# Meeting the Oxygen Requirements of an Isolated Perfused Rat Liver
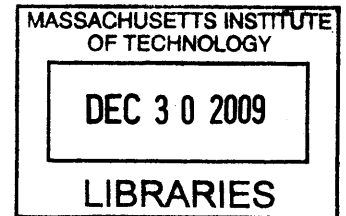
by

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Submitted to the Department of Mechanical Engineering in partial
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## Abstract

Liver perfusion systems can be used as organ culture platforms for metabolic, genetic and systems engineering, tissue regeneration, pharmacokinetics, organ storage and marginal donor reconditioning for transplantation. The primary requirement of such a system is that it maintain ex vivo organ function in a stable manner for indefinite periods of time. The more physiologically relevant the perfusion system is, the lower the likelihood of incurring functional instability, and the greater the clinical relevance of the data obtained. Currently, a major limiting factor in achieving such a design is the absence of organ-specific in vivo data to standardize, evaluate and optimize the state of perfused livers. Oxygen uptake rate, the primary indicator of organ metabolism and therefore a likely important marker of organ viability, was chosen as one such parameter to be investigated. A systematic review and reproduction of numerous oxygenation conditions cited in the literature in addition to *in vivo* data sampling across the rat liver shows a significant paucity of oxygen uptake in perfusion compared to *in vivo*. The reasons stem largely from the biological and mechanical flow properties of the perfusate, but also from the altered metabolic state of the organ in perfusion. In the absence of an oxygen carrier, the liver became oxygen starved and lost functionality. The addition of erythrocytes significantly improved oxygen delivery rates while reducing the flow rate and damaging shear stress. However, as hematocrit approached physiological values perfusate viscosity became detrimentally high, causing severe structural and ultimately functional damage. Large strains of erythrocytes similarly impacted the liver. The addition of sodium heparin reduced erythrocyte-erythrocyte and erythrocyte-parenchyma interaction, thereby reducing sheer stress on the liver. The optimal oxygen carriers were small heparinized erythrocytes, such as goat or rat erythrocytes, at a hematocrit of 12%-20%. These conditions continued to provide adequate in vivo oxygen delivery rates and sustained organ structure and function.

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# Acknowledgements

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# Chapter 1

# Introduction

## 1.1 Background

Stable isolated organs provide results which are substantially better controlled and more quantitative than organismal studies and much more clinically relevant than cellular/molecular scale analyses. As such isolated organ perfusion may be regarded as a highly sophisticated cell culture technique with which to conduct clinically relevant investigations. Applications that would require such a tool include metabolic, genetic and systems engineering, tissue regeneration, pharmacokinetics, organ storage and marginal donor reconditioning for transplantation. As applications for a competent model grow in number and complexity, it is highly desirable to establish the liver perfusion as a robust and ubiquitous tool that can generate well-preserved organ-defined data. However, unlike the organism that has well-defined measures of viability, or a cell culture where the well-defined needs are provided for by a standardized protocol, a fundamental limiting factor of all current perfusion systems is the significant, and often polarizing, variability in deciding what the basics needs of that organ are and how they should be met. Inherent in this problem is the inability to exactly define an index of organ viability derived from the parameters critical to its stability. There are as many studies regarding what the optimal system should be as there are reporting findings with one, but no single study with a numerical model capable of identifying and relating the common key parameters and the boundary conditions that define them. Subsequently, perfusion systems are fraught with numerous developments into dead ends: 1) They are frequently reserved only for purposes of relative comparisons to each other, with minimal reproducibility from venue to venue, 2) No account of systematic error has been established as there is no normal with which to compare them to, 3) Advances are piecemeal and not oriented toward developing a single gold standard.

## 1.1 Objective

The aim of this project has been to initiate the groundwork upon which to identify and evaluate the governing parameters of a liver perfusion system. The ultimate future goal of this work is to numerically predict the response of the liver to manipulation of any of these parameters, thereby facilitating correlations between studies and advancing perfusions toward clinical applications. In order to do this, it is imperative to first develop

a liver perfusion system that has the ability to support and maintain an *ex vivo* liver in a stable manner for prolonged periods of time. *The major hypothesis to be tested is that a liver must be sustained with a physiologically relevant perfusion system for it to maintain both its structural and functional integrity and if either component is compromised then the system will no longer be stable.* The focus of this body of work therefore is to reduce the impact of the perfusion system upon the liver by manipulating parameters that affect this mechanical intervention.

The requirements inherent in developing this animal model include:
1) A thorough review of the literature to determine the evolution of the limitations of perfusion systems to date.
2) A comprehensive understanding and quantification of the organ physiology and in vivo conditions to be modeled and conserved. This will provide a "physiologically relevant" standard to which all systematic errors and acceptable boundaries of the system can be evaluated.
3) The development of an ethical, cost-effective, highly reproducible perfusion apparatus that allows for ample data collection and manipulation to study multiple conditions.

# 1.3 Applications

The motivation to develop a liver perfusion system that accurately articulates the differences between a healthy liver and one that is diseased or injured is fueled by its numerous applications. The results of such a model will have the ability to integrate current cell culture systems and gear the outputs to an organism scale. An inherent quality of perfusion systems therefore is the ability to detect the organ's response to any insult. What is implicitly stated here is that for a physiologically relevant description of the organ's response, however subtle, to be elicited by the system, the system itself must have an inconsequential effect on the organ's performance. The likelihood of this achievement is large enough to expand the uses of perfusion systems into developing technologies that will contribute significantly to the current organ donor shortage. By developing a system that is physiologically similar so as to be able to enhance the time of organ storage and transport distances, organ preservation protocols may be radically altered. Tapping into currently unused resources is another option via the organ's susceptibility to manipulation in a perfusion system that lends itself to the notion of similarly being treated *in situ*. This suggests the possibility of using livers not normally feasible for transplantation. It may be possible to de-fat steatotic livers, which currently comprise a quarter of all livers obtained from an otherwise healthy donor pool. Non-heart-beating-donor livers, if obtained before irreparable ischemic damage has occurred, may also be resuscitated through appropriate perfusion. Other constant needs for liver perfusions are in the study of metabolic pathways of the liver, which represents systemic metabolism by encompassing all metabolic processes observed in the body. This is especially useful in elucidating disease processes such as cachexia or sepsis and is therefore capable of suggesting treatment alternatives that could restore homeostasis. Similarly, perfusion systems are invaluable as a technique to determine how the liver may

respond to a particular drug, or exposure to a particular chemical. Thus the applications of such a system gratifyingly extend from "bench to bedside" with very real potential in research, pharmaceutical and clinical fields.

# 1.4 Liver Perfusion Systems

The basic concept behind a perfusion system is that the perfused organ can be physically modeled as a black box governed by a set of external circuitry. To achieve this, the organ's circulatory system is replaced with a wholly artificial one thereby isolating the liver from the rest of the organism. A closed loop connects the organ's inflow and outflow to a reservoir. Contained in this reservoir is the blood substitute, or perfusate, the chemical constituents of which are defined. This perfusate is subsequently mixed in the reservoir, pumped through an oxygenator and a heat exchanger prior to entering the liver. The effluent subsequently re-enters the reservoir, completing the circuit.

The perfusate flow properties, such as rate, temperature, and viscosity are a function of the pump speed, heat exchanger and perfusate content respectively, while the value of the portal pressure is governed by the state of the organ.

1) Rate: The liver receives two blood supplies; one from the portal vein (1.2 to 1.5mL/min per g liver) and one from the hepatic artery (0.2 to 0.3mL/min per g liver). Although the hepatic artery perfuses 9-12% more hepatic tissue than the portal vein [2], frequently dual vessel perfusion is avoided for simplicity based on the decision that this flow rate is small by comparison and that liver transplantations in rats have been performed successfully without the hepatic artery reconstruction [3]. The chosen value of flow rate in perfusion has previously been based on measured in vivo rates [4, 5] or on the desire to achieve a physiological delivery rate of substrate, such as oxygen. In the absence of an efficient oxygen carrier such as erythrocytes, dependence upon the marginal amount dissolved in perfusate has necessitated a flow rate of 3 to 5 times the physiological value.

2) Temperature: While temperature of the perfusate plays a role in the fluid properties particularly as some perfusions take place at 4°C (for cold storage purposes), its primary effect is dictating the degree of active metabolism that occurs – the higher its value above normal, the more heat shock and hypermetabolism there is, whilst lowering it reduces liver metabolism and slows down or stops many reactive pathways. As the purpose of this project was to investigate differences in oxygen uptake rates, physiological temperatures of 37°C were used to ascertain the demand of a completely functioning metabolic state.

3) Viscosity: The viscosity is primarily affected by the presence or absence of erythrocytes and the perfusate constitution:
   a. Plasma substitute. Typically water-based with a variety of chemical constituents to mimic the content and osmolarity of whole blood and is therefore on the order of ~1.2 times the viscosity of water ($\mu_{water} = 0.001$

kg/(m·s) at 20°C). This value decreases negligibly with a temperature rise to 37°C but is otherwise constant.

    b. Plasma with red blood cells. The addition of red blood cells causes the perfusate to no longer behave in a Newtonian fashion. The viscosity of whole blood at 20°C can be stated as being 0.0027 kg/(m·s) for a hematocrit of 40%. Changing the hematocrit ratio, the size and flexibility of the red blood cells, the geometry of the vasculature, the osmolarity, and the velocity at which they are flowed largely impact the effective viscosity. Moreover, as they are biologically interactive, the dynamics of the system may change their behavior with time.

4) Pressure: The difference between portal (inflow) pressure and central (outflow) pressure is a function of the resistance of the liver and flow rate into the liver. As the flow rate enters with a pressure of value $P_i$ and exits with atmospheric pressure $P_o$, the flow diverges into hundreds of sinusoids or micro-channels in parallel and series with each other such that every hepatocyte is partially directly in contact with the fluid (a sponge-like maze for fluid flow). The sum of the flow rates through each parallel sinusoid is the total flow rate into the liver; subsequently the flow along each sinusoid is reduced significantly in magnitude. The resistance depends on perfusate viscosity ($\mu$) and the permeability ($\mathcal{P}$) or geometry of flow of organ vasculature. In vivo, the viscosity of blood is low enough, and a healthy liver so permeable that $P_i$ - $P_o$ is less than 6cmH$_2$0. For a given organ architecture, too low a pressure drop, as a result of inefficient flow and/or minimal viscosity, can cause shunting within the organ leaving the periphery hypo-perfused. Too high a flow pressure from excessive inflow rates or increased viscosity causes shear stress ($\tau$) damage to the organ. This results in architectural deformation by disruption of the internal vasculature thereby increasing its internal resistance to flow. Subsequent congestion of the organ escalates a further rise in pressure difference across it. Physiologically, the portal pressure is maintained at a relatively constant value despite variations in the volumetric flow rate from the splanchnic blood supply. Changes in flow trigger a reciprocal hemodynamic response of the vascular bed so as the flow increases, the hepatic vascular resistance decreases and vice versa.

$$P_i - P_o \propto Q \times R \tag{1.1}$$

$$R = f\left(\mu, \mathcal{P}\right) \tag{1.2}$$

Conversely, the chemical constituents do change, as the organ is metabolically active. The change in concentration over time of various metabolites can be measured and used to provide information of the metabolic fluxes within the organ. Naturally, only a select number of metabolites can be measured that appear in the perfusate reservoir, thus for information of metabolic activity within the black box itself, the remaining metabolites are accounted for using mass balance equations. These are described by stoichiometric

formulas that account for all known metabolic reactions within the organ and using metabolic flux analysis (MFA), the relative change in the these fluxes can describe the metabolic state of the organ. This is useful when comparing different states as would be observed in hypermetabolism, sepsis or the effects of a particular drug; providing information on the mechanisms of those insults upon the liver and pathways of intervention if they are to be addressed. Subsequently, the content of the perfusate is important:

1) Volume: The circulating volume should be able to act as a reservoir that can dilute the changes in concentration of the perfusate constituents by the liver so that while they are still measurable, they do not affect its performance. It also needs to accommodate volume losses due to measurements taken over time.

