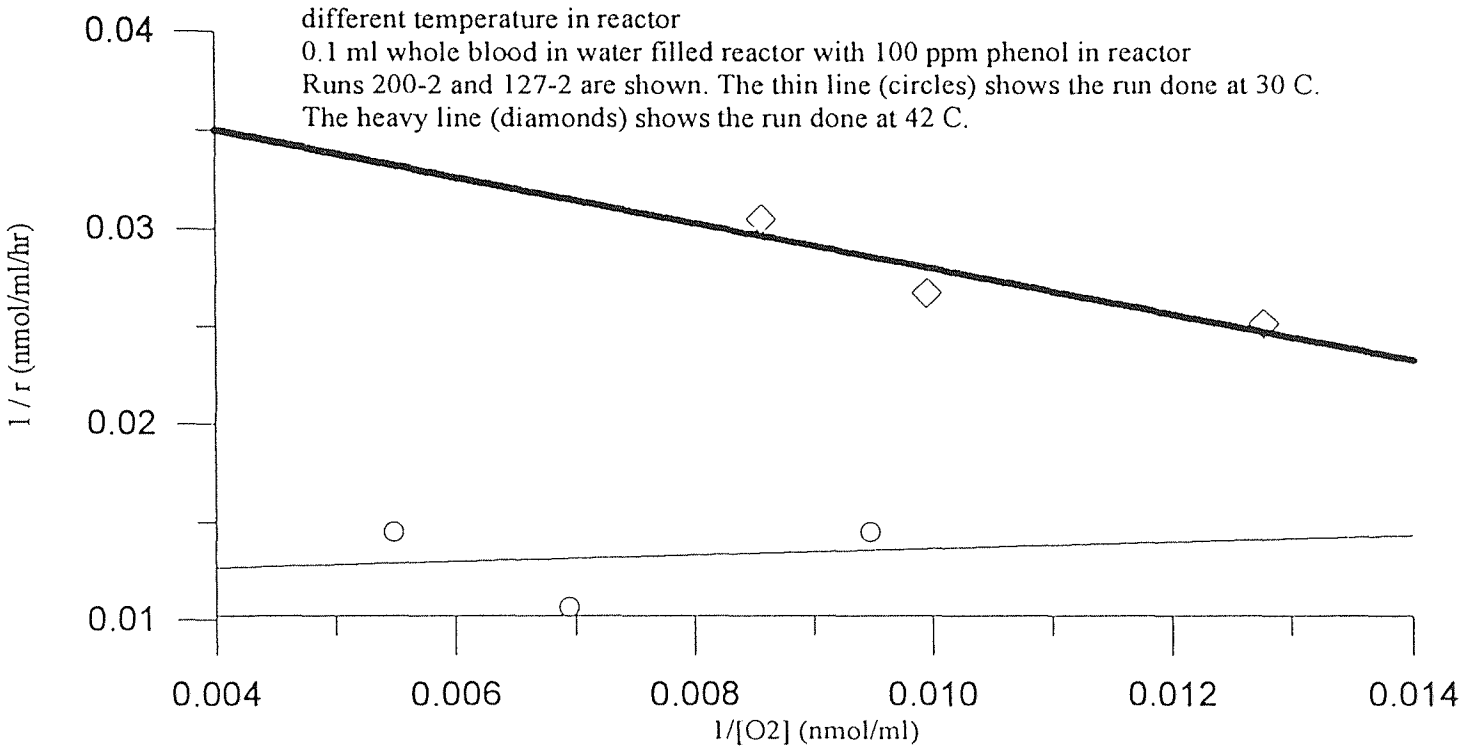


Figure 5.8 Effect of temperature on DO consumption for 0.1 ml whole blood in 100 ppm phenol in water filled reactor.

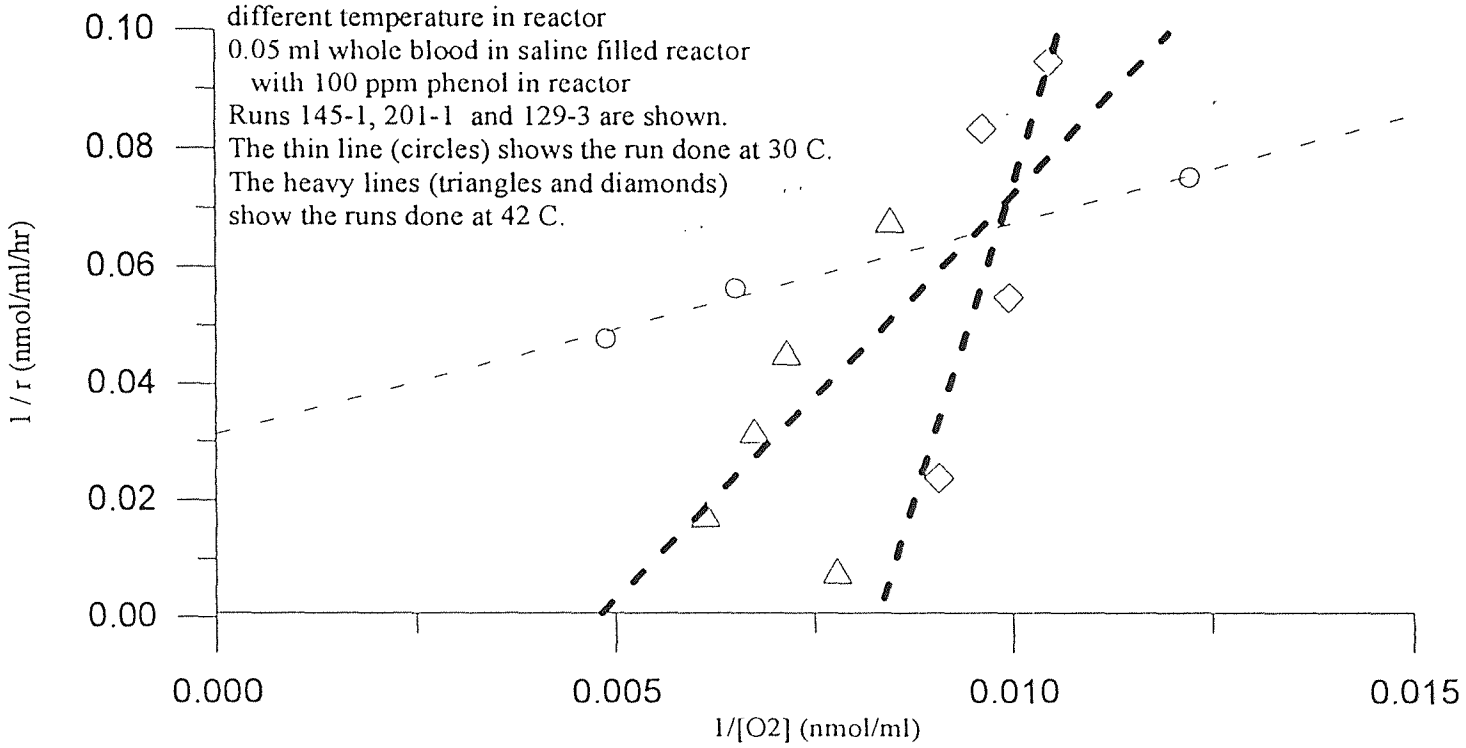


Figure 5.9 Effect of temperature on DO consumption for 0.05 ml whole blood in 100 ppm phenol in saline filled reactor.

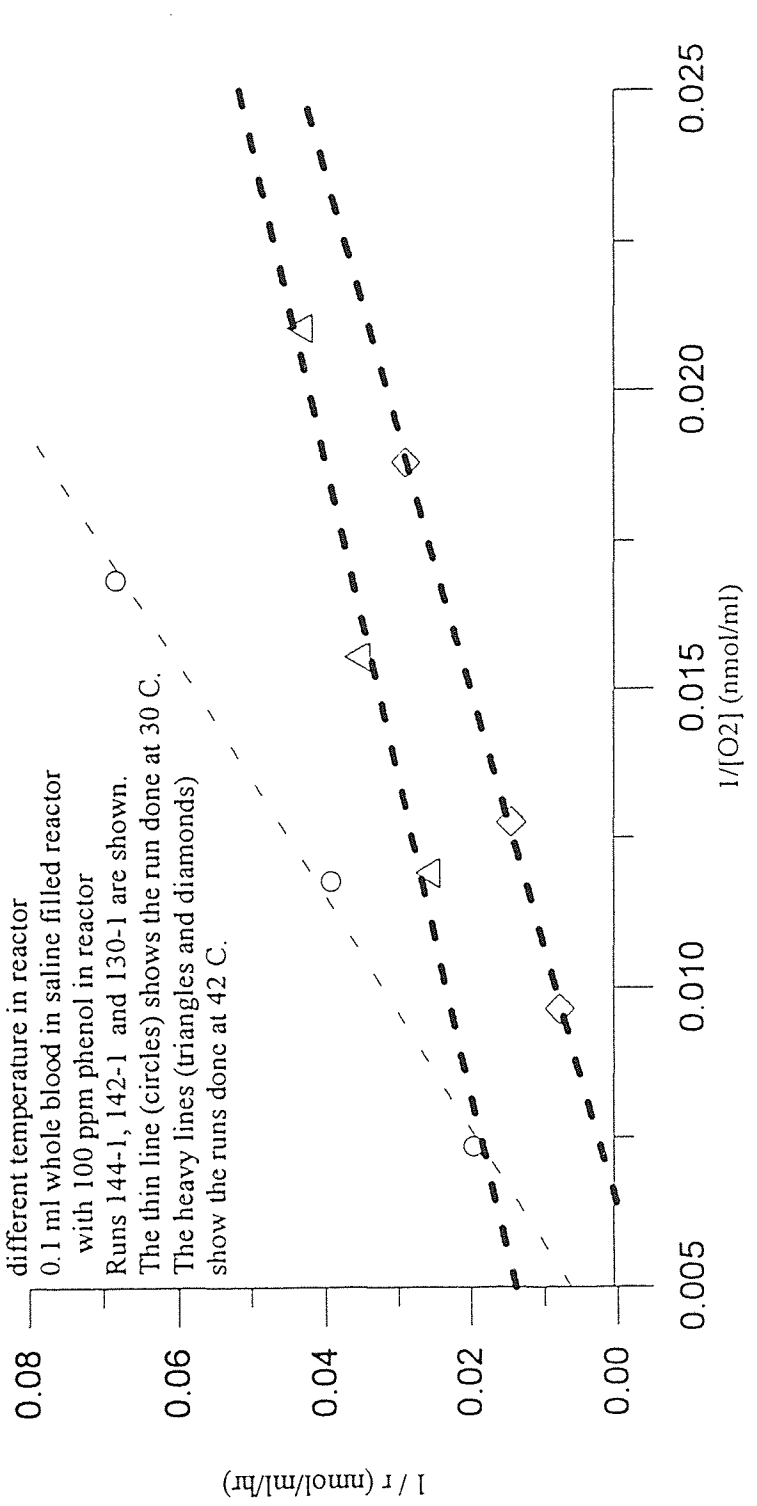


Figure 5.10 Effect of temperature on DO consumption for 0.1 ml whole blood in 100 ppm phenol in saline filled reactor.