2) Metabolites: The perfusate must contain enough substrate to allow for stable metabolism over the analytical period. Several assumptions regarding what those initial values should be have been made, based upon common values observed in the blood during fasting and fed states and then adjusting for depletion of anabolic substrates and the accumulation of by-products.

3) Perfusion gas analysis: For more transient changes in concentration, such as oxygen uptake by the organ, samples must be taken immediately prior to entry and post exit of the liver. The differences in values are then used to determine the oxygen uptake rate (OUR). Oxygenation of the perfusate (using the "lungs" of the external circuit) during each cycling of the perfusate should ensure that the oxygen delivery rate (ODR) is constant. Perfusate gas analysis of flow across the organ through information on oxygen delivery and uptake, carbon dioxide levels and pH and is an acute marker of organ function in direct response to the perfusion.

4) Osmolarity: This is adjusted at the beginning of any perfusion to be between 280-300mOsm/L and remains buffered by the reservoir of perfusate over the duration of the perfusion.

Finally, post-perfusion histological studies will show to what extent organ architecture has been preserved throughout the perfusion and is and provides a qualitative measure of organ function.

Thus a perfusion system is designed to provide real-time information regarding organ viability, and projects a snapshot of organ function and structure at some pertinent point in the organ's research history.

$$O_2 \text{ UptakeRate } (OUR) = \dot{V}_{O_{2,in}} - \dot{V}_{O_{2,out}}$$

$$= \left[ O_{2,in} \right] \times \dot{V}_{perfusate,in} - \left[ O_{2,out} \right] \times \dot{V}_{perfusate,out}$$

$$c(t) = \sum_{i=Aext}^{Dext} c_{0,i} \pm dc_i(t)$$

Sample at t=(0⁻,0⁺, 10, 20..., 60min)
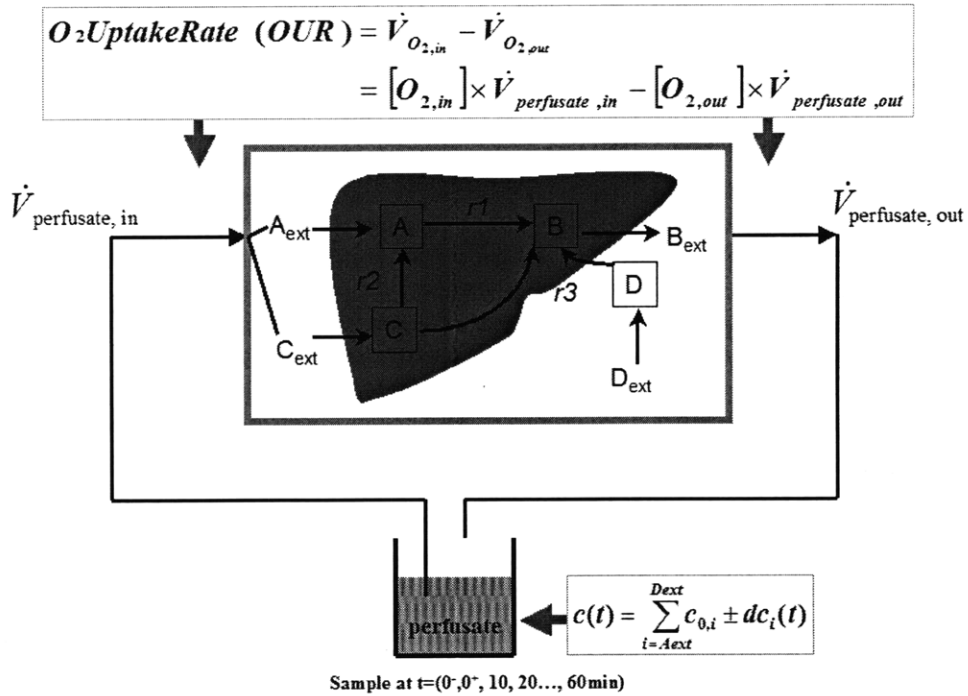
**Closed loop, cumulative time course experiment**

Figure 1-1: Data collection points in a perfusion circuit. A schematic of the liver as a black box governed by external quantified parameters. Any changes in these parameters describe perfusion stability and provide metabolic information on the effects of the pertinent study at hand, relative to a normal liver.
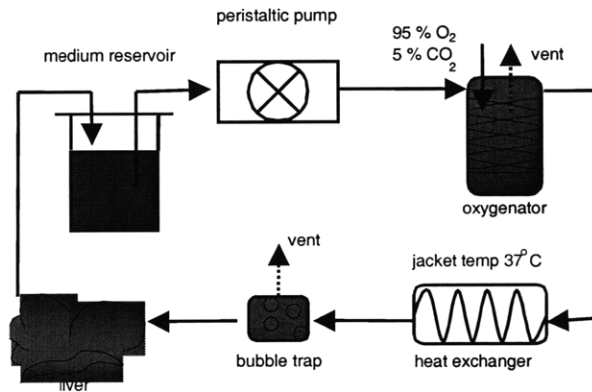


Figure 1-2: Essential requirements of a perfusion circuit. A pump flows the perfusate through an oxygenator, a heat exchanger and a bubble trap into the liver after which it flows back into the original reservoir.

# Chapter 2

# Perfusion Timeline: A Review of the Literature

Some of the earliest available information on rat liver isolation and perfusion dates back to work done by Brauer [6] wherein he makes references to earlier work still by Kellaway (1947) and Bernard (1934) who established such techniques that were "carried out in general, as parts of biochemical investigations." Brauer's work however was seminal to the development of the field of long-term liver preservation and was accompanied by Miller, Bly, Watson & Bale (1951), Mortimore (1961), Hems and Krebs (1966).

Briefly, an outline of Brauer's perfusion system will be given to provide a sense of where the starting point of concept integration began and what the limiting factors were. This is helpful in following the resolution of these issues over the decades in the tables below, particularly in making the point that the perfusion system begot a better perfusion system. In so doing, a clearer understanding of the requirements and hence the physiology of the liver has been achieved.

## 2.1 Early System Design

### 2.1.1 Biomechanical considerations

Brauer's perfusions were based upon the knowledge of liver physiology at the time which guided preparations in the mechanical setup: 1) Survival of hepatic cells in culture was possible for periods of several days at least, so this was a feasible project 2) The liver architecture was known to be very sensitive to perfusion pressures and unless they were sufficiently low at inflow, some function and most structural integrity would be lost 3) Hepatic arterial blood supply was not essential for normal function of the rat, nor even of the dog liver, provided adequate antibiotic levels were maintained in the blood supply (presumably to prevent infection due to possible tissue decay at site of anastomosis and subsequent non-perfused sections of liver) 4) Heart-lung-liver preparations in the dog – in contrast to saline perfusate – would produce bile thus erythrocytes were necessary for its production, while innervation played a marginal role. Brauer's perfusion system comprised a pump to direct the perfusate from its reservoir through flaring connections (found to preserve erythrocytes) after which it was passed through a falling film aerator

into a nylon bag filter back to the reservoir. The blood supply to the organ was tapped from the point of pressure regulation and cycled back to the system via the aerator.

## 2.1.2 Perfusate considerations

Brauer acknowledged the difficulty of designing a perfusate that would adequately meet all the criteria of blood that are necessary in organ preservation. Thus he devised three solutions which were then added together: "A" contained crystalline bovine plasma albumin (BPA) for maintenance of osmotic pressure, sodium carbonate for buffering pH, and salts for sodium, potassium, calcium and chloride levels, dissolved in distilled water. "B" contained dibasic sodium phosphate, a laxative, and potassium phosphate, a diuretic for the purposes presumably of vascular dilatation and relaxation. "C" comprised whole rat blood with heparin. A fourth solution "D" was infused throughout the experiments for "topping up" elements of A and B, but also dextrose for constant glucose supply, terramycin hydrochloride as an antibiotic, and a dye for observation of flow. For an appropriate oxygen carrier, Brauer made use of whole blood.

## 2.1.3 Liver preparation

Sprague-Dawley rats were used as the animal model for both liver and blood harvesting. A serious drawback in his technique was the insertion of the explanted liver into the perfusion stream, which frequently implied significant handling and ischemic damage. In order to compensate for this, Mortimore suggested an in situ method of perfusion, which adequately solved the problem of manipulation and ischemia. Variations of both methods are still in use today.

## 2.1.4 Results

Despite a very primitive system, Brauer noted uniformity in organ perfusion, bile formation, and microscopic organ preservation after a short period of perfusion, however his system was not stable and was incapable of prolonged, consistent perfusion without causing damage.
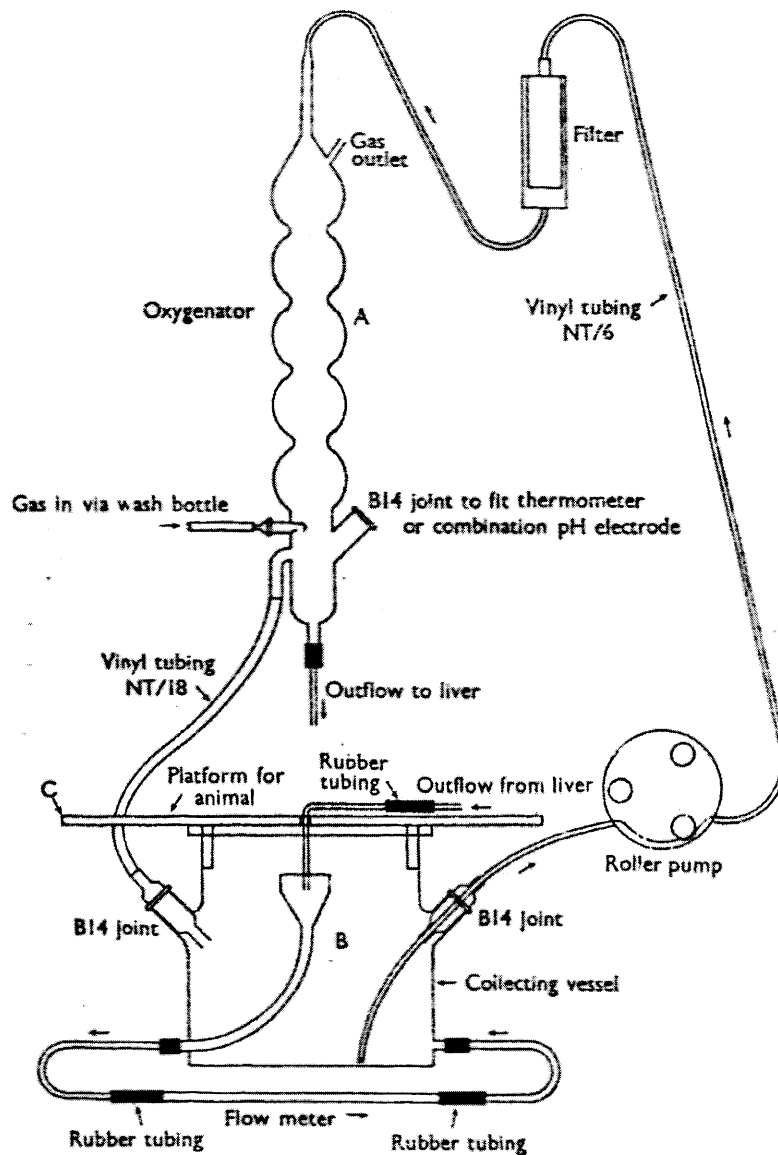
Figure 2-1: Early perfusion system. The adaptation of Brauer and Mortimore's techniques to a slightly modified system by Hems, Berry and Krebs [7], yet the fundamental components are distinctly similar to the original system, and what is being used today. It is at this early point in the development of physiological perfusion systems that they were rapidly being used to characterize various aspects of the organ despite any drawbacks the system might have. The "make do" paradigm governs the trend in this field as developing the ultimate perfusion system is an ongoing quest.

## 2.2 Development of Current Perfusion Systems

As seen in the tables below, several other groups became involved in developing perfusion systems over the years. The tables are by no means an exhaustive list of studies done using liver perfusions; rather they are of select authors whose work went into improving an existing system or utilizing an optimized one. The tables highlight several important points:

1. The technique for isolating the liver and cannulating it to the perfusate flow has not changed dramatically since the 1950s. Two basic differences exist:
   a. Perfusion in situ: allows immediate access to, and cannulation of, the organ. This has the advantage of minimal-to-no-ischemic time and marginal organ manipulation. The disadvantage is that for longer experiments (several hours) this becomes less desirable as a "healthy" environment in which to keep the liver.
   b. Organ bath: the liver is explanted from the animal and placed in a bath, which may be of saline or effluent perfusate. This is preferable for long-term liver perfusions however it requires significantly more handling and subsequent tissue damage, and greater care must be taken to ensure proper cannulation with minimal ischemic time.
2. The choice of liver for perfusion shows a preference towards a murine model. Rats are smaller and thus require significantly less effort to prepare, are cost-effective and manageable. They are considered more ethically appropriate to work with, and as there is little variability among them (with the choice of inbred or not for added genetic flavor) various parameters can be reproducibly tested. Porcine models are much more clinically relevant, and as such are used for the purposes of establishing a relationship to humans based on organ size and volume capacity (eg. Extracorporeal liver assist devices).
3. Temperatures are all averaged around physiological, except if there is a preference of cold storage machine perfusion at 4-5°C.
   Flow rates and perfusion pressures vary, either as a requirement of the experiment or as a goal of having one parameter constant to watch how the other varies.
4. There is a significant difference between groups as to what the preferred perfusate basis should be, and what should be added to supplement it. The current trend is to use off-the-shelf cell culture mediums such as Krebs Henseleit, Ringer's or University of Wisconsin (UW) solution. Newer variations such as histidine-tryptophan-ketoglutarate (HTK) or a milder UW solution such as Williams E have gained favor. Nevertheless, none of these solutions have been tailor-made for the purposes of normothermic liver transplantation and subsequently form a serious point of contention.
5. The most prominent distinction among the groups however, is whether they used an additional oxygen carrier or not. If an oxygen carrier was used, then the variations extend further to:
   a. Autologous vs. xenogeneic blood supply
   b. Whole blood vs. isolated serum/ plasma/ red blood cells (RBCs)

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Table 2-1: Chronological Review of Perfusion Systems

| Year | Author | Species | Technique | Perfusate | Erythrocytes | Hct (%) | Oxygenation | pO2 (%) | T ( C) | (ml/min) | P (mmHg) | t(H) |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1951 | Brauer | Sprague-Dawley (SD) A. (160-200g), B. (200-233g), C. (233-260g), D. (266-300g) | Organ bath | BPA | Autologous rat heparin | ~30% | Falling film aerator | Carbogen | 37C | ND | ND | A. 4H B. 6.6H C. 8.3H D. 10.4H |
| 1966 | Miller | SD | Miller's | Heparin in Ringers Glucose, leucine Tyrode 3% BSA | Clotting-factor-free serum Autologous rat | 28% | Multi-bulb glass oxygenator | Carbogen | ~37C | ND | ND | >7H |
| 1966 | Hems | Murine | Mortimore's in situ | Saline 2.6% BSA | Human | 2.50% | Multi-bulb glass oxygenator | Carbogen | 38C | 15-25 | 20-30cmH2O | 3H |
| 1973 | Tolman | Wistar | Mortimore's in situ | KHB 3% BSA, glucose | Ovine/ Bovine | 20-22% | ND | Carbogen | 37C | 7.2 | ND | ND |
| 1974 | Miller | SD I. (350-400g) II. (250-300g) | Miller's | Krebs-Ringer 3%BSA, glucose, Streptomycin, Penicillin | Bovine heparin | 36% | Oxygenator | Carbogen | 37C | ND | ND | 24H |
| 1975 | Schmucker | SD (250-300g) | Organ bath | Krebs-Ringer 3% BSA, glucose Penicillin | none | none | Silastic tubing | Carbogen | 38C | 20-25 | ND | 2-5H |
| 1977 | Lee | AS (126-180g) | Mortimore's in situ | Krebs-Ringer 7% BSA, glucose Penicillin | none | none | Multi-bulb glass oxygenator | ND | 5C 35C | 8 (5C) 22 (35c) | ND | 24, 48, 72H |
| 1983 | Riedel | SD (150-200g) | Mortimore's in situ | Krebs-Ringer 3% BSA, glucose | Human & Canine | I) 10-40 II) 80-10% | Oxygenator | I) Carbogen II) Air | 37C | Constant Flow | Constant pressure | ND |
| 1984 | Alexander | SD (250-300g) | Organ bath | Heparinized human blood derivative | Expired Human | 36% | Silastic tubing | Carbogen | ND | 26 | 15-20mmHg | 24-48H |
| 1988 | Hoeper | Wistar (200g) | Mortimore's in situ | Krebs-Ringer 3.5% BSA | none | none | Tubing Self-devised | Carbogen | 22C | ~4/g | 15cmH2O | 3H |
| 1992 | Mischinger | Lewis (250-350g) | Organ bath | Krebs 2%BSA, glucose Taurocholic acid | Autologous rat heparin | 15-20% | Silastic tubing | Carbogen | 37C | 3.5/g | 10-15cmH2O | 3H |

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| Table 2-1: Continued | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Year | Author | Species | Technique | Perfusate | Erythrocytes | Hct (%) | Oxygenation | pO2 (%) | T ( C) | (ml/min) | P (mmHg) | t(H) |
| 1993 | Bell | Porcine | Orthotopic | FLUSHING SOLUTION 1. Control 2. UW (6H) 3. BSA (6H) 4. UW (18H) 5. BSA (18H) | Autologous Porcine | 38 | Membrane-exchange oxygenator | ND | 37C | ND | ND | 2H |
| 1995 | Alexander | SD (250-300g) | Organ bath | KHB 6g BSA, glucose, insulin | Rat heparin | 10% | Silastic tubing | Carbogen | 37C | ~2/g | 11.4mmHg | 6H |
| 1995 | Grossman | Wistar (250-300g) | ND | KHB 2.5%BSA Sodium nitroprusside | Rat | 30% | ND | Carbogen | 37C | 0-7/g | Relate flow with portal resistance | 1.5H 5H |
| 1996 | Cheung | SD (200-275g) | Mortimore's in situ | Krebs-Ringer w or w/o BSA, glucose | Canine | 15% | Silastic tubing | 576mmHg | | 6/g w/o RBC 2/g w/ RBC | 5-20mmHg | 3H |
| 1997 | Adham | Landrace pigs (25-30kg) | Organ bath | FLUSHING SOLN: 1. Ringer's lactate 2. ELOHES 3. Ringer's lactate 4. UW | Autologous Porcine | ND | Hollow fiber dialyzer | 1. Air, 5% CO2 2. As (1) 3. Carbogen 4. As (1) | 38C | 1/g PV 0.2g/g HA | PV 11-20 HA 37mmHg | 6H |
| 1998 | De Godoy | Lewis (250-450g) | Orthotopic | Saline, buffered Insulin | Autologous rat heparin | 20% | Membrane-exchange oxygenator | Carbogen | 37C | 1.5/g | 3.6mmHg | 2.4H |
| 1998 | Alexander | SD (250-300g) | Organ bath | KHB | Rat | 10% | Silastic tubing | | 37C | 2/g | 11-15mmHg | 6H |
| 1998 | Pastor | SD (240-410g) | Organ bath | KHB NH4Cl or glutamine | none | none | Oxygenator | Carbogen | 37C | >3/g reduced) | ND | ND |
| 2002 | Grosse-Siestrup | Landrace pigs (109-111kg) | ND | Eurocollins flush | Autologous Porcine Heparin | 30% | Hollow fiber dialyzer | Carbogen | 37C | PV & HA 200 | PV 14-18 HA 70mmHg | 3H |
| 2004 | Chaib | Hartley guinea pigs (223-339g) SD (230) | Miller's | KHB Albumin, glucose, calcium, AAs | none | none | Oxygenator | Carbogen | 37C | HGP ~4.3/g SD 3.1/g | Constant pressure | 1.5H |
| 2004 | Jourdan | SD (by age: 3mo vs 24 mo) | Miller's | KHB Albumin, glucose, calcium, AAs | none | none | Oxygenator | Carbogen | 37C | 45 vs 65 | Constant pressure | 1.5H |

    c.  Age of RBCs (expired vs. fresh)
    d.  Oxygen delivery rate (dependent on hematocrit, oxygenation mechanism, oxygen tension, flow rates)

As this is a fundamental issue of basic perfusion requirements, and the implications of using each different oxygenation modality are significant, it was determined that this would be a desirable starting point for the evaluation of pertinent perfusion parameters.

*A significant fraction of this work is a comparison of the currently available oxygenation mechanisms used in liver perfusion systems in an attempt to determine their effects on the organ structure-function relationship. It subsequently serves to illustrate the empirical relationship between the suggested mechanical parameters set forth at the beginning of this thesis.*

Artificial blood substitutes have not been included in this review for various reasons. While blood substitutes would eliminate all variability abovementioned in using blood and its components, they are themselves not very well characterized. Concerns over their cytotoxicity, requirements of use, relations to physiological oxygen supply, and cost segregated them into a separate category that merits a study of its own. For the purposes and goals of this thesis, they were not felt to be an appropriate addition.

6.  Not one study was able to claim stability for extended periods of time; all livers showed deterioration that was largely a function of how far-removed the perfusion conditions were from normal. Livers perfused with autologous whole blood could be perfused longer than livers perfused with anything else.

In order to perform the comparison of oxygenation techniques, it is necessary to establish what the *in vivo* oxygen requirements of the rat liver are first.
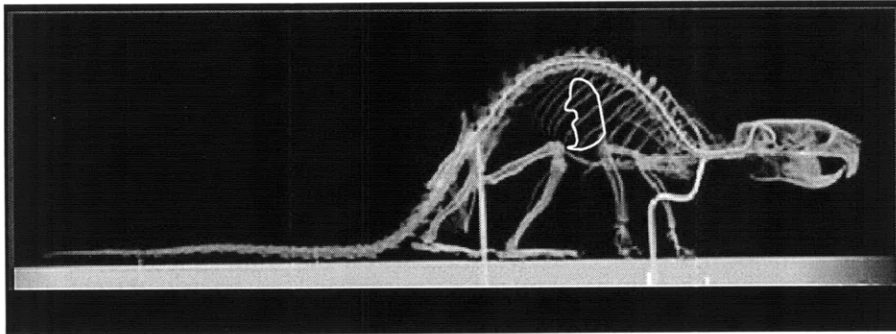
# Chapter 3

## Rat Liver Anatomy



Figure 3-1: [8] Illustrates an X-ray image of the sagittal plane of the rat (*Rattus sp.*) and the area occupied by the liver superimposed upon it.