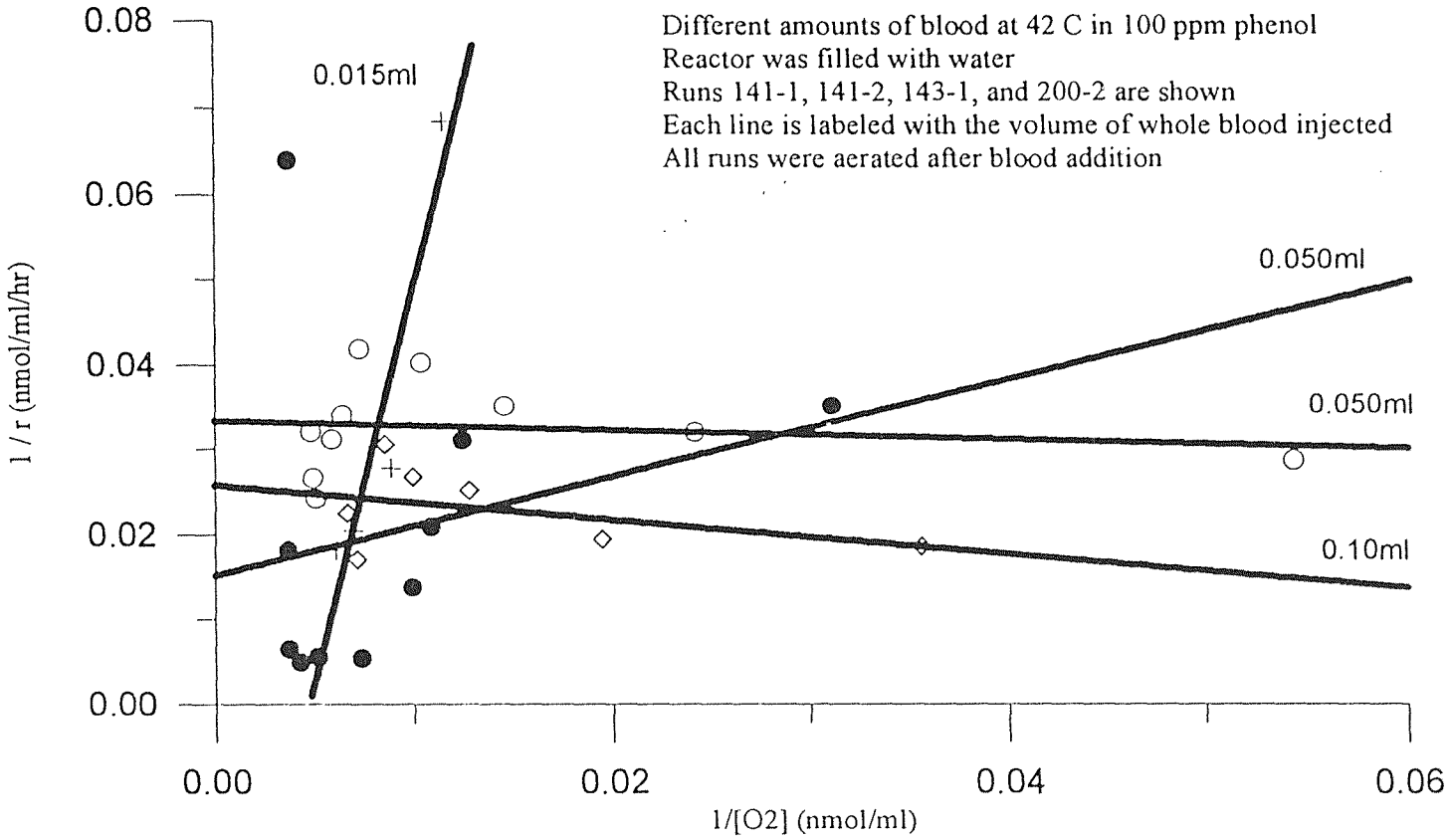


Figure 5.12 Effect of blood amount on DO consumption in 100 ppm phenol in water filled reactor at 42 °C.

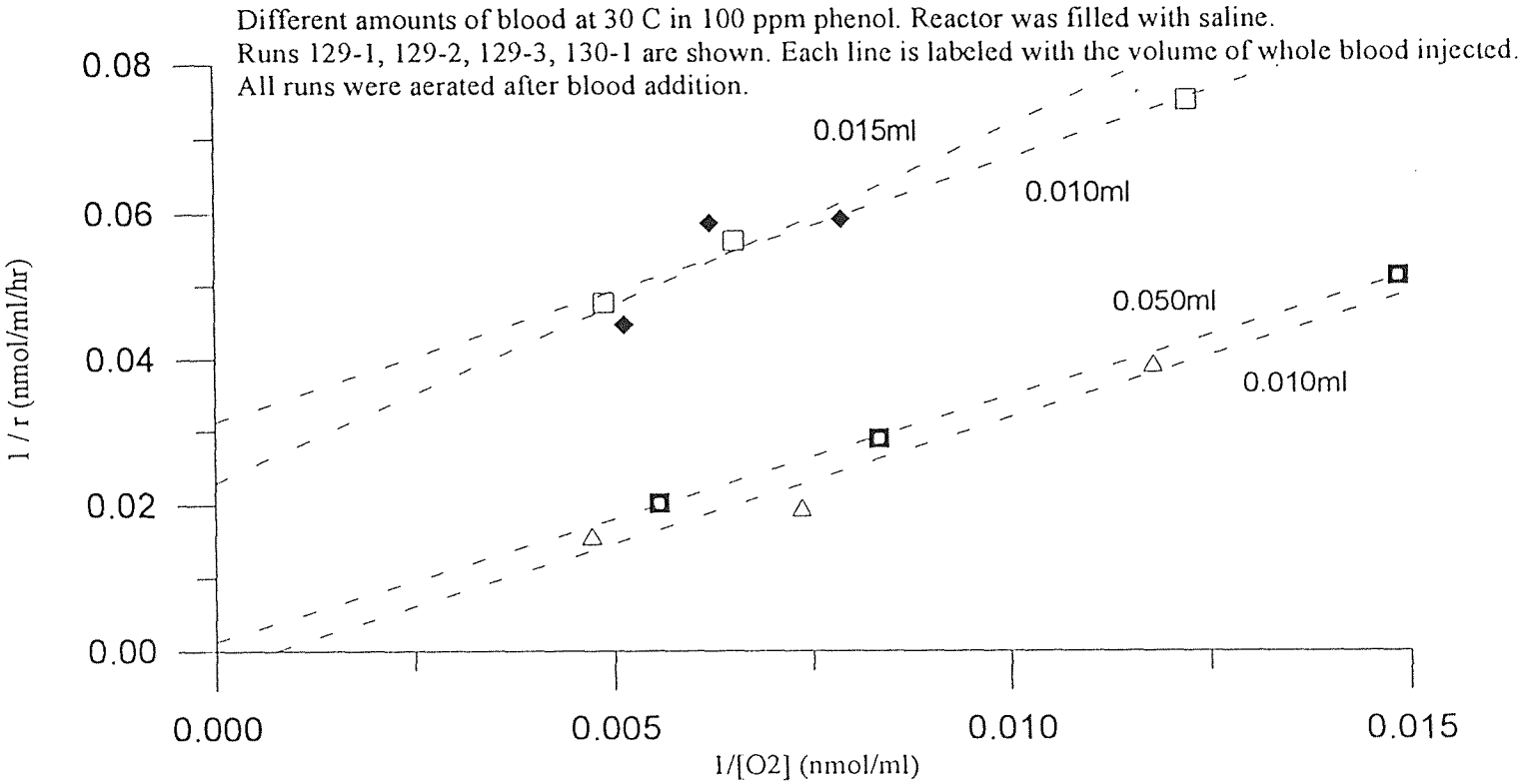


Figure 5.13 Effect of blood amount on DO consumption in 100 ppm phenol in saline filled reactor at 30 °C.

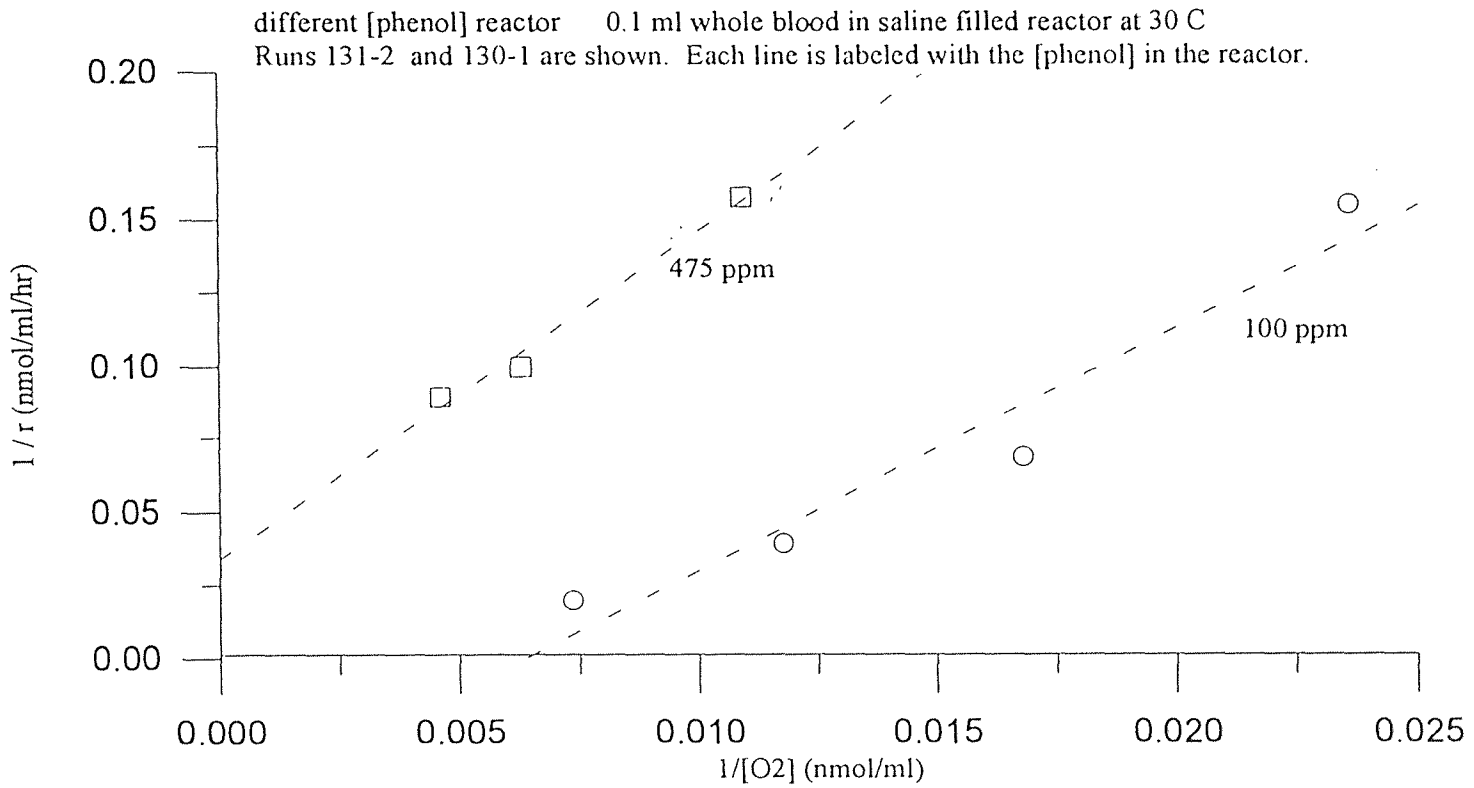


Figure 5.15 Effect of phenol concentration in the reactor on DO consumption for 0.1 ml blood in saline filled reactor at 30 °C.

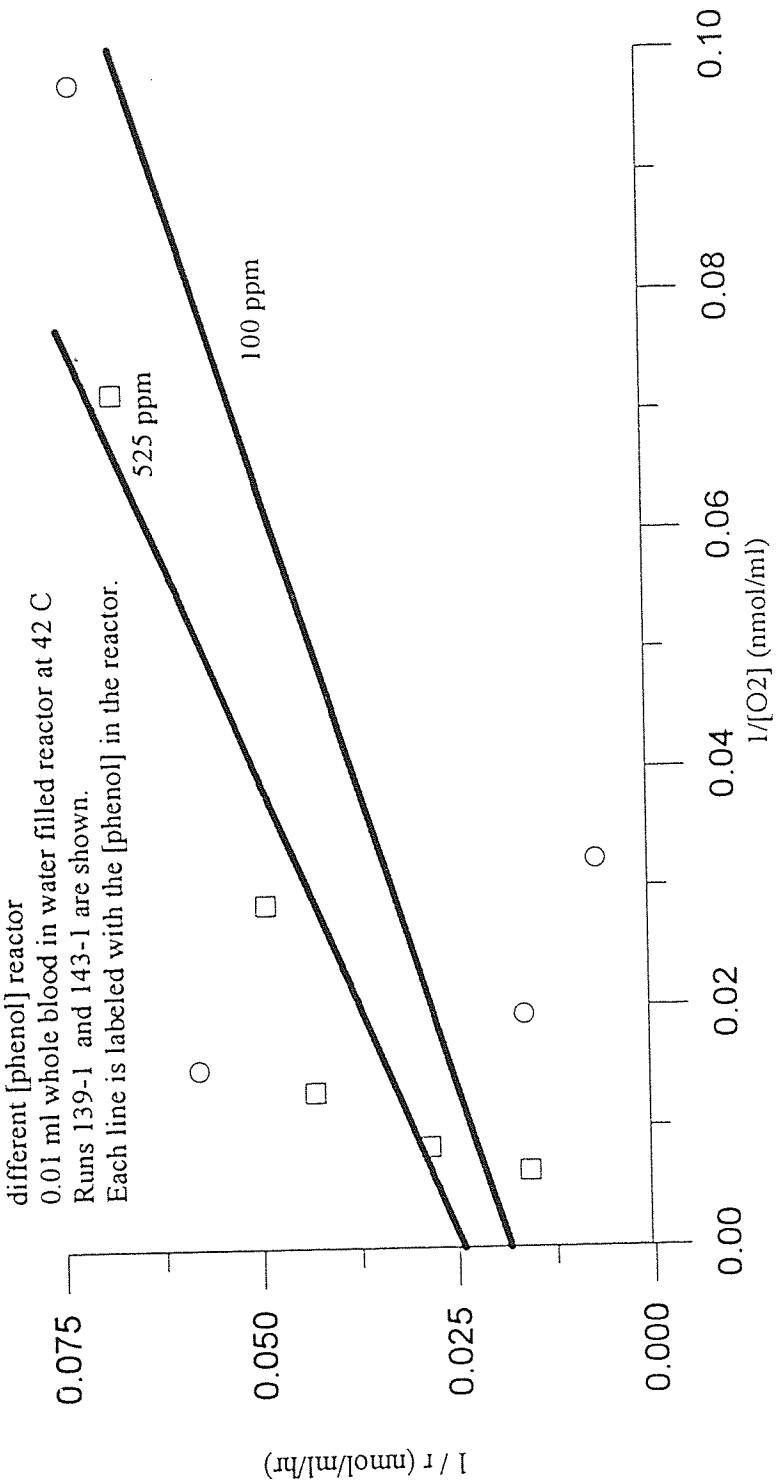


Figure 5.16 Effect of phenol concentration in the reactor on DO consumption for 0.01 ml blood in water filled reactor at 42 °C.