The rat liver makes up approximately 4% of the body weight and most of it is situated in close contact with the diaphragm within the rib cage; ventrally it extends along the abdominal wall beyond the ribs; while the visceral surface contacts the stomach, descending duodenum, transverse colon, jejunum and spleen; and the caudate process meets the right kidney. Unlike the human organ, the rat liver is comprised of several distinct lobes; a left, middle and right lobe can be identified and subdivided. The left consists of a great lateral lobe joined to the other lobes only by means of interstitial tissue and vessels, and has a single smaller medial lobe cranial to it. It is separated from the middle lobe by a deep fissure, which receives the insertion of the falciform ligament. The narrow intermediate lobe extends to overlap the right part of the liver. From the visceral surface arises the pointed caudate process, which projects dorsally. Two further disc-like papillary processes arise left of the hepatic portal. The small right lobe of the liver is not subdivided. The lobes are maintained in position by very delicate diaphanous ligaments originating from the lesser omentum, falciform ligament and coronary ligament [9].
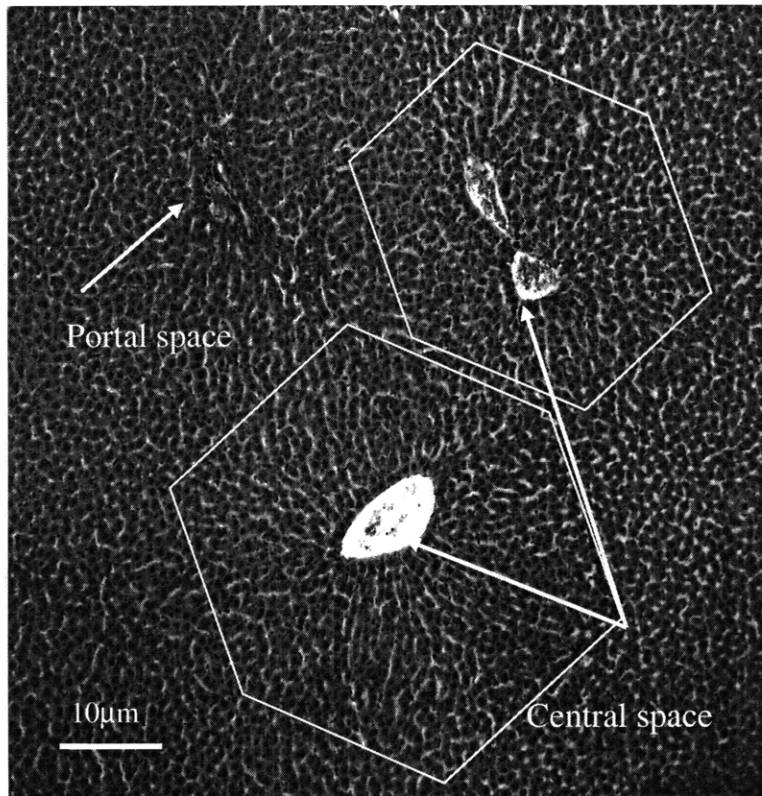
Figure 3-2: Histological specimen of a normal rat liver with schematized lobules. The portal space comprises the hepatic triad (portal vein, hepatic artery, bile duct) bringing in blood and removing bile. The blood flows through sinusoids, the sinuous white spaces akin to magnetic field lines, bringing in nutrients and collecting waste as the hepatocytes filter it. The blood then passes out the central spaces to collect in hepatic veins prior to exiting the organ via the SHVC. It is clear from this photograph that every cell is in direct contact with blood flow.

At the caudomedial margin of the caudate process the vena cava caudalis enters the liver and receives seven segmental veins (three of which arise from the left and ventral part of the liver, merging before the vena cava) before its exit on the craniodorsal aspect of the supraportal lobe. Ventral to the site of entrance of the vena cava, the hepatic artery and portal vein enter the hepatic portal and divide in an almost identical pattern.

The bile ducts, which run in portal channels forming triads with branches of the hepatic artery and portal vein, unite in a corresponding way, and flow together to form the hepatic duct. It exits the liver as a tube with a length of 12-45mm and a diameter of 1mm, which crosses the beginning of duodenum dorsally and opens distally to the pylorus. The rat does not have a gallbladder.
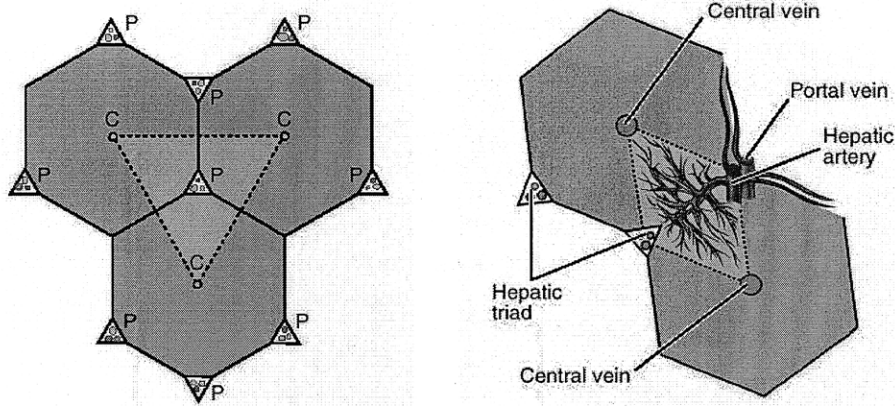
Figure 3-3: Schematics of the hepatic lobules: idealized as a 2 dimensional representation of planar hexagonal "stacks" of hepatocytes along which run the portal space (P) connected via the hepatic triad or rhomboid acinus to the central spaces (C) into and out of the page.

Portal lobulation is the pattern in the liver produced by bands of interconnecting fibrous tissue. A single portal lobule is a polygonal mass of liver tissue, larger than a liver acinus, containing portions of three adjacent hepatic lobules, and having a portal vein at its center and a central vein peripherally at each corner. There is modest lobulation in the rat liver primarily explained by its low content of interstitial tissue. Lobules can generally only be determined with reference to the portal spaces carrying the hepatic triad and the central spaces to which they drain via sinusoids.

The nerves of the liver are derived from the pneumogastric and sympathetic systems and enter at the transverse fissure to accompany the vessels and ducts to interlobular spaces. The medullated fibres are distributed almost exclusively to the coats of the blood vessels, while the non-medullated enter the lobules and ramify between the cells.Subsequently, entrance to the organ is achieved solely through the portal system vasculature comprising the portal vein and hepatic artery; and exit via the suprahepatic vena cava (SHVC) ensures complete drainage of the hepatic veins. Complete vascular isolation of the organ requires stifling flow from the inferior vena cava. Explantation can be achieved by careful severing of ligamentous ties to the diaphragm and anterior abdominal wall via the falciform ligament and other minor attachments to the peritoneum. Transplantation procedures suggest that the restoration of the innervation and lymphatics are not necessary for normal organ function.

# Chapter 4

# Measurement of *in vivo* Parameters

While the laboratory rat has been used for decades as an animal model for research purposes, detailed information on the physiology of each strain, which changes slightly over the years as strains become more robust or their weaknesses more pronounced, is very difficult to come by. Mostly in part due to the fact that an animal model is frequently chosen because it is only the relative differences before and after insult that are of interest, or the information regarding its physiology is considered so old and well-known that it is not well published and updated. Thus it was considered necessary to detail the governing parameters of a perfusion system by quantifying them in the strain of choice, with the goal of obtaining as much *in vivo* information as possible relating specifically to oxygen delivery and uptake rates of the liver.

## 4.1 Materials and Methods

*Animals.* Male Sprague-Dawley rats (Charles River Laboratories, Boston, MA) weighing 150-200g were housed in a temperature- (25°C) and light- (12 hour light cycle) controlled room. The animals were cared for in accordance with the National Research Council guidelines. Experimental protocols were approved by the Subcommittee on Research Animal Care, Committee on Research, Massachusetts General Hospital. Water and rat chow were provided *ad libitum*. The animals were allowed at least 2 days to adjust to their new surroundings before any experimental work was conducted.

*Blood sampling.* Animals were anesthetized using a mixture of ketamine (62.5mg/kg body weight) and xylazine (12.5mg/kg body weight). Body temperature was measured using a thermocouple rectal probe. A midline abdominal incision was made, and the intestines gently moved aside to expose the portal and hepatic vessels. Using a transit time flowmeter (TTFM Type 700, March-Hugstetten, Germany), volumetric flow rate through the portal vein and hepatic artery was recorded. Two 23-G needles were attached to tuberculin syringes and sodium heparin (100 u/ml) drawn into the head of the syringe. Another 10ml syringe was prepared the same way with a 20-G needle. The inferior vena cava (IVC) was clamped gently proximal to the renal venous return and allowed to drain for approximately 1 minute to ensure that the blood in the suprahepatic vena cava (SHVC), distal to the diaphragm, was receiving blood only from the hepatic veins. Using one of the 23-G prepared syringes, the liver was depressed with a cotton-tip

to reveal the SHVC and 0.5ml of blood was gradually drawn and immediately placed in the blood gas analyzer for evaluation. The liver was flipped back into position, the pressure of which ensured no blood loss from the SHVC. The IVC was slowly unclamped. The portal vein was then isolated and blood drawn from it by careful entry with the remaining 23-G syringe through one of the connecting splanchnic veins draining into the vein. Then, using the 10ml syringe, the abdominal aorta was isolated and cannulated, thereby exsanguinating the animal. As all blood entering through the arteries is directly from the aorta, these values were used to describe hepatic artery blood gas characteristics. The liver was then removed from the animal and weighed. It was then placed in 0.9% formalin for histological preparation of a normal rat liver. Portal pressure was not determined, rather based on literature values was expected to be ~14mmHg/19cmH$_2$O. This was used merely as a guideline during perfusions as portal pressures varied based upon perfusate composition and flow rates used.
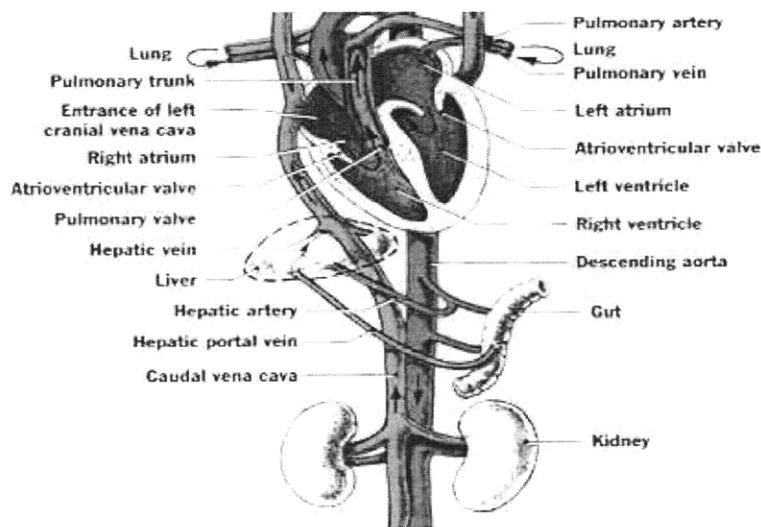


Figure 4-1: [9] A schematic of the blood supply to and from the liver

## 4.2 Results

### 4.2.1 In vivo measurements

Results of the in vivo analysis (n=13) provided a mean body temperature of 37.6°C. Portal flow rate was determined to be 1.4ml blood/min/g liver (± 0.2), and hepatic artery flow rate was determined to be 0.38ml blood/min/g liver (± 0.2); the total flow into, and therefore out of, the liver was thus 1.78ml blood/min/g liver (± 0.4).

## TABLE 4-1

### Blood Gas Analysis

| | pH | pCO$_2$ (mmHg) | pO$_2$ (mmHg) | ctHb (g/dL) | Hct (%) | O$_2$sat (%) | FO$_2$Hb (%) | FCOHb (%) | FMetHb (%) | FHHb (%) |
|---|---|---|---|---|---|---|---|---|---|---|
| Liver VC (n=6) | 7.25±0.1 | 67.83±11.4 | 14.45±2.8 | 14.52±1.0 | 42.67±3.2 | 4.17±4.0 | 11.98±4.0 | 4.28±0.2 | 0.42±0.2 | 83.32±3.9 |
| PV (n=13) | 7.26±0.0 | 66.94±9.7 | 70.85±7.9 | 14.72±0.8 | 43.23±2.4 | 90.76±3.4 | 79.29±4.6 | 6.41±0.3 | 0.00 | 14.30±4.9 |
| AA (n=11) | 7.30±0.1 | 56.55±14.2 | 137.48±18.0 | 13.49±1.4 | 39.45±4.3 | 98.35±0.5 | 93.62±0.7 | 6.23±0.4 | 0.00 | 0.15±0.3 |

*Note.* FO$_2$Hb = fraction of oxyhemoglobin
FCOHb = fraction of carboxyhemoglobin
FmetHb = fraction of methemoglobin
FHHb = fraction of deoxyhemoglobin

## 4.2.2 Theoretical Analysis

Using in vivo analysis to determine input conditions for the perfusion system.