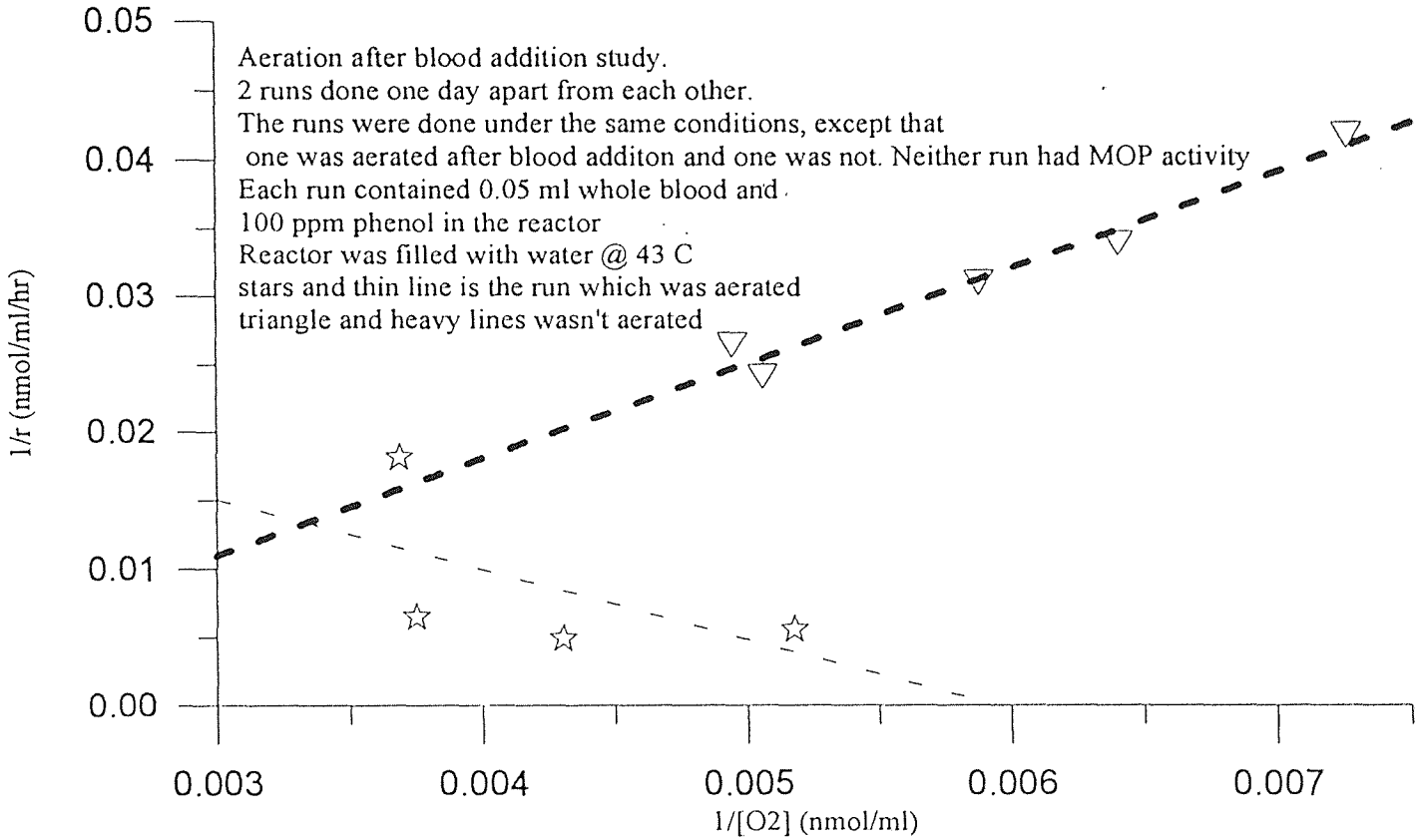
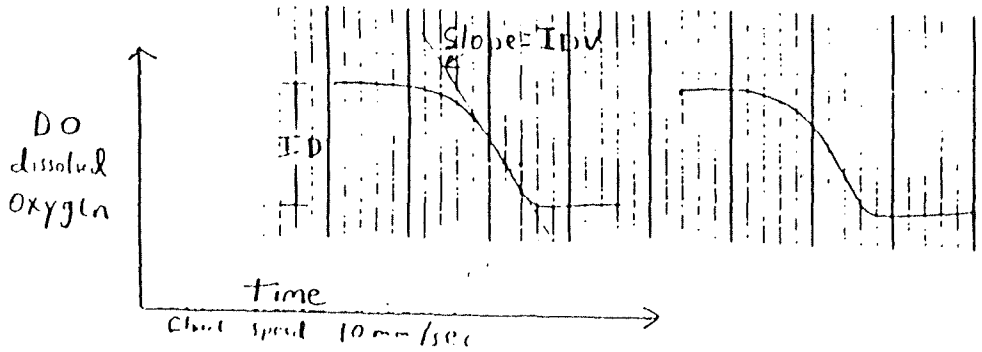


Figure 5.17 Comparision of aerated vs. non-aerated whole blood.

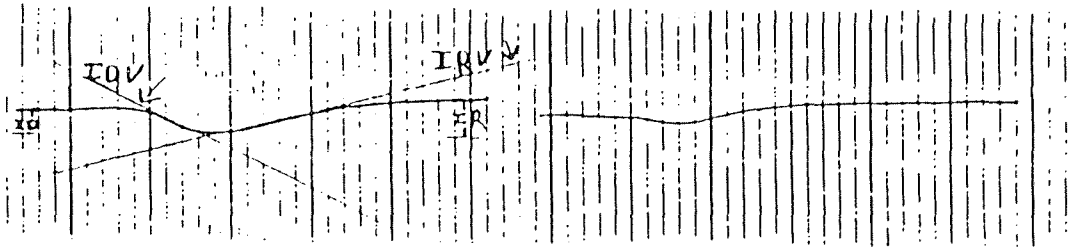
APPENDIX 6

FIGURES FOR CHAPTER 6

The initial response in the runs shown to the right have an ID (initial drop) but no IR (initial rise). The ID and IDV (initial drop velocity) are labeled.



The initial response in the runs shown to the right have both an ID and an IR. The ID, IDV, IR and IRV (initial rise velocity) are labeled.



The initial response in the runs shown to the right have an IR but no ID. The IR and IRV are labeled.

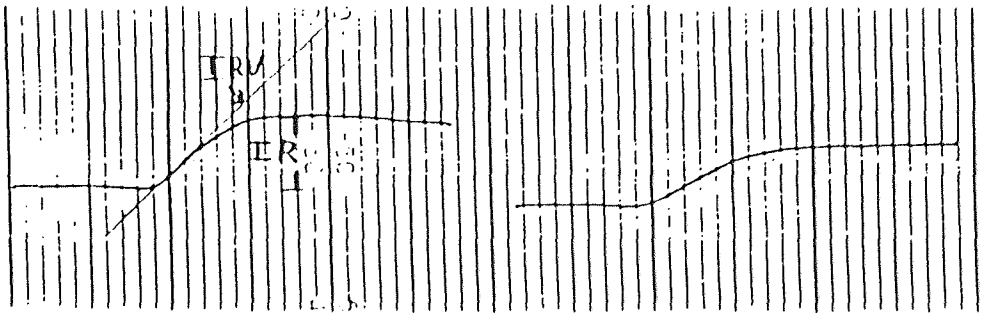


Figure 6.1 Typical Oxygraph Recordings of Initial Response.

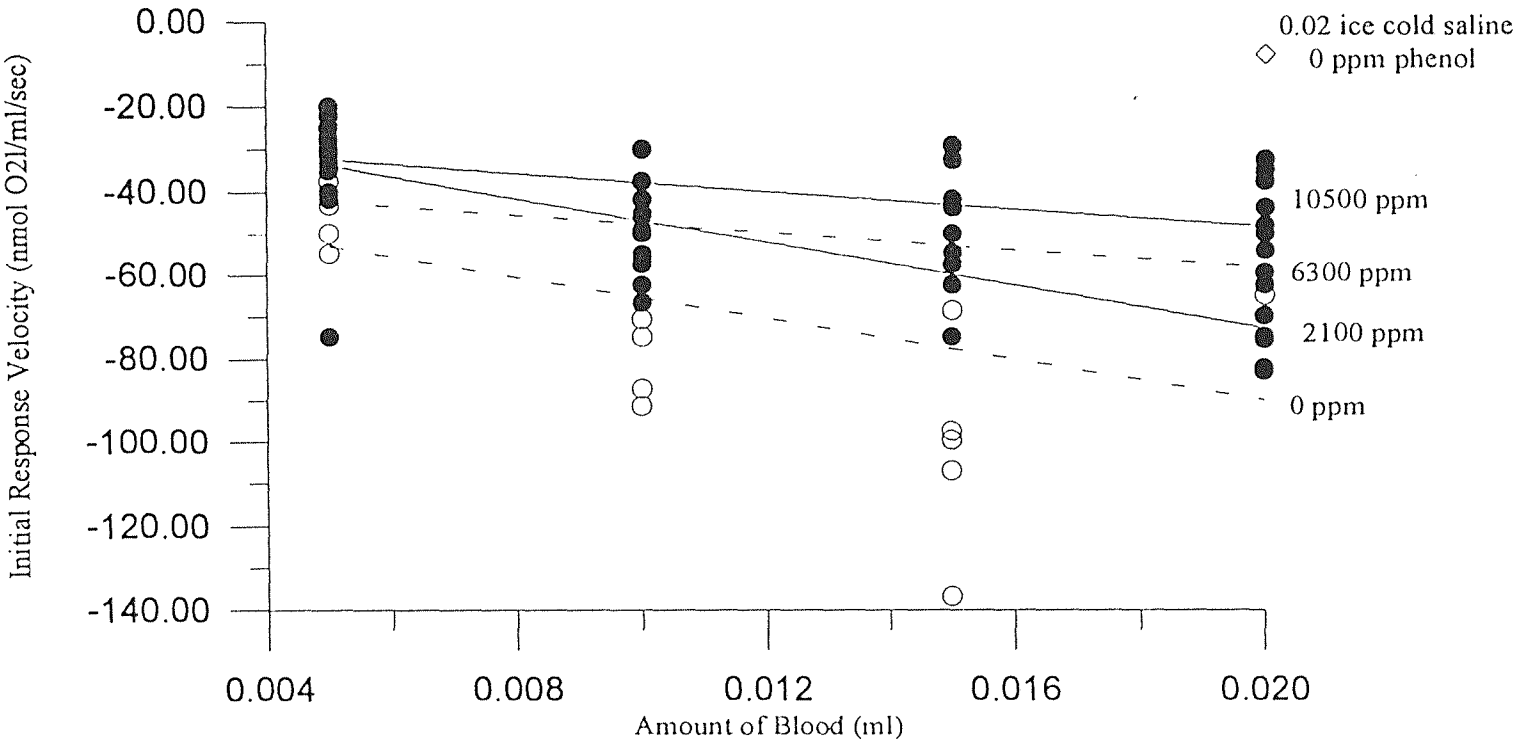


Figure 6.3 Initial response velocity vs. amount of blood added on 7/19/96. The open circles are runs with no phenol in the reactor; the closed circles are runs with various amounts of phenol in the reactor; the open diamond is ice cold saline injected into the reactor as a control.