The primary goal of the in vivo analysis is to establish the required oxygen delivery and oxygen uptake rate to a normal liver. This is used for comparison to the current degrees of oxygenation provided by different perfusion systems in use today, and also as a desirable parameter to be achieved in developing a physiologically relevant model. As oxygen consumption is tantamount to organ viability, it is the primary focus of improvement and the ultimate marker of success in this project.

Oxygen content is the concentration of the total oxygen carried by the blood, including oxygen bound to hemoglobin as well as oxygen dissolved in plasma and in the fluid within red cells. Using NCCLS recommendations[10]:

$$[O_2] = FO_2Hb \times 1.xx \times [Hb] + 0.00314 \times pO_2$$

(4.1)

$$\left(\frac{mlO_2}{100mlBlood}\right) = (\%) \times \left(\frac{mlO_2}{gHb}\right) \times \left(\frac{g}{dL}\right) + \left(\frac{mlO_2}{100mlBlood \bullet mmHg}\right) \times (mmHg)$$

Where 1.xx represents the oxygen-binding factor of hemoglobin and is a value in the range of 1.30 to 1.40. If the fraction of oxyhemoglobin is unavailable, a less accurate measure can be obtained using oxygen saturation estimates:

$$[O_2] = O_{2,sat} \times 1.39 \times [Hb] + 0.00314 \times pO_2$$

(4.2)

Oxygen content of each of the vessels entering and draining the liver can be determined using the results of the blood gas analyzer.

The total oxygen concentration entering the liver is the sum of the contribution by the portal vein and the hepatic artery and is dependent upon their flow rates.

Consequently, from equation [4.1] above:

$$[O_{2,portal\_vein}] = 79.29 \times 1.39 \times 14.72 + 0.00314 \times 70.85$$

$$= 16.5 \frac{mlO_2}{100mlBlood}$$

$$[O_{2,hepatic\_artery}] = 93.62 \times 1.39 \times 13.49 + 0.00314 \times 137.48$$

$$= 18.0 \frac{mlO_2}{100mlBlood} \tag{4.3}$$

$$[O_{2,vena\_cava}] = 11.98 \times 1.39 \times 14.52 + 0.00314 \times 14.45$$

$$= 2.5 \frac{mlO_2}{100mlBlood}$$

It should be noted here that the fraction of oxygen that is dissolved in plasma is only approximately 2% of the total contribution to the organ. Oxygen flow into the liver (ODR) is subsequently the product of the oxygen content and the blood flow rate in each vessel:

$$\forall_{O_{2,in}} = [O_{2,portal\_vein}] \times \forall_{blood,portal} + [O_{2,hepatic\_artery}] \times \forall_{blood\_arterial} \tag{4.4}$$

$$= \frac{16.5}{100} \times 1.4 + \frac{18.0}{100} \times 0.38$$

$$= 0.30 \frac{mlO_2}{min \bullet gliver}$$

Arterial contribution is observed to be ~23% of the total oxygen supply to the liver. Oxygen outflow is:

$$\forall_{O_{2,out}} = [O_{2,vena\_cava}] \times \forall_{blood,venous} \tag{4.5}$$

$$= \frac{2.46}{100} \times 1.78$$

$$= 0.04 \frac{mlO_2}{min \bullet gliver}$$

Subsequently, oxygen uptake rate (OUR) is 0.26ml $O_2$/min/g liver and is the minimum ODR that can be physiologically expected, if it were possible for the partial oxygen pressure and the fraction of oxyhemoglobin to be zero at the exit to the liver. Careful study of the literature indicates that there is a negligible volume of liver that is perfused exclusively by the hepatic artery [2, 11], thus for ease of perfusion technique it is entirely possible to perfuse solely through the portal vein and provide 100% of the oxygen requirements through this vessel. Working backwards then, it is possible to determine what the oxygen content of the perfusate should be, knowing what the oxygen requirements are. An assumption is made here that because there is marginal oxygen content in the vena cava outflow, the delivery to the organ should still be 0.3ml $O_2$/min/g liver:

$$[O_2] = \frac{\forall_{O_{2,in}}}{\forall_{blood,portal+arterial}}$$ (4.6)

$$= \frac{0.3}{1.4 + 0.38}$$

$$= 17.0 \frac{mlO_2}{100mlBlood}$$

So in returning to the original equation [4.1] it possible to observe that the variables are perfusate hemoglobin, percentage oxyhemoglobin/oxygen saturation, and partial oxygen pressure. In a system designed to meet physiological requirements, it is reasonable to assume that maximum oxygen saturation would be attained if using a capable oxygenator. Physiological partial oxygen pressures rarely exceed arterial capacity of ~140mmHg in this case, or 18% of the gas mixture (carbon dioxide would subsequently occupy ~60/760 or ~7%, with the remainder made up by nitrogen). The hemoglobin content required then is:

$$[Hb] = \frac{[O_2] - 0.00314 \times pO_2}{1.39 \times FO_2Hb}$$ (4.7)

$$= 12g/dL$$

The simplified relation of conversion to hematocrit 12x2.941=37% of the total perfusate volume.


## 4.3 Conclusions

The rat liver requires a total of 0.26ml $O_2$/min/g liver for optimal functionality at 37.6°C. If a physiological flow rate is sustained through the portal vein of ~1.7-1.8ml/min/g liver, then for a maximally oxygenated perfusate, it must contain the equivalent of a hematocrit that is ~37% of the total perfusate volume.

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# Chapter 5

# Liver Perfusions

## 5.1 Materials and Methods

Male Sprague-Dawley rats (Charles River Laboratories, Boston, MA) weighing 150 to 200 gm were housed in a light-controlled room (12-hour light-dark cycle) at an ambient temperature of 25 ~ C. The animals were cared for in accordance with the guidelines set forth by the Committee on Laboratory Resources, National Institutes of Health, and Subcommittee on Research Animal Care and Laboratory Animal Resources of Massachusetts General Hospital. Tap water and stock standard laboratory food were provided ad libitum for 5 days before experimental procedures.

## 5.1.1 Experimental Groups

The selection of groups was designed to illustrate the spectrum of currently utilized perfusions and provide a basic framework within which to compare those outcomes with a unique modification intended to overcome their limitations. The type of oxygenation mechanism in the perfusate used defined them:

> *Group A* (n=6) was the aforementioned normal in vivo rat liver.

> *Group B* (n=5) contained no erythrocytes with the ODR maximized by increasing both portal flow rate (35ml/min) without surpassing physiological portal pressure of ~14mmHg, and percentage oxygen (95%) dissolved in the perfusate.

> *Group C* (n=4) contained isolated and washed porcine erythrocytes at half the physiological hematocrit to serve as a determinant of the effect of increased viscosity on the medium as well as provide a gauge of the effect of inappropriately sized erythrocyte. Porcine erythrocytes (6μ) are twice the size of rat erythrocytes (3μ), but are smaller in diameter than other frequently used red blood cells from canines (8-9μ) or humans (6-8μ), and approximately .5 microns larger than the average bovine erythrocytes. Thus the use of porcine erythrocytes is the smaller of the currently used oxygen carriers from xenogeneic resources.

*Group D* (n=4) mimicked C but with the introduction of sodium heparin to the perfusate with the expectation of an increased propensity by the perfusion system to aggravate the clotting cascade. Although the rat liver is flushed with plain perfusate prior to exposure to cold-stored (hence inactivated platelets) and washed erythrocytes of a different species, total elimination of platelets and preservation of the endothelium is never guaranteed. In the normal homeostatic process two sources of coagulation and thrombosis come into play: factors released by the damaged endothelium and the activation of platelets, which adhere to denuded extracellular matrix at sites of endothelial damage. Heparin intervenes indirectly by activating antithrombin III, which inhibits the activity of thrombin and other serine proteases-factors IXa, Xa, XIa, and XIIa that form intrinsic and extrinsic components of the coagulation cascade. Hence by removing as many platelets as possible and eliminating the effect of injured endothelium to trigger further thrombotic potential, it is possible to reduce this potentially hazardous effect.

*Group E* (n=4) examined the effect of equally readily available goat erythrocytes, which most closely approach rat erythrocyte diameter.

*Group F* (n=4) served as a positive control to determine whether the use of rat erythrocytes could sustain stable long-term perfusion.

## 5.2 Perfusion Circuit

*Perfusate.* The perfusate contained Eagle's minimum essential medium (MEM, formulated to contain 9.5g of powder per liter of medium) (Sigma Chemical Co., St. Louis, MO) supplemented with several amino acids (Table 5-1) so that their concentrations were approximately two times that reported in post-absorptive rat plasma [12, 13]. Bovine serum albumin was added to reach a final concentration of 3% (Fraction V; Sigma Chemical Co.) and sodium bicarbonate used as a pH buffer. The pH was adjusted using hydrochloric acid or potassium hydroxide to 7.3. The perfusate was filter sterilized with a Nalgene disposable filter unit (Nalge Company, Rochester, NY) and stored in sterile vials at 4°C, ready for use.

*Erythrocytes.* Whole blood was purchased (Lampire, Pipersville, PA) preserved in acid citrate dextrose (ACD) and stored at 4°C for no more than one week [14, 15]. Erythrocytes were isolated by centrifuging the whole blood in 50ml vials at 3000g for 15 minutes using a swinging bucket centrifuge. They were then washed three times using a 0.9% sodium chloride and 0.2% dextrose solution. Assessing the final volume of spun erythrocytes and adding the appropriate volume of perfusate to them determined hematocrit. This was verified during perfusion using the blood gas analyzer.
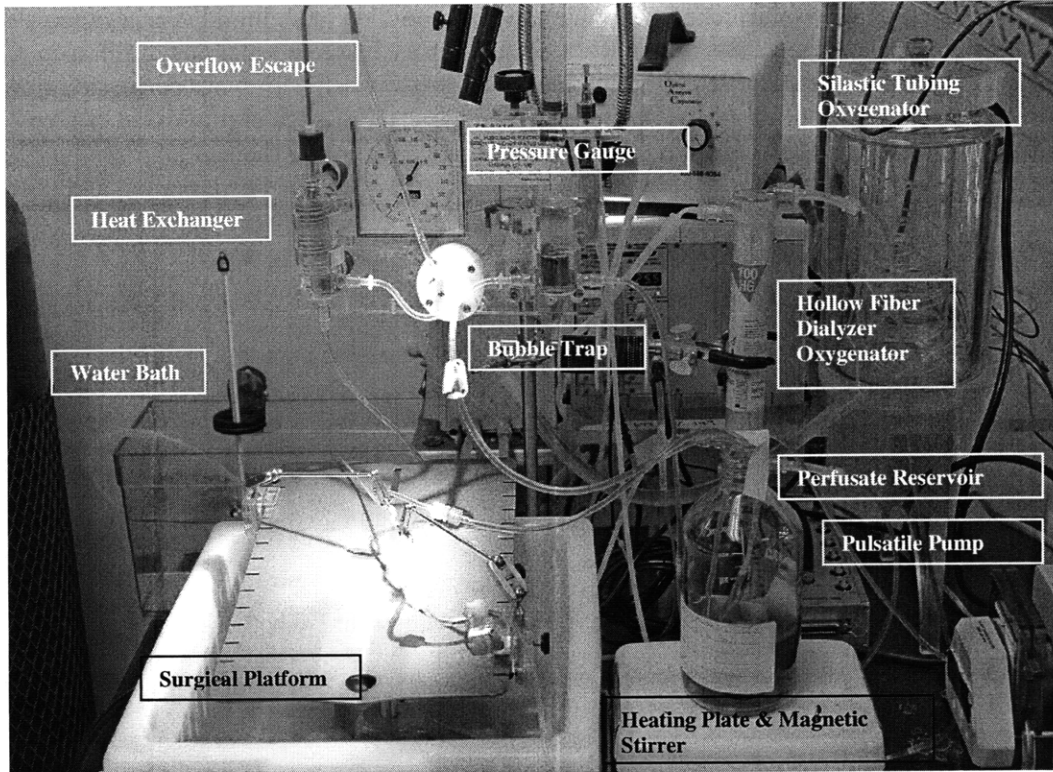
Figure 5-1: Perfusion apparatus. The essential requirements of a perfusion system have varied very little since the early 1950s: The perfusate reservoir with stirbar is mounted on a heating plate and magnetic stirrer at a height below the surgical platform to ensure minimal hydrostatic pressure on the influx to the liver. A roller-pump passes perfusate to one of two different oxygenators. The hollow fiber dialyzer is used in the presence of erythrocytes for superior surface area in contact with the gas supply, while the silastic tubing is preferred otherwise. The perfusate then passes through a bubble trap into a heat exchanger connected to a pressure gauge set at a maximum accepted perfusion pressure (excess leads to escape). This then passes down to a final bubble trap prior to the cannulae. The perfusion system is allowed to equilibrate before introducing the liver.

*Apparatus without erythrocytes.* Approximately 4m of tubing were used with a total surface area of ~0.0126m$^2$, exposed to 95% $O_2$ and 5% $CO_2$ (Carbogen) implying a partial oxygen pressure (p$O_2$) of 722mmHg could be expected at the inflow to the liver. The flow rate through the perfusion chamber under these settings would be 35ml/min, providing a maximum possible ODR of 0.08ml$O_2$/min. The perfusate would then pass through a bubble trap and a final heating coil to set the temperature to 37°C, both of which connected to a flow resistance regulator. This served to set a particular perfusion pressure such that if the pressure of the perfusate exceeded the set value, an overflow port would take the return back to the reservoir. While this was used to set an upper limit of acceptable perfusate pressure, it could also serve as a crude measure of real-time pressure

if the resistance was increased to the point of overflow. Alternatively, it also indicated pathological build-up of resistance in a damaged liver if overflow occurred while set at the minimum normal resistance. The set pressure is indicated on the gauge as being between 0 and 300mmHg. In practice there are certain restrictions on achieving 0mmHg. One is the height of the organ relative to the reservoir. Ensuring that the organ is higher than the remainder of the tubing can eliminate The hydrostatic pressure of the reservoir. There is also a pressure build up by virtue of the pump causing flow against tubing resistance and during laminar flow this pressure is proportional to flow. For low flow pressure, the pump output should be as low as possible, and the closer the perfusion flow is to pump discharge the more the perfusion pressure is determined by the vascular resistance of the organ, as opposed to the characteristics of the flow resistance. These are important considerations for accurate pressure measurements as these experiments are constant flow experiments. The perfusate is therefore flowing at a desirable flow rate, temperature, oxygen saturation and pressure, dictated by the vascular resistance of the organ. This can also be measured by the head of perfusate that is observed at the opened measurement port just prior to the liver, which acts as a makeshift manometer. This is a section of tubing that connects to the main stream via a small bubble trap, and serves to provide a last escape for trapped air, as well as a siphon for perfusate samples taken immediately prior to entry of the organ to determine gas characteristics. The perfusate then enters the liver via the portal system and exits through the venous return where a second sample is taken for gas uptake calculations before finally returning to the reservoir. Care with both sample ports must be taken to not eliminate flow to the liver or cause a suction effect.

**Table 5-1**: Concentrations of Components in Perfusate (g/100ml)

| | |
|---|---|
| Alanine | 48.4 |
| Arginine | 72.3 |
| Asparagine | 78.0 |
| Aspartic acid | 6.3 |
| Cysteine | 25.8 |
| Glutamic acid | 32.8 |
| Glycine | 38.0 |
| Histidine | 27.1 |
| Lysine | 49.6 |
| Proline | 41.6 |
| Serine | 63.2 |
| Tyrosine | 28.6 |
| | |
| Lactic acid | 622.2 |
| Pyruvic acid | 49.8 |
| | |
| Sodium Bicarbonate | 2618.7 |

*Apparatus with erythrocytes.* The addition of erythrocytes to the perfusion system occurred between t=0- during the organ flushing phase, and t=0+. The latter time point reflected the perfusate in its initial state with red blood cells before entering the liver. This was usually achieved by pre-marking the level of desired perfusate that should remain in circulation after flushing to achieve the required hematocrit upon adding the red blood cells. Before pouring the erythrocytes into the perfusate, approximately one unit of heparin was added to them in groups D, E and F. The primary difference between systems with and without erythrocytes is the requirement of a much more effective oxygenator. For this purpose, a dialyzer was installed (Gambro Polyflux Dialyzer, Harvard Apparatus, Holliston, MA) which has a surface area of $0.22m^2$ and is capable of fully restoring oxygen saturation levels upon counter-flow of the gas mixture to the

perfusate. A small degree of ultra-filtrate is produced during this process, which must be recycled to the perfusate reservoir. The gas composition in these experiments was reduced to 21% $O_2$, 5% $CO_2$ with the remainder taken up by nitrogen for the purpose of reducing the oxygen tension to physiological values. Immediately upon adding the erythrocytes, the pH was adjusted for as frequently a large enough shift occurred that was not initially buffered against and had the propensity to cause organ damage and prevent oxygen dissociation.

## 5.1.3 Hepatic Isolation

After each animal was anesthetized by ketamine and xylazine as aforementioned and its abdomen shaved, it was placed on a heated perfusion plate in a supine position, and the abdominal cavity was cut open by means of a midline incision. Ten units of heparin were administered by cardiac puncture through the diaphragm. A 2.3mm OD cannula (Harvard Apparatus, Holliston, MA) was inserted into the portal vein by small incision of the vessel, and the hepatic artery was ligated. The perfusate was introduced into the liver via the portal vein catheter at a constant rate of 35 ml/min (3.5 -+ 0.2 ml/gm wet liver/min). The inferior vena cava was immediately severed at a site distal to the junction of the renal vein to facilitate the washing of blood from the liver; a loose ligature was placed around the proximal end of the severed vein, above the kidneys. The chest cavity was then opened, and another 2.3mm OD cannula was inserted into the thoracic portion of the inferior vena cava via the right atrium for collection of the liver outflow from the hepatic veins. At this point the loose ligature around the lower part of the inferior vena cava was tightened to ensure a unidirectional outflow of perfusate from the hepatic vein. After residual blood was flushed for 15 minutes, the tip of the outflow catheter was placed into the perfusate reservoir and perfusion recycling was initiated. This point in time is experimentally considered t=0+.

## 5.1.4 Analytical Studies

At time t=0-, considered to be the point before closure of the circuit during which the perfusate was passed through the cannulated liver and allowed to run off into a separate catch basin, a sample point was taken from the perfusate reservoir and stored on dry ice for later metabolic analysis, and would serve as the experimental control. As soon as the liver was completely flushed and the circuit closed by restoring the liver outflow to the perfusate reservoir, a second time point at t=0+ marked the initial time point with the liver in place and would also be the initial time point with the addition of red blood cells in the perfusions that were primed with them. Three samples of perfusate were taken from different ports every ten minutes thereafter for analysis. Samples were drawn from the reservoir as aforementioned, and again directly before and after the liver. The latter two were taken to the blood gas analyzer RapidLab 800s (Bayer Diagnostics, Tarrytown, NY) for immediate observation of oxygenation status of the organ: perfusate entering the liver was always to be at the maximum possible oxygen delivery rate (ODR) capable while the degree of oxygenation at the outflow provided information on oxygen uptake

rate (OUR). At the end of one hour of perfusion, the livers were completely isolated from the rat and the wet weight was determined before final storage in 0.9% formalin for later histological studies. The total final perfusate volume was recorded.

# 5.2 Results

| Group | Oxygenation Mechanism | Flow Rate (ml/min/ g liver) | Blood | Hct (%) | Heparin | ODR (mlO2/min/ g liver) | OUR (mlO2/min/ g liver) | Histology |
|---|---|---|---|---|---|---|---|---|
| A (n=6) | Lungs | 1.78 | Autologous | 40 | Autologous | 0.30 | 0.26 | Normal |
| B (n=5) | Silastic tubing 95% $O_2$ 5% $CO_2$ | 4.0 | None | — | — | 0.075 | 0.054 | Gradients of hypoxic necrosis |
| C (n=4) | Hollow fiber dialyzer 21% $O_2$ 5% $CO_2$ 63% $N_2$ | 1.36 | Porcine | 20 | — | 0.15 | 0.07 | Extreme congestion. Micro - Macro vesicular steatosis |
| D (n=4) | Hollow fiber dialyzer 21% $O_2$ 5% $CO_2$ 63% $N_2$ supplemented with 100% $O_2$ for pH control | 1.0 | Porcine | 30 | Yes | 0.18 | 0.10 | Architecture preservation. Homogeneous perfusion. Mild sinusoidal congestion. |
| E (n=4) | Hollow fiber dialyzer 21% $O_2$ 79% $N_2$ | 1.8 | Goat | 35 | Yes | 0.29 | 0.04 | Architecture preservation. Homogeneous perfusion. |
| F (n=4) | Hollow fiber dialyzer 21% $O_2$ 5% $CO_2$ 63% $N_2$ | 1.8 | Rat (CD male) | 20 | Yes | 0.15 | 0.052 | 6 hour perfusion showing "fatigue" |