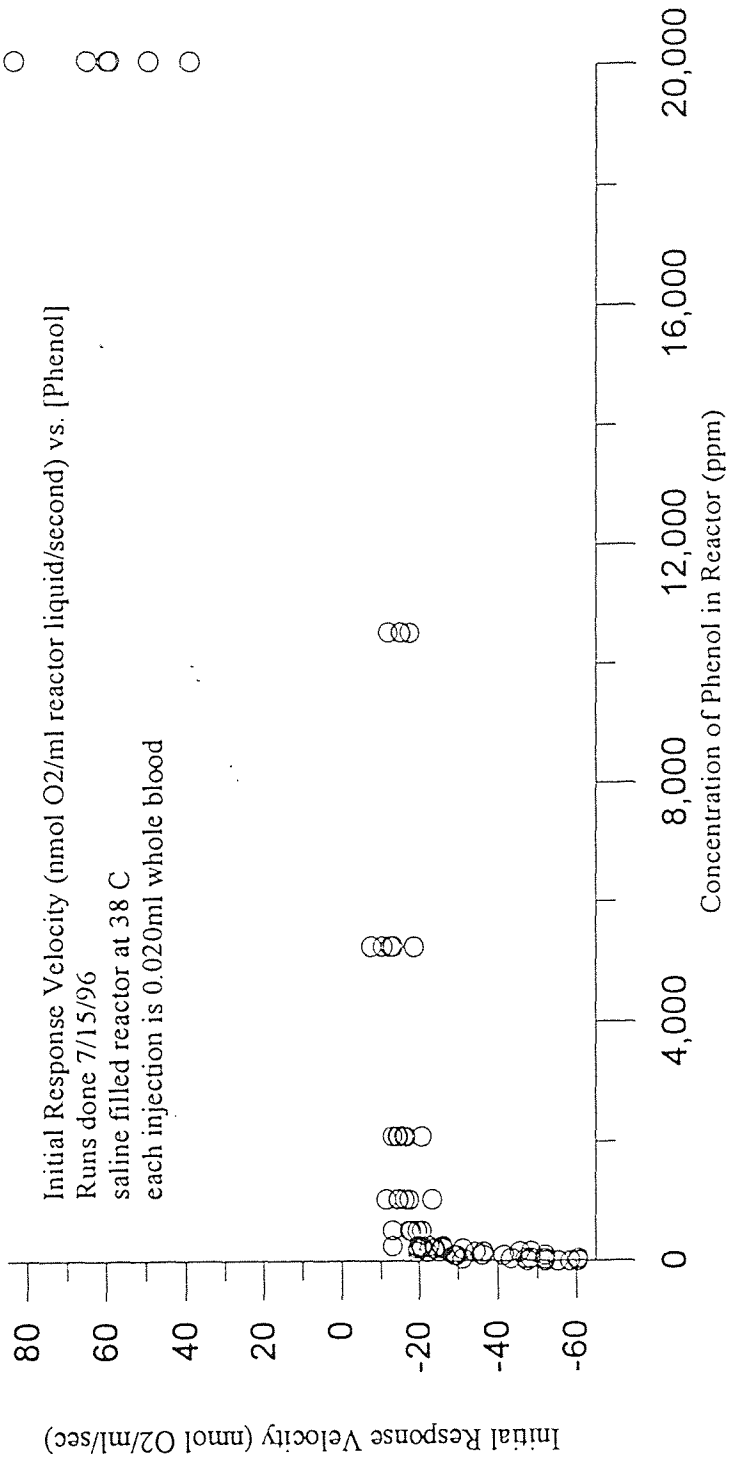


Figure 6.7 The dose response curve of the initial response velocity of 20µl aliquots of blood stressed with a wide range of phenol.

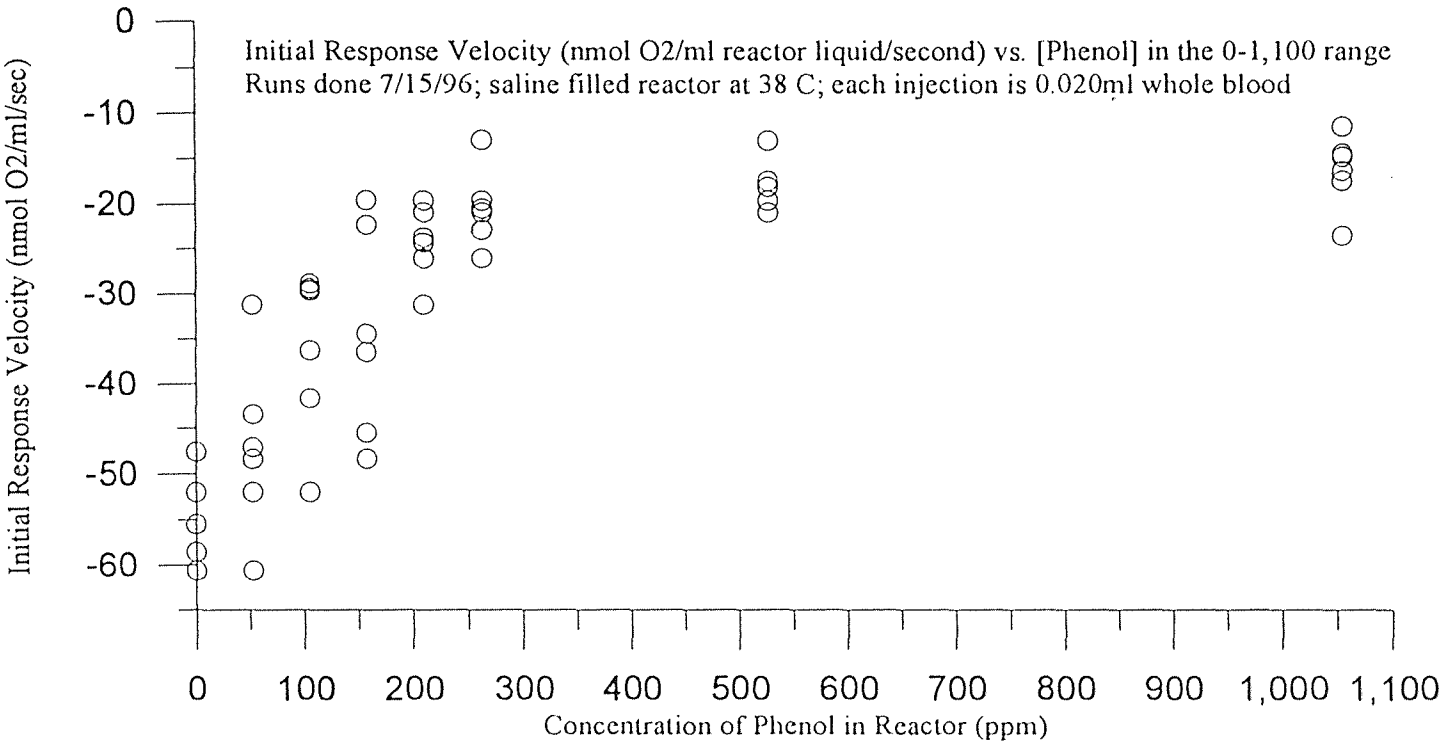


Figure 6.8 The 0-1,100 ppm phenol range of the dose response curve shown in Figure 6.7.

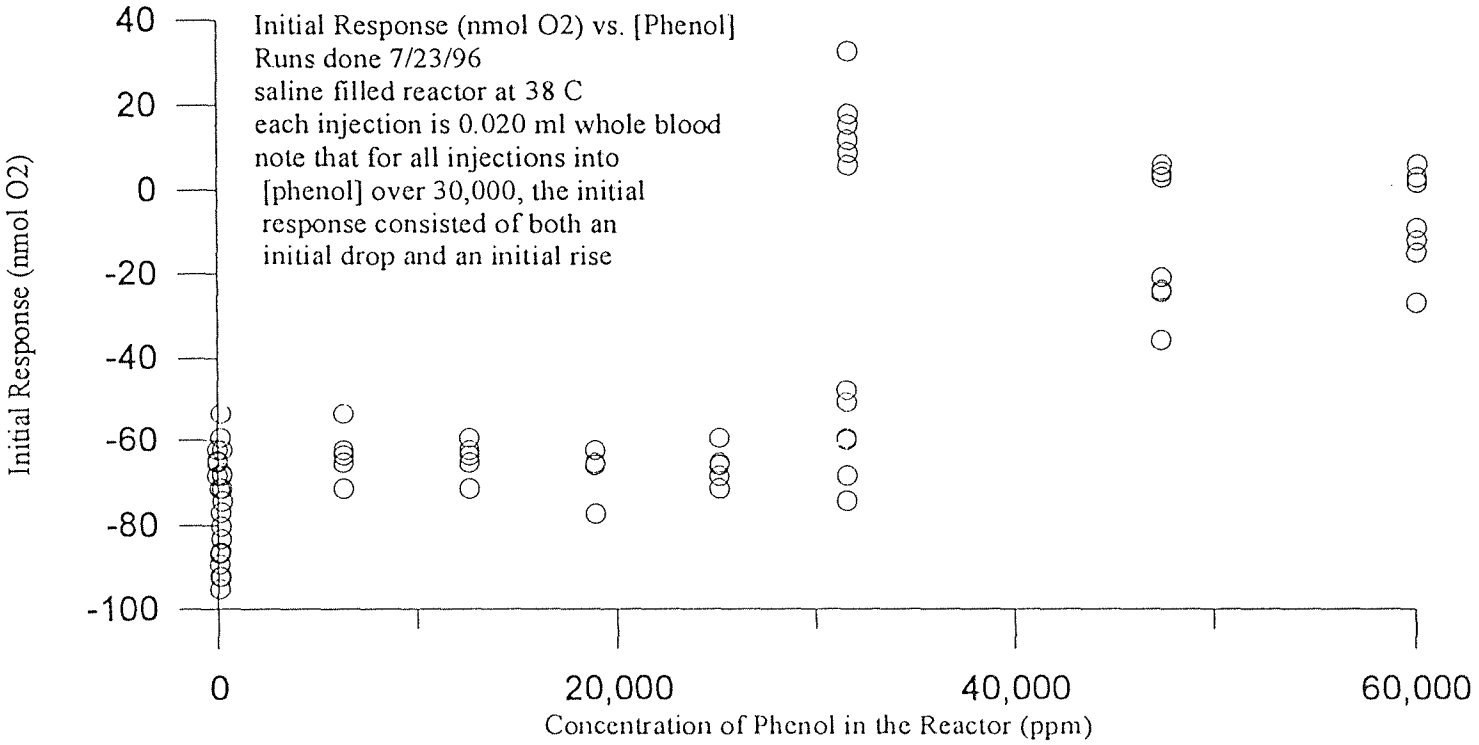


Figure 6.9 The dose response curve of the initial response of 20µl aliquots of blood stressed with a wide range of phenol on 7/23/96.