Table 5-2: A systematic comparison between currently used oxygenation mechanisms, with the introduction of a novel source of erythrocytes from goat. These results compare variations in hematocrit, flow rate, presence of anticoagulation, oxygen tension, pH and size of erythrocyte, to a normal liver.
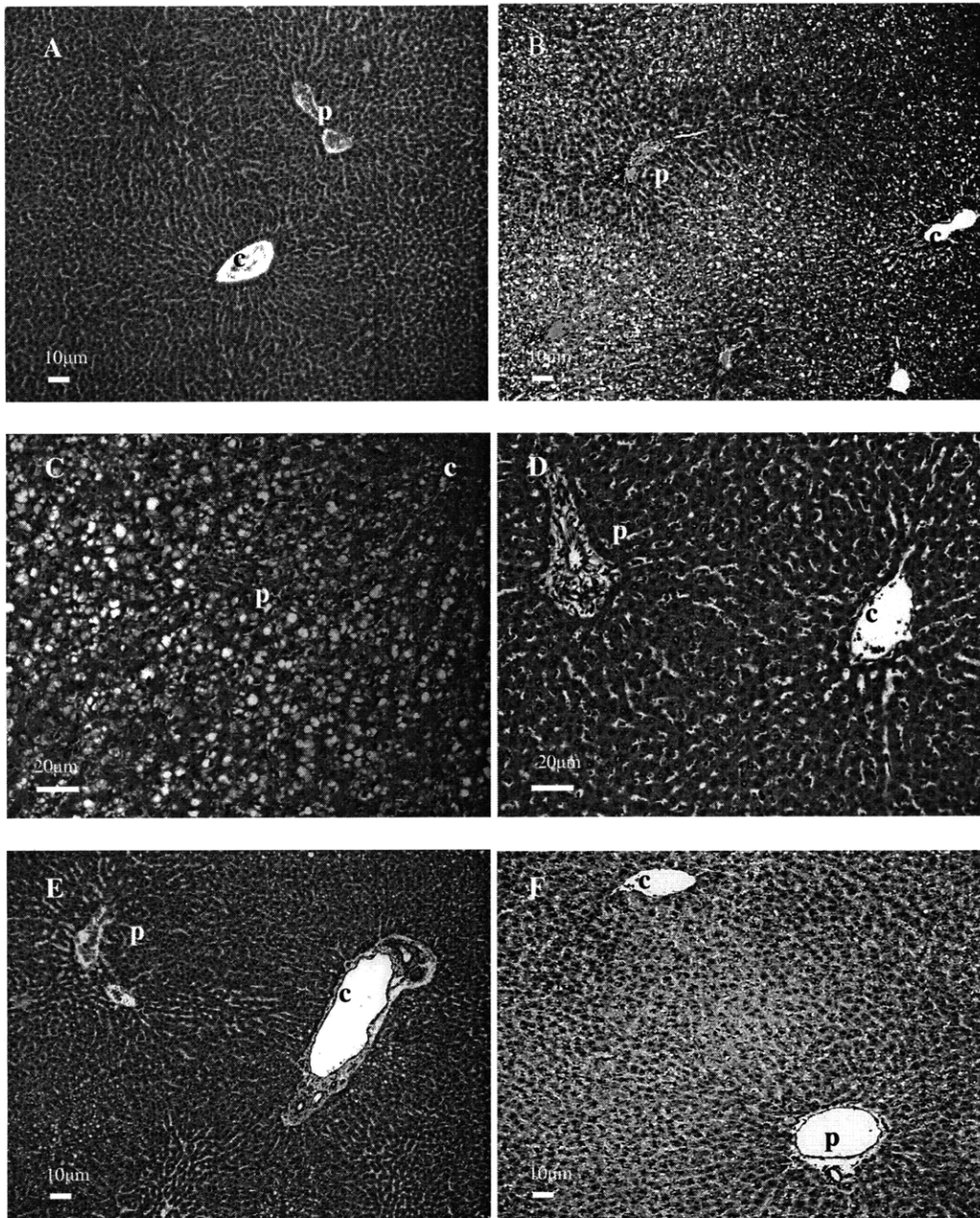
Fig. 5-2: Histological specimens of all the groups after 1hr of perfusion using different oxygenation methods. A-F Normal rat liver (A) showing architectural homogeneity, intact vessel endothelium in both portal (p) and central (c) spaces, minimal erythrocyte stasis and non-dilated sinusoids, compared with livers under different perfusion conditions: B: One hour perfusion with no oxygen carriers showing a steep gradient of viable cells around portal space to ischemic cells toward central space. C: Replication of (B) with the addition of porcine erythrocytes resulting in obliteration of liver architecture through congestion and steatosis. D: Replication of (C) with the addition of sodium heparin to the perfusate resulting in a viable healthy liver with minor congestion due to

size of porcine erythrocytes. E: Replication of (B) with the addition of smaller caprine erythrocytes and sodium heparin showing remarkable preservation of architecture. F: Five-hour perfusion with similarly-sized murine erythrocytes indicating architectural preservation with inception of perfusion "fatigue."

# 5.3 Discussion

$$[O_2] = O_{2,sat} \times 1.39 \times [Hb] + 0.00314 \times pO_2$$

$$\forall_{O_{2,in}} = [O_{2,in}] \times \forall_{perfusate\_portal} - [O_{2,out}] \times \forall_{perfusate\_central}$$

## 5.3.1 Absence of an oxygen carrier

*Group B.* Numerically, in the absence of an additional oxygen carrier in the perfusion system, and the only supply coming from the oxygen dissolved in the perfusate alone, even by increasing the flow rate and oxygen tension to compromise for this deficit, it is not possible to achieve normal ODR. Physically, the absence of erythrocytes affects the viscosity and subsequent viscous drag on the liver. Poiseuille's law states an inverse relation between flow rate and viscosity therefore, as a simple order of magnitude calculation, by removing the erythrocytes, the viscosity is reduced approximately from 0.0027 kg/(m·s) to 0.001 kg/(m·s) while the flow rate is increased from 1.78ml/min/g liver to 4 ml/min/g liver – resulting in no order of magnitude difference. It is possible to assume therefore, that the absence of erythrocytes, in combination with a high oxygen tension, has a largely hypoxic effect upon the liver resulting in central areas of necrosis. Knowledge that the liver can withstand 20-30 minutes of warm ischemia before becoming necrotic [16], it is not unreasonable to assume that by perfusing it, this effect is marginally delayed, but it certainly does not make for a stable model.

## 5.3.2. Addition of an oxygen carrier

Isolated red blood cells were the oxygen carriers of choice in these experiments. Whole blood was not used as it introduced too many variables as well as the obvious risk of an immediate immune response by the presence of foreign platelets, white blood cells and clotting factors. The use of rat erythrocytes in analytical perfusions is the most desirable option, however the volume of perfusate required makes the process of obtaining the erythrocytes unethical and expensive. Alternative sources are subsequently preferred, and freshness is important for preserving 2,3-diphosphoglycerate levels, pH, and red blood cell flexibility [14, 15]. Even within a week of storage, the red blood cells largely affected pH levels of the perfusate, and it is unclear to what extent 2,3-DPG levels were

depleted, and flexibility compromised. The complexity of the parameter relations increases with the addition of RBCs. In figure [5-3] below, a shift of the oxygen
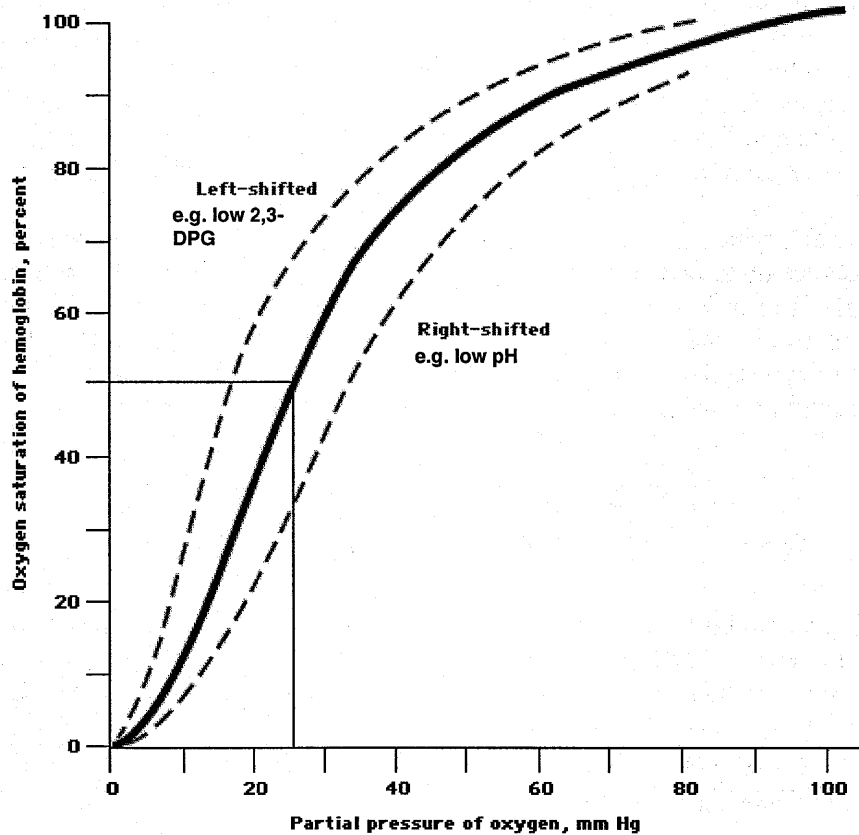


Figure 5-3: [17]. The dissociation curve of hemoglobin depicted here is for humans. In a study done by Hall [18], the p50 value for the albino laboratory rat was 38±2 mmHg, shifting the normal curve above to the right. Oxygen dissociation curves are related to body size; therefore it can be assumed that the goat is normal-to-right shifted but with a p50 less than rat, while porcine is normal-to-left shifted.

dissociation curve to the right means that more oxygen is released at higher oxygen tensions. Such a shift can be caused by a decrease in pH. A shift to the left is caused by depleted 2,3-DPG levels, making stored erythrocytes a concern in how reliably they are capable of releasing oxygen. While pH can be adjusted for using 0.9% sodium bicarbonate to restore a value of 7.4, rejuvenating 2,3-DPG levels is more challenging, thus it was estimated that blood less than a week old would have sufficient supplies and be rejuvenated during the perfusion. One other consideration was to reduce the oxygen tension to normal levels as high pO2 tension was associated with ultrastructural damage [19]. The amount of circulating volume, the capability of the oxygenator to keep up with oxygenation of the blood, and pH levels were important considerations throughout and

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variations in their modulation were largely affected by the hematocrit, oxygen tension and flow rates used. In general, as small a circulating volume as possible should be used to reduce the load on the oxygenator. It was found that in doing so, the hollow fiber dialyzer was capable of keeping up with the oxygen demands.

Porcine erythrocytes

*Group C.* An initial attempt to determine the effect of the addition of erythrocytes was done by adding half the normal hematocrit to the perfusate. This was also done without the addition of any extra heparin as was observed in some of the studies documented. While ODR was half the normal, as expected, oxygen uptake was minimally enhanced and the liver architecture was completely destroyed. In the absence of heparin, residual endogenous clotting factors, white blood cells or platelets react to endothelial injury or activation causing extensive congestive tissue damage.

*GroupD.* A second series of experiments with porcine erythrocytes with .75 the normal hematocrit and extensive application of heparin caused significantly less obvious macroscopic congestion. This was aided by reducing the flow rate, which subsequently compromised both oxygen delivery rates and the maximum oxygen uptake. A far more homogeneous microscopic architecture was obtained however, despite an improvement in oxygen delivery by only 0.05mlO2/min/g liver compared to Group B. Moreover, the degree of congestion observed microscopically appeared not to be associated so much with clot formation as an increased time of residence of the erythrocyte within the vascular bed. This could be due to two interrelated effects: 1. Hepatocyte swelling, possibly due to the onset of ischemic damage, causing a decrease in effective sinuisoidal space, and an increase in total liver size, and 2) Size of the porcine erythrocytes, which are twice the size of murine erythrocytes getting "stuck" as a consequence and being unable to perfuse well.

Goat erythrocytes

*Group E.* The use of goat erythrocytes, which are similar to rat, was capable of completely minimizing the mechanical impact of the flow on liver architecture. The liver appeared homogeneously perfused and well preserved. One additional variation to these sets of experiments was the removal of the 5% buffering $CO_2$ content with the goal of avoiding the initial periods of acidosis that could prevail in perfusions due to the erythrocyte addition (frequently reaching 7.0). Unfortunately, this had the undesirable effect of being too basic, with values ~7.5, thereby shifting the dissociation curve to the left and markedly reducing oxygen delivery. In fact, peripheral vaso-constricition appeared to occur (evidenced by darker pink-rimmed livers with bright pink centers), causing arterio-venous shunting thereby effectively shuttling unattainable oxygen straight through the organ.

Rat erythrocytes

*Group F*. The data obtained for this group was from a modified perfusion system designed for the purposes of transplantation, and hence the need to use rat erythrocytes (circulating volume 60ml). These livers were perfused for 6 hours. The average oxygen uptake rate was 0.05 mlO2/min/g liver, similar to oxygen uptake rate without erythrocytes and also with goat erythrocytes. These histological preparations provide evidence that longer perfusions will begin to be limited by such things as the perfusate content as the cytoplasm of the cells appear depleted of glycogen granules.

The sum of these findings confirm several important considerations for optimum organ perfusion: 1) Livers in perfusion do not take up as much oxygen as in vivo, 2) A lower hematocrit (20%, or approximately half of normal) is acceptable for adequate oxygen delivery and sustenance of organ architecture by reducing the shear stress on the liver, 3) Though oxygen supply rate delivered without erythrocytes is achievable, the high flow rates result in architectural damage, 4) Goat erythrocytes are a feasible and ethical alternative to rat erythrocytes.

# 5.4 Conclusions and Future Work

The results of these experiments provide useful information regarding the effects of, and relations between, the external flow parameters. The preservation of the architecture is an adequate indication of suitable flow mechanics, and is an obvious measure of organ stability. Ensuring that the liver is functioning in a physiologically similar manner however is a slightly more complex problem, as it is clear the liver may *look* well, but it may not be experiencing the optimal conditions required for "normal" metabolism.