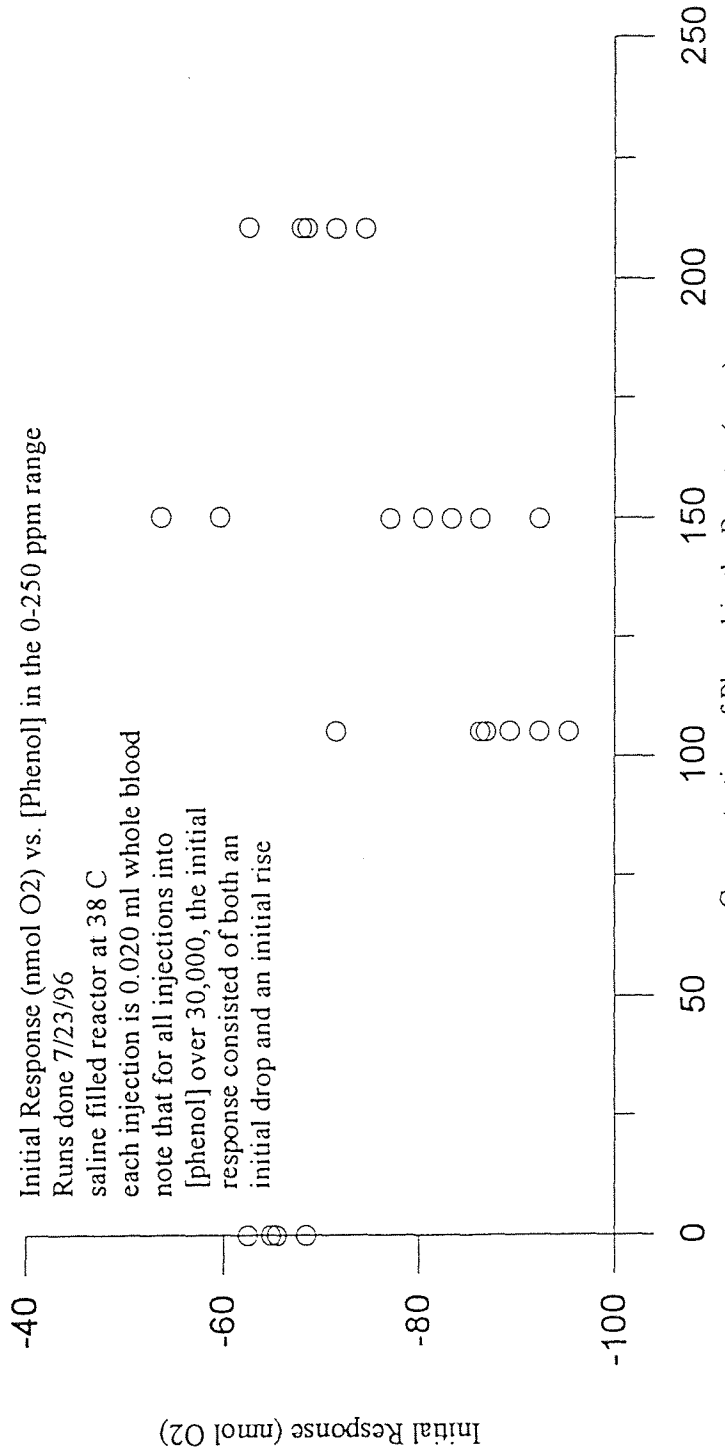


Figure 6.10 The 0-250 ppm phenol range of the dose response curve shown in Figure 6.9.

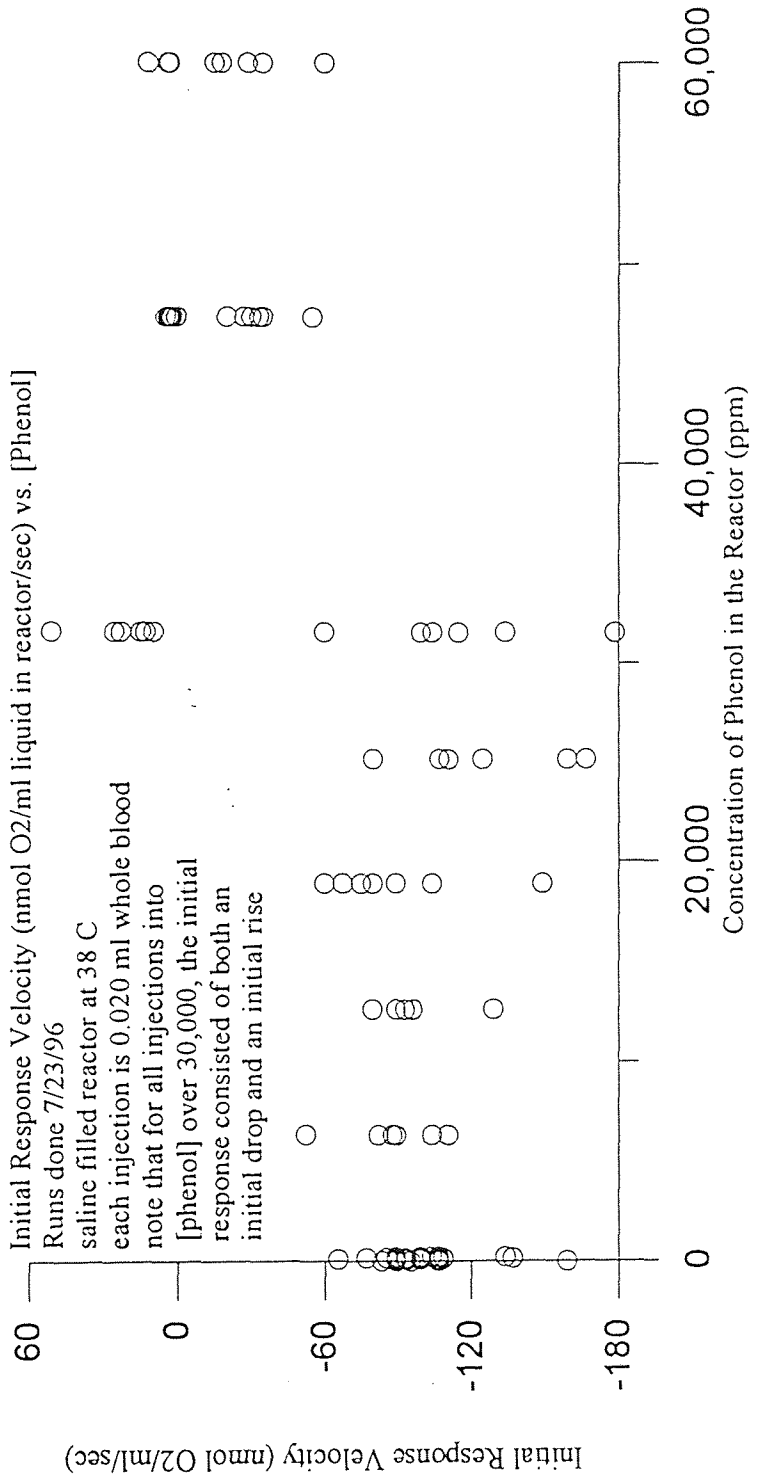


Figure 6.11 The dose response curve of the initial response velocity of 20µl aliquots of blood stressed with a wide range of phenol on 7/23/96.

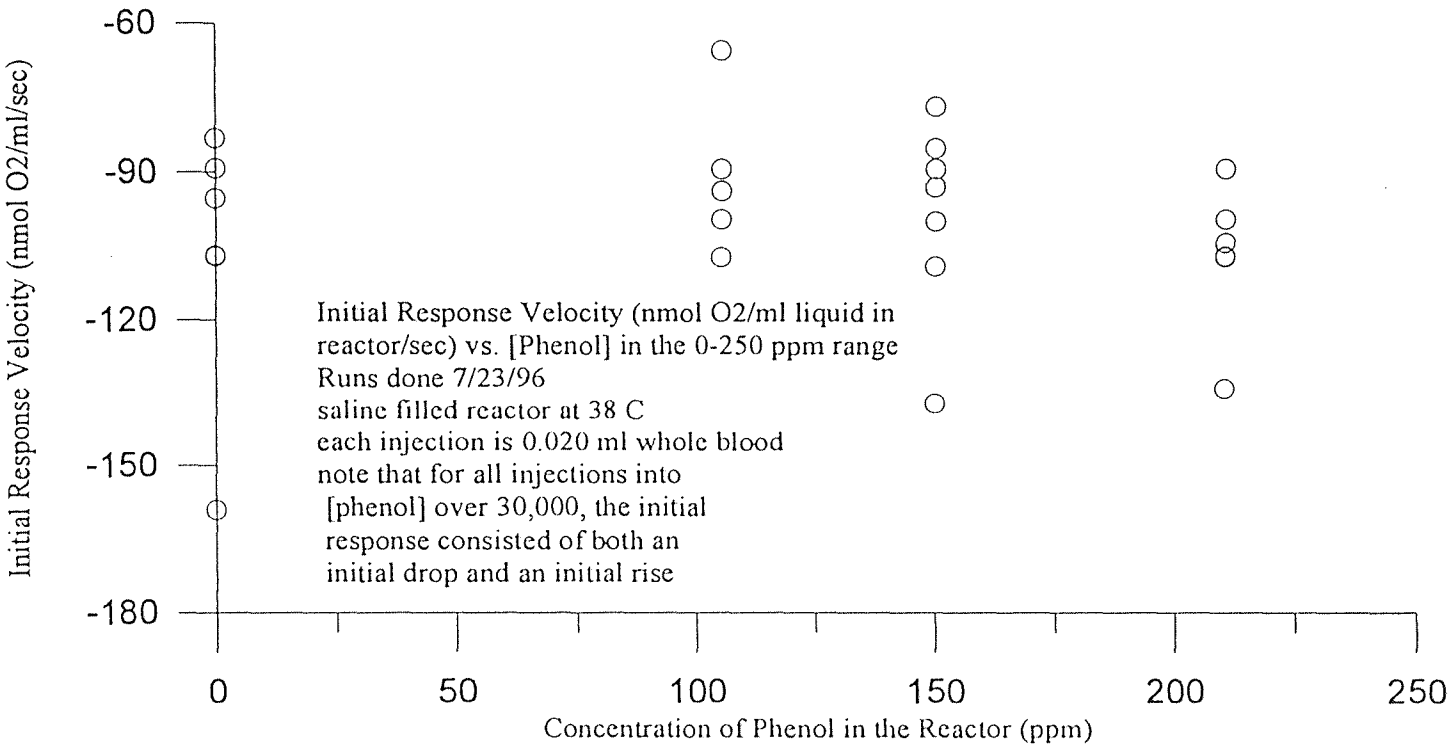


Figure 6.12 The 0-250 ppm phenol range of the dose response curve shown in Figure 6.11.

APPENDIX 7

FIGURES FOR CHAPTER 7

Table 7.3 K_R Values for pairs of runs each done on the same day under the same conditions (Figs 7.3-7.6, 7.8). All runs contain 1,050 ppm phenol in saline at 43 °C. The two most interesting pairs are the pair on 6/10 and the pair on 6/14 because they allow comparison of K_R before and after phenol addition for two identical runs, one which gave a peak and one which did not. The K_R before phenol addition is smaller for the reactor which later gave a MOP in each run. However, the K_R after phenol addition is greater for the reactor which later gave a MOP in one pair but less in the other.

		before phenol addition		**after phenol addition**	
Date	**Amount of blood**	***K_R for run with MOP activity***	***K_R for run w/o MOP activity***	**K_R for run with MOP activity**	**K_R for run w/o MOP activity**
(1996)	**(ml)**	***(1/hr)***	***(1/hr)***	**(1/hr)**	**(1/hr)**
6/10	0.5	0	0.12	-0.02	0.10
6/13	0.75		-0.03		0
			-0.03		-0.05
			(two runs)		(two runs)
6/14	0.2	0.55	0.91	1.22	0.87
6/15	0.2	-14.53		-34.50	
		-36.04		6.63	
		(two runs)		(two runs)	
7/14*	0.4			115.00	86.17

* This run was done in 20,000 ppm phenol at 37.5 °C. The phenol was in the reactor before the blood was added.

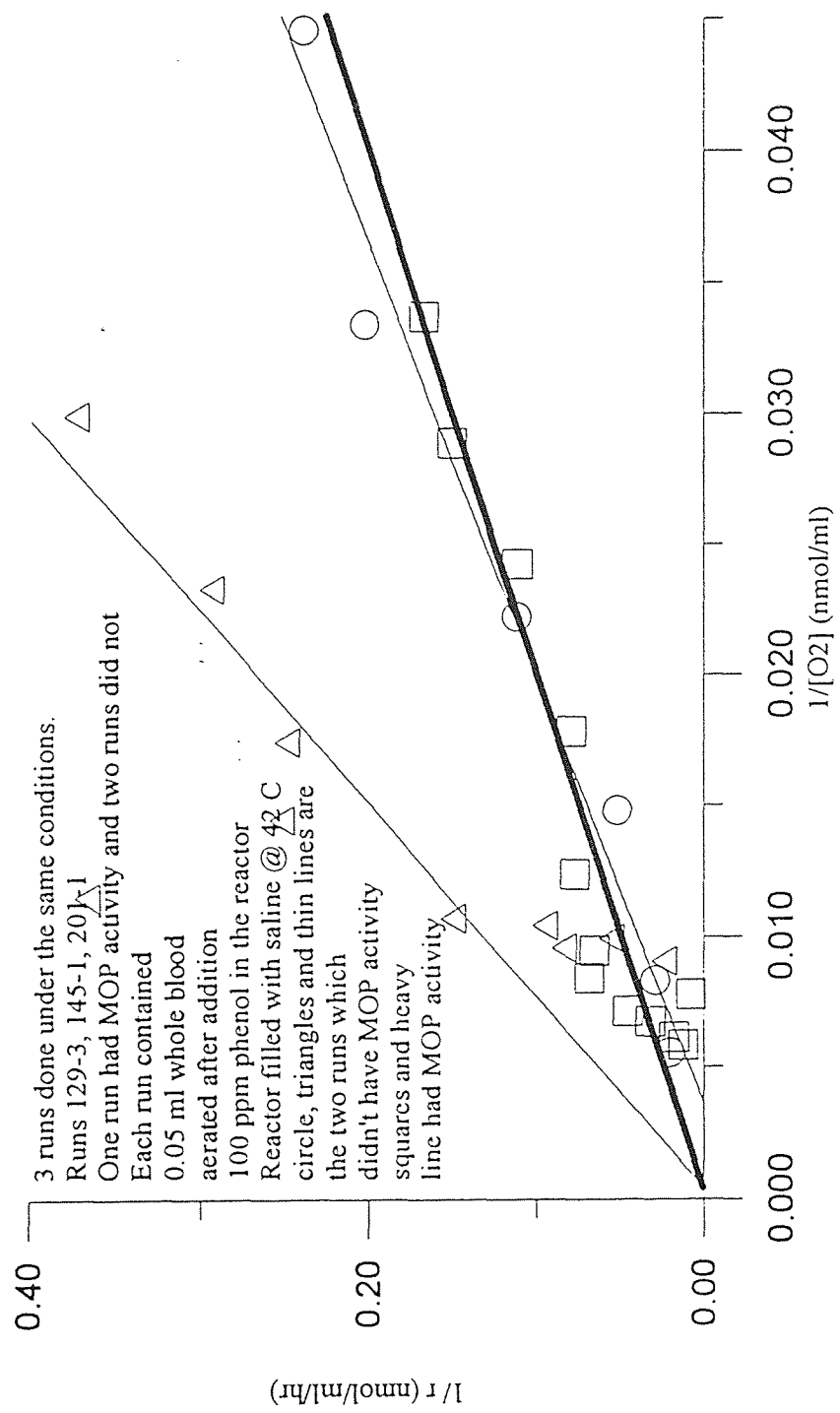


Figure 7.2 Three runs with the same reactor conditions. One run has MOP activity, two do not.

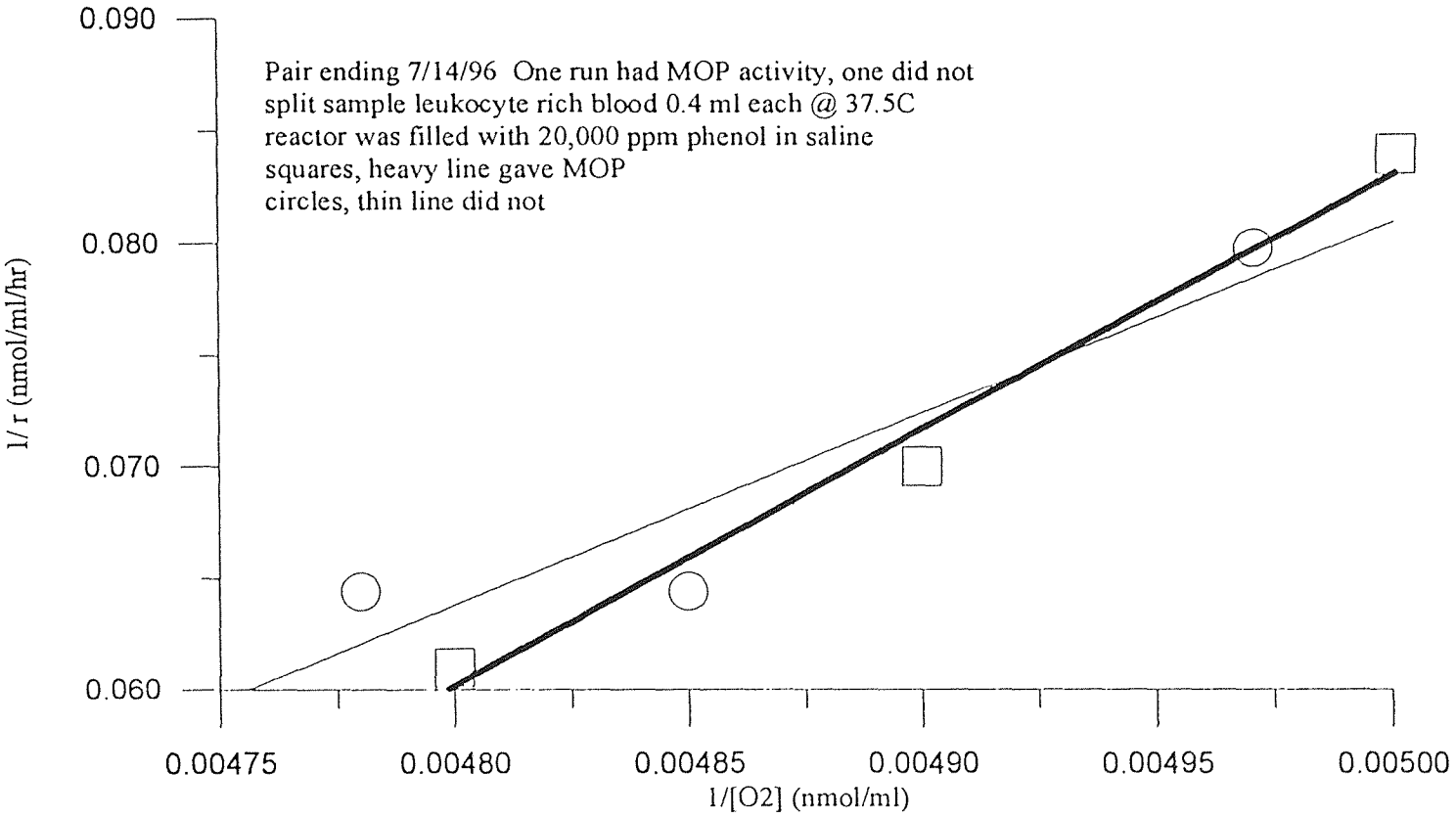


Figure 7.3 Two runs with the same reactor conditions on 7/14/96. One run has MOP activity, one does not.

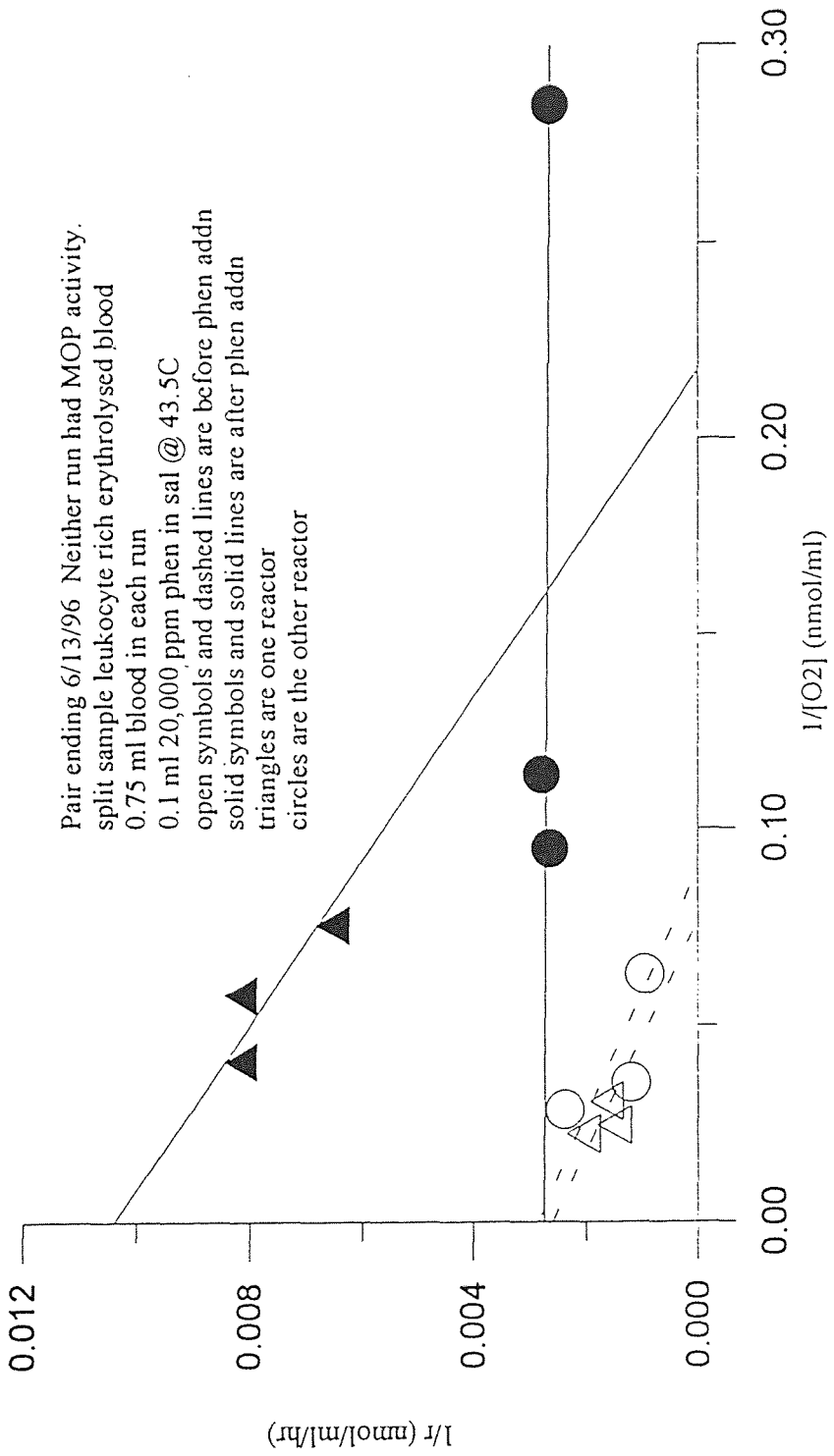


Figure 7.5 Two runs with the same reactor conditions on 6/13/96. Neither has MOP activity. Note the similar slope before phenol addition.

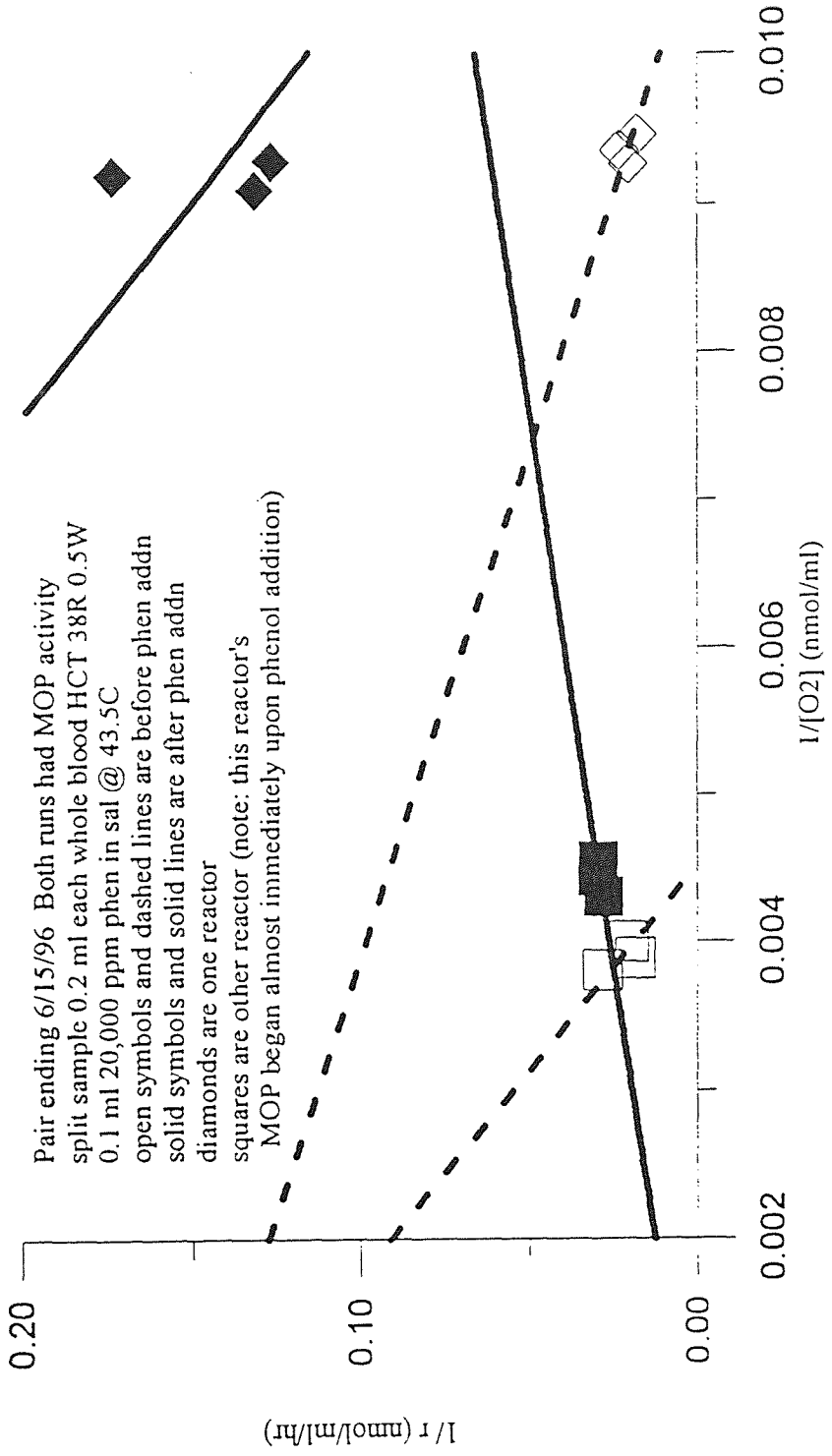


Figure 7.8 Two runs with the same reactor conditions on 6/15/96. Both runs have MOP activity.

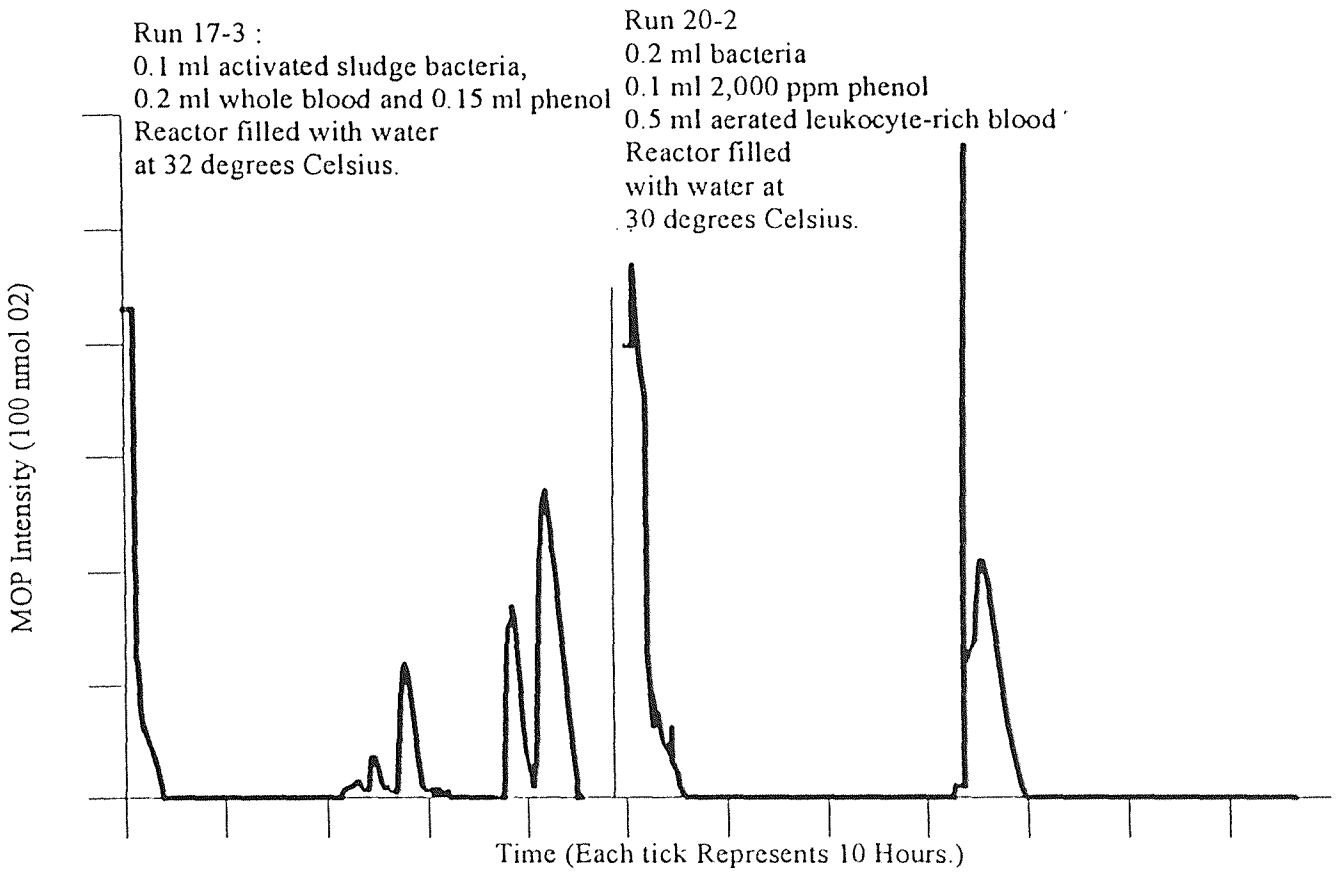


Figure 7.9 Oxygraph recording of runs 27-3 and 20-2.

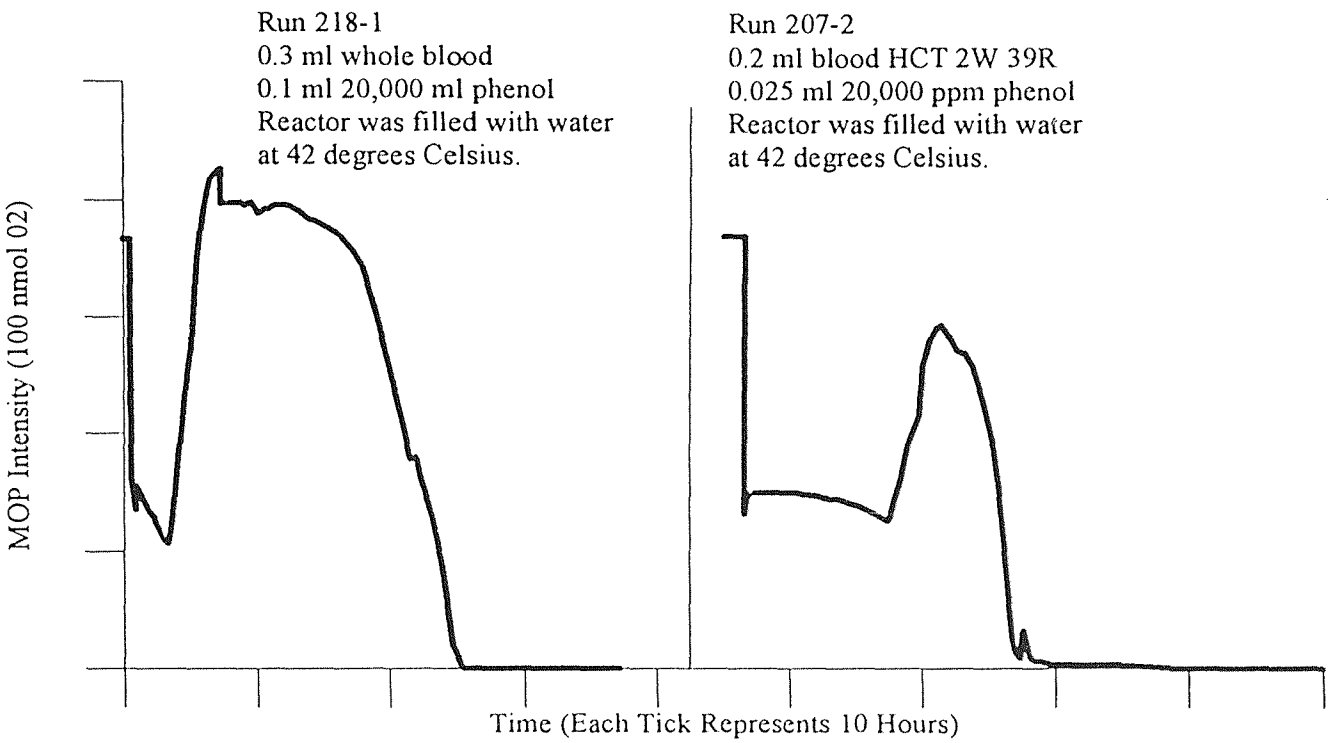


Figure 7.10 Oxygraph recording of runs 218-1 and 207-2.

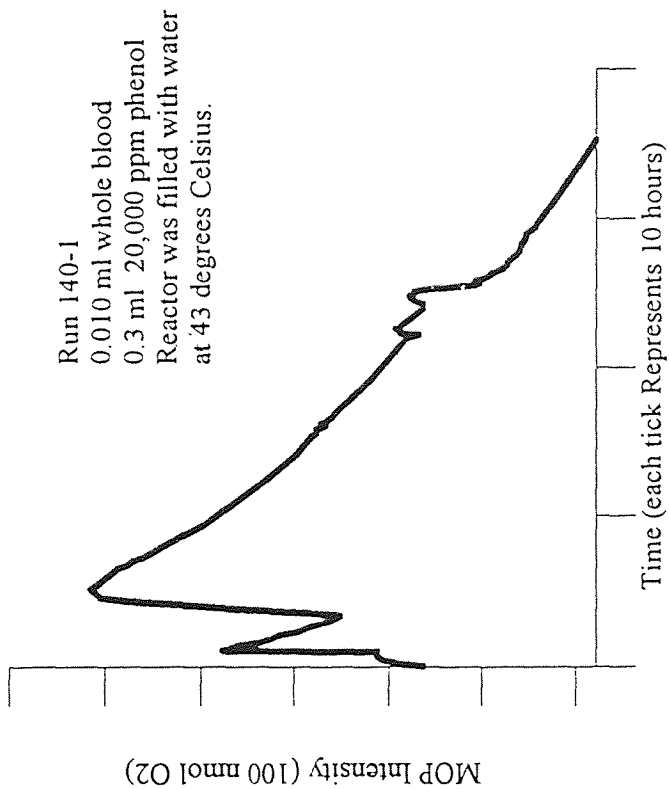


Figure 7.11 Oxygraph recording of run 140-1.

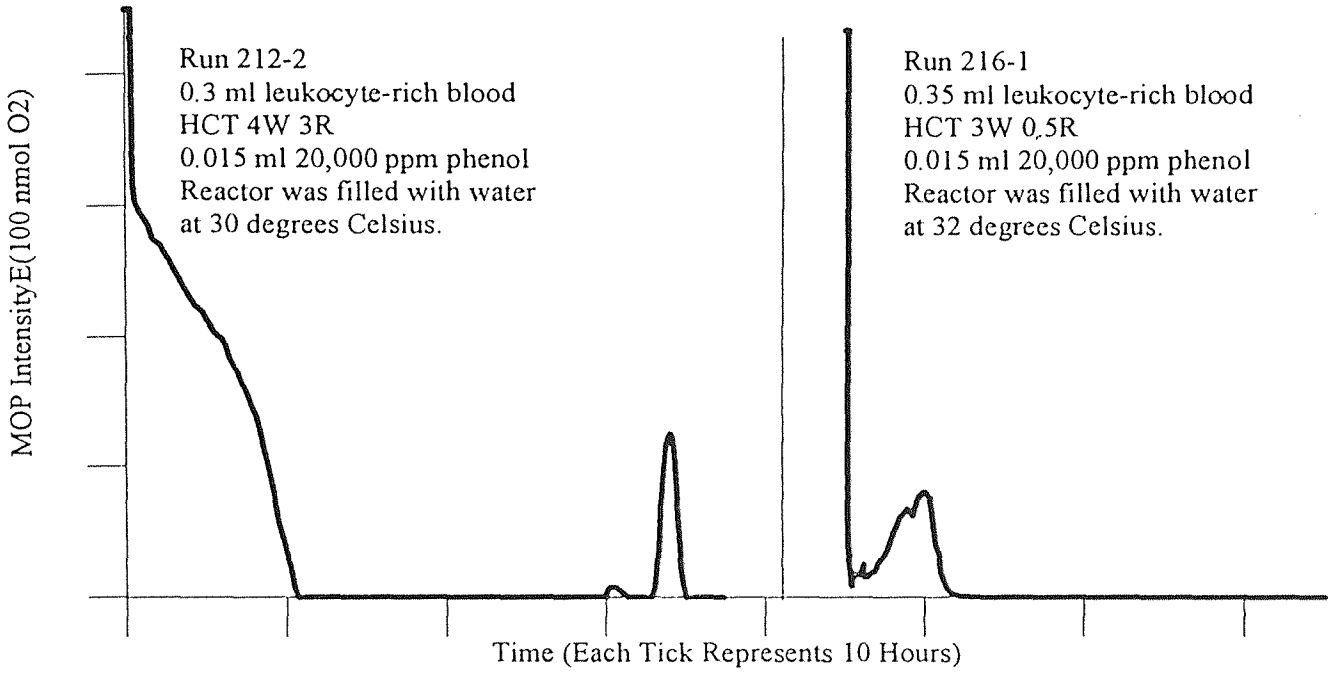


Figure 7.12 Oxygraph recording of runs 212-2 and 216-1

REFERENCES

Adema, CM; van Deutekom-Mulder, EC; van der Knaap, WP; Sminia, T. 1993. "NADPH-oxidase activity: the probable source of reactive oxygen intermediate generation in hemocytes of the gastropod Lymnaea stagnalis." *J Leukoc Biol* . 54(5) 379-83.

Arms, K. and P. Camp. 1987. *Biology*. 3rd ed. Harcourt Bruce Coll. Pubs. Orlando, FL 1113.

Aukrust, P., F. Muller, S.S. Froland. Aug. 1994. "Enhanced generation of reactive oxygen species in monocytes from patients wih common variable immunodeficiency." *Clin. Exp. Immunol.* 97(2): 232-8

Barbior, B. 1991. "Oxidants From Phagocytes: Agents of Defense and Destruction." *Blood.* 959-966

Chen, TP; Roberts, RL; Wu, KG; Ank BJ; Stiehm ER. 1993. "Decreased superoxide anion and hydrogen peroxide production by neutrophils and monocytes in human immunodeficiency virus-infected children and adults." *Pediatr. Res.* 34/4 (544-550)

Ciarn,M. 1992."Beta-naphthylamine induces anion superoxide production in rat peritoneal macrophages." *Biochem-Pharmacol.* Apr 12; 43(8):1841-5

Courreges, MC; Benencia, F; Coto, CE; Massouh, EJ; Couloumbie, FC. 1994. "In vitro antiphagocytic effect of Melia azedarach leaf extracts on mouse peritoneal exudate cells." *J Ethnopharmacol.* 43(2): 135-40.

Daniels, RH; Elmore, MA; Hill, ME; Shimizu, Y; Lackie, JM; Finnen, MJ. 1994. "Priming of the oxidative burst in human neutrophils by physiological agonists or cytochalasins B results from the recruitment of previously non-responsive cells." *Immunology.* 82(3):465-72

Erslev, A. 1985. *Pathophysiology of blood.* W.B. Saunders Company. Philadelphia, PA

Garby, L, Meldon, J. 1977. *The Respiratory Functions of Blood.* Plenum Publishing Corp. Plenum Medical Book Company New York and London

Greenberg, A.E., R. R. Trussell, L S. Clesceri. *Standard Methods for the Examination of Water and Wastewater*. 1985. American Public Health Association. Washington, DC

Gressder, B; Lebegue, S; Brunet,C; Luyckx,M; Dine,T; Cazin,M; Cazin,JC. 1994. "Pro-oxidant properties of methotrexate: evaluation and prevention byan anti-oxidant drug." *Pharmazie*. 49(9): 679-81

Gridley, D.S., S.N. Hammond, B.H. Liwnicz. May-Jun 1994. "Tumor necrosis factor-alpha augments radiatiohn effects against human colon tumor xenografts." *Anticancer Res.* 14(3A) : 1107-12

Grzybowski, A., R. Matusiewicz, K. Grzybowska. Jan-Feb 1994. "The migration, phagocytosis and reduction ability of granulocytes from skin infiltrations of patients with atopic asthma." *J Investig Allergol Clin Immunol.* 4 (1) : 18-22

Harrison, J., X. Shi, L. Wang, J.K. Ma, Y. Rojanasakul. 1994. "Novel delivery of antioxidant enzyme catalase to alveolar macrophages by Fc Receptor-mediated endocytosis." *Pharm Res.* Aug; 11(8) 1110-4

Haynes, DR; Wright, PF; Gadd SJ; Whitehouse, MW; Vernon-Roberts, B. 1993. "Is aspirin a prodrug for antioxidant and xytokine-modulating oxymetabolites?". *Agents Actions.* 39(1-2) 49-58.

Hellstrand, K., A. Asea, C. Dahlgren, S. Hermodson. 1/12/1994. "Histaminergic regulation of NK cells. Role of monocyte-derived reactive oxygen metabolites." *J Immunol* 153(11):4940-7

Hofstra, AH; Uetrect, JP. 1993. "Myeloperoxidase-mediated activation of xenobiotics by human leukocytes." *Toxicology.* 82/1-3 (221-242).

Klegeris, A; McGeer, PL; 1994. "Rat brain microglia and peritoneal macrophages show similar responses to respiratory burst stimulants." *J-Neuroimmunol.* 53(1):83-90.

Kuby, J.1994. *Immunology.* W.H. Freeman and Company. NY, NY.

Lakhwala, Fayaz. "A Combined Gel-Entrapped and Membrane-Attached Microbial Process for Biodegrading Organic Compounds." Dept of Chemistry, Chem. Eng., and Env. Science. NJIT. 1991.

Laskin,D. 1994. "Activation of alveolar macrophages by native and synthetic collagen-like polypeptides." *Am. Journal Respir Cell Mol Biol.* Jan; 10(1):58-64

Little, R., M.R. White, K.L. Hartshorn. Oct 1994. "Interferon-alpha enhances neurtophil respiratory burst responses to stimulation with influenza A virus and FMLP." *J Infect DIs.* 170(4): 802-10

Mathews,C; van Holde, K. 1990. *Biochemistry.* The Benjamin/Cummings Publishing Company, Inc. Redwood City, CA

Noel D, E. Bach 'ere, E. Mialhe. 1993 .Nov-Dec. "Phagocytosis assiciated chemiluminescence of yhemocytes in Mytilus edulis (Bivalvia)." *Dev Comp Immunol.* 17 (6) 483-963

Ottonello, L., P. Dapino, M. Scirocco, P. Barbera, F. Dallegri. 1994 Mar. "Triggering of respiratory burst by tumor necrosis factor in neutrophils adherent to fibronectin. Evidence for a crucial role of CD18 glycoproteins." *Agents Actions* 41(1-2): 57-61

Passwell, JH; Shor, R; Smolen, J; Jaffe, CL. 1994. "Infection of human monocytes by Leishmania results in a defective oxidative burst." *Int J Exp Pathol.* 75 (4) : 277-84.

Pipe, R. K. Mar-Jun 1992. "Generation of reactive oxygen metabolites by the haemoctes of the mussel Mytilus edulis." *Dev Cop Immunol* 6 (2-3) 111-22

Poulsen, L; Ziegler, D; Sofer, S. 1976. "Properties and Application of an Immobilized Mixed-Function Hepatic Microsomal Drug Oxidase" *Methods.Enzymol.* Vol 44. Mosbach, K (Ed).

Rabe, KF; Perkins, RS; Dent, G; Gustmann, H; Barnes, PJ. 1994. "Inhibitory effects of sulfonsted shale oil fractions on the oxidative burst and CA++ mobilization in stimulated macrophages." *Arzneimitelforschung.* 44(2): 166-70

Rautelin, H; von-Bonsdorff, CH; Blomberg, B; Danielsson, D. 1994. "Ultrastructural study of two patterns in the interaction of Helicobacter pylori wit neutrophils." *J Clin Pathol.* 47(7) : 667-9

Rickinger, O., H. Sheriff, R. Hirschelmann. Mar 1993. "Effects of (D-Ala2) methionine enkephalinamide in PMA-induced mouse ear edema." *Agents Actions* 41 (1-2): 118-9

Rosenkranz,AR; Templ, E; Traindl, O; Heinzl, H; Zlabinger, GJ. 1994. "Reactive oxygen product formation by human neutrophils as an early marker for biocompatibility of dialysis membranes." *Clin-Exp-Immunol.* 98(2): 300-5.

Sanguinetti, C. 1992. "Oxidant/antioxidant imbalance: role in the pathogenesis of COPD". *Respiration.* 59 Suppl 1:20-23.

Schulte,P. 1991. "Contribution of biological markers to occupational health." *Am. Journal of Industrial Medicine.* 20(4): 435-46

Schalm, O.W., N.C. Jain, E.J. Carroll. 1975. *Veterinary Hematology.* 3rd ed. Lea& Febiger. Philadelphia, PA.

Shakespeare, William. *Hamlet.* Act IV, Scene III Lines 20-30.

Shen, X., W. Mei, X. Xu. Oct 15 1994. "Activation of neutrophils by a chemically separated but optically coupled neutrophil populatioh undergoing respiratory burst." *Experientia.*; 50(10): 963-8

Simons, JM; Hart, BA; Ip Vai Ching, T; Van Dijk H; Labadie, RP. 1990. "Metabolic activation of natural phenols into selective oxidative burst agonists by activated human neurophils." *Free Radic. Biol. Med.* 8/3 (251-258).

Singh, A., M.L. Failla, P.A. Deuster. Jun 1994. "Exercise-induced changes in immune function: effects of zinc supplementation." *J Appl Physiol* 76 (6): 2298-303

Sozzani, S., D.E. Agwu, M.D. Ellenburg, M. Locati, M. Rieppi, A. Rofas, A. Mantovani, L.C. PęPhail. Dec 1 1994. "Activation of phospholipase D by interleukin-8 in human neutrophils." *Blood*; 84(11): 3895-901

Subrahmanyam,V. VV. Ross, D. Eastmond, DA. Smith , MT. 1991. "Potential role of free radicals in benzene-induced myelotoxicity and leukemia." *Free Radic Biol Med.* 11(5) 495-515

't Hart B. A. and J. M. Simons. 1992. "Metabolic activation of phenols by stimulated neurtophils: a concept for a selective type of anti-inflammatory drug." *Biotechnol Ther.* 3 (3-4) 119-35 Ref: 64

Turner, E., C. E. Somers, B. M. Shapiro. Oct 25, 1985. "The relationship between a novel NAD(P)H oxidase activity of ovoperoxidase and the CN- resistant respiratory burst that follows fertilization of sea urchin eggs." *J Biol Chem.* 13163-71

Turyna, B; Bos, M; Jurek, J. 1994. "The influence of in vivo stimulation on functional activity of rat macrophages." *Folia Histochem Cytobiol.* 32(2) : 101-5

Waddell,TK; Fialkow,L; Chan,CK; Kishimoto, TK; Downey,GP. 1994. "Potentiation of the oxidative burst of human neutrophils. A signaling role for L-selectin." *J Biol Chem.* 269(28) : 18485-91

Watson, F., G.M. Lowe, J.J. Robinson, D.W. Galvani, S.W. Edwards. Apr 1994. "Phospholipase D-dependent and -independent activation of the neutrophil NADPH oxidase." *Biosci Rep.*, 14(2): 91-102

Wolf, HM; Vogel, E; Fischer, MB; Rengs, H Schwarz; Eibl, MM.1994. "Inhibition of receptor-dependent and receptor-independent generation of the respiratory burst in human neutrophils and monocytes bu human serum IgA." *Pediatr-Res.* 36(2): 235-43.

Zeller, JM; Sullivan, BL. 1992. "C-reactive protein selectively enhances the intracellular generation of reactive oxygen products by IgG-stimulated monocytes and neutroophils." *J Leukoc Biol.* 52(4)449-55