Normal liver function is a term that now needs further clarification: It is quite possible that by virtue of being in a perfused state, the liver may necessarily be occupying a different metabolic state. In order to test this hypothesis and ascertain organ stability, several additional experiments will be carried out:

1.  The results of Group F are from a system that perfuses livers using the measured *in vivo* parameters, and has now proven successful in transplantation after 6 hours. It will be possible to evaluate what the OUR is in a perfused liver suitable for successful transplantation, and re-evaluate the oxygen needs for future perfusions.
2.  In vivo measurements of the metabolites in the hepatic artery, portal vein and suprahepatic vena cava will be made to provide a metabolic map of a normal murine liver during a fasting and fed state using metabolic flux analysis. This will be compared to the results of the measured metabolites obtained for these perfusions (not shown here).

While the data in this thesis shows incremental progress toward developing the optimal perfusion system and a means of numerically modeling it, several components remain that will determine the longevity of organ viability outside the realm of mechanical flow

considerations. These primarily relate to the need to account for diurnal changes, metabolic needs and waste disposal. An accumulation of the data regarding metabolic activity over time will provide a good indication as to which point in the perfusion history the chemical content begins to affect organ stability. Determining the appropriate perfusate and methods to sustain its content is, however, left as another thesis.

# Appendices

## A. Flow Probe

Measurement of blood flow into the liver through the portal vein and hepatic artery was done using a HSE Transonic Transit Time Flowmeter TTFM Type 700 (Hugo Sachs Elektronik, March-Hugstetten, Germany).

*Operating Principles*
The flowmeter allows for real-time measurement of blood flow within vessels by means of a perivascular probe. The data is displayed digitally for direct reading of mean flow and an analogue display shows flow, signal quality and scale factors.

The flow probe consists of a perivascular probe body which houses two ultrasonic transducers and a fixed acoustic reflector. The transducers are positioned on one side of the vessel and the reflector positioned midway between the two transducers on the opposite side. An electrical excitation causes the downstream transducer to emit a plane wave of ultrasound. This ultrasonic wave intersects the vessel in the upstream direction to the acoustic reflector whereupon it is reflected back through the vessel and received by the upstream transducer. This provides the transit time it took for the wave of the ultrasound to travel from one transducer to the other. The upstream transducer emitting in the downstream direction repeats the same sequence and obtains a second transit time. One ray of the ultrasonic beam undergoes a phase shift in transit time proportional to the average velocity of the liquid times the path length over which this velocity is encountered. The flowmeter subtracts the downstream transit time, from the upstream transit time utilizing wide-beam ultrasonic illumination. The difference in transit times is a measure of volume flow rather than velocity as the transducer integrates over the vessel's full width to obtain a mean reading and thus a volumetric flow rate. Ultrasound waves that do not pass through the vessel are not time shifted and therefore do not contribute to the readings, subsequently allowing vessels smaller than the probe to be measured.

*Calibration and Use*
For the purposes of *in vivo* analysis and real-time measurements that are not associated with computer-aided output, the flow probe comes pre-calibrated from the manufacturer and no further calibration is required. It should however, be zeroed while in "measure" mode by placing the probe in a *plastic* beaker of warm, still water and pressing "zero."

Vessels are acutely sensitive to external stimuli including pressure and temperature differences. Consequently, vessels must be carefully isolated from surrounding connective tissue without touching the vessels themselves. The probe, pre-warmed from the plastic beaker, should be suspended and positioned appropriately using an alligator clip and stand. Coupling gel, also pre-warmed, can be applied post-probe positioning to ensure good quality reading (CAL: 1; Gd). The probe should be left in position for as long as 1-2 minutes to ensure vessel stability. Vessels should not be combined as this gives an erroneous reading due to an inability of the probe to accurately sense different flow regimes. As the hepatic artery is much smaller than the portal vein and experiences pulsatile flow, several measurements must be made over an adequate time span to ensure an accurate average recording.

# B. Blood Gas Analyzer

Blood gas analysis is performed with a RapidLab 800 series (Bayer Healthcare LLC Diagnostics Division, Tarrytown, NY).

*Operating Principles*
The measurement technology used for critical blood analytes is based on electrochemical, biochemical and optical tools.

Molecular recognition occurs selectively in each unique sensor through a membrane, which is sensitive only to the analyte being measured. A transducer converts the potential generated by the molecular analyte to an electrical signal, which is developed either through potentiometry or amperometry.

Potentiometry measures the voltage or potential generated between two electrodes in the electrochemical cell when no external current is applied; the cell is in a state of equilibrium. The electrochemical cell is comprised of two electrodes (the measuring or indicator electrode and a reference electrode), an electrolyte solution (sample), and a voltmeter. It is capable of measuring the concentration or activity of a substance in solution. Each electrode develops a half-cell potential that is directly related to the concentration or activity of the specific analyte. This is compared to the reference electrode, which completes the cell and provides a steady unchanging potential. Consequently:

$$E_{cell} = E_{measured} - \left( E_{reference} + E_{liquid\_junction} \right)$$

The liquid junction potential is a small but significant voltage that develops at the liquid junction between the sample solution and the reference electrode. The reference sensor contains a silver wire coated with a layer of silver chloride and an ion permeable polymer surrounded by a solution of saturated potassium chloride. By ensuring that the concentration of $Cl^-$ remains unchanged in the solution, the reference sensor maintains a constant electrical potential and forms the reference for several ionic species. Consequently, the liquid junction occurs because of the different rates at which the

various chemical species diffuse across the boundary between the two liquids giving rise to a charge separation that generates the potential. The chemical species measured here are ions: $H^+$ (and hence pH), $K^+$, $Na^+$, $Cl^-$, $Ca^{2+}$.

The sensor's recognition mechanism is dependent upon the ion-selective membrane which also physically establishes a liquid junction potential between the sample and the sensor, which comprises its own inner reference element immersed in a fixed electrolyte solution. Subsequently,

$$E_{measured} = E_{sensor\_reference\_element} + E_{membrane}$$

So the membrane potential is the only variable which drives the cell potential. This can also be constitutively expressed using the Nernst equation [20]:

$$E_{cell} = K + (2.3RT/ZF)\log a_i$$

Where K represents a constant from various sources such as the liquid junction, R is the universal gas constant, T the absolute temperature, Z is the inductance (effective ionic charge), F is Faraday's constant and $a_i$ is the activity of the ion in the sample and the only independent variable. The activity is the numerical value of the concentration of the ion M (mol/L) times the activity coefficient. The latter is a measure of the interaction that ion has with other ions in solution and is dependent upon the ionic strength (I) of the solution where

$$I = \frac{1}{2}\sum M \times Z^2$$

Normal ionic strength of blood plasma water is 160mmol/kg. Because ionic strength is the primary variable affecting the activity coefficient of ionic species in solution, controlling the ionic strength of calibrating solutions to 160mmol/kg [21] sets the activity coefficients of ionic species in the calibrating solutions equal to those of blood plasma water at sample ionic strengths close to normal. This allows for calibrations and the expression of measured quantities to be expressed in units of concentration rather than activity [10].

Amperometry determines the amount of a specific substance in solution by applying a fixed voltage between two electrodes in an electrochemical cell and then measuring the current generated as a result of a reaction, which produces or consumes electrons (oxidation or reduction respectively). This method of detection is used in detecting oxygen and carbon dioxide tension ($pO_2$ and $pCO_2$ respectively). The $pCO_2$ sensor is based upon the Severinghaus and Bradley electrode [22]. It is a complete electrochemical cell that consists of a measuring electrode and an internal reference electrode. The measuring electrode, which is a pH electrode, is surrounded by a chloride bicarbonate solution. A membrane permeable to gaseous $CO_2$ separates this solution from the sample. The internal reference electrode, which contains a silver/silver chloride electrode surrounded by the chloride-bicarbonate solution, provides a fixed potential. As the sample comes in contact with the membrane, $CO_2$ diffuses into the chloride-bicarbonate

solution, which causes a change in hydrogen ion activity. The internal pH electrode detects the change in hydrogen concentration occurring in the chloride bicarbonate solution and generates a half-cell potential. This potential, when compared to the fixed potential of the reference electrode, results in a measurement that reflects pH change in the chloride bicarbonate solution. The change in pH is related to the log of the partial pressure of $CO_2$. Gaseous $CO_2$ exists in dynamic balance with carbonic acid and carbonate ion, and the ratio of the last two is directly proportional to pH. Using the Henderson-Hasselbach equation, the measured pH and $pCO_2$ values can be used to determine that ratio. The $pO_2$ sensor is based upon the Clarke amperometric electrochemical cell [23]. The sensor consists of a platinum cathode and silver anode, an electrolyte solution and a gas permeable membrane. A constant polarizing voltage is maintained between anode and cathode. As dissolved oxygen from the sample passes through the membrane into the electrolyte solution, it is reduced at the cathode. The circuit is completed at the anode, when the silver is oxidized. The amount of reduced oxygen is directly proportional to the number of electrons gained at the cathode. Therefore, by measuring the change in current between the anode and the cathode, the amount of oxygen in the electrolyte solution is determined.

The blood gas analyzer has a separate CO-oximeter module, which has the ability to detect and quantify absorbance spectra to detect hemoglobin derivatives, and interfering substances such as bilirubin, cyanmethemoglobin, turbidity and dyes. The CO-ox lamp passes light through lenses, a series of filters and a fiber optic coupler to the sample chamber. The light is coupled from the sample chamber by a second pair of lenses to the polychromator. In the polychromator, light is collimated by the first mirror and is diverted to the grating, which separates the light into a continuous spectrum of wavelengths. The spectrum of light is focused by the second mirror on to the diode array where the intensities at several wavelengths are measured. The spectral absorption method determines concentrations using matrix equations. For each substance or fraction the absorbance at a specific wavelength is equal to the product of the path length, concentration of the fraction or substance, and the molar absorptivity or the extinction coefficient for that substance [24-26]. Of particular importance are the fractions of oxyhemoglobin, deoxyhemoglobin, methemoglobin, carboxyhemoglobin all of which contribute to total hemoglobin. Hematocrit is a calculated value using total hemoglobin:

$$Hct = [Hb] \times 2.941$$

Where 2.941 is a factor calculated by dividing 100g/dL by a normal mean corpuscular hemoglobin concentration of 34%. Oxygen saturation is a ratio expressed as a percentage of the volume of oxygen carried to the maximum volume that can be carried by the hemoglobin. It is estimated by the blood gas analyzer as a function of the base excess (BE) of blood, which depends on total hemoglobin, the concentration of carbonate and pH:

$$O_{2,sat} = \frac{N^4 - 15N^3 + 2045N^2 + 2000N}{N^4 - 15N^3 + 2400N^2 - 31100N + (2.4 \times 10^6)} \times 100$$

$$N = pO_2 \times 10^{[0.48(pH-7.4)-0.0013BE]}$$

$$BE = (1 - 0.014 \times [Hb])[([HCO_3^-] - 24.8) + (1.43 \times [Hb] + 7.7)(pH - 7.4)]$$

Since oxygen saturation also depends upon the level of carbon monoxide and 2,3-DPG levels in the blood, the calculated value of oxygen saturation may not be equal to the measured value if these levels are abnormal. The above equations do not account for CO or 2,3-DPG implying that it is only useful as an estimation of the actual saturation value.

*Calibration and Maintenance*

The blood gas analyzer auto-calibrates to set-point values. In the event that these values are not achievable the system is either not ready, or will not measure the parameter that is considered out of bounds. Regular maintenance is subsequently required: 1) On a weekly basis all electrolyte solutions must be topped up, the sample pathway and associated tubing cleaned, the CO-ox module calibrated by insertion of a pre-made sample standard 2) On a monthly basis all gas tanks must be checked to ensure appropriate pressure readings for $pO_2$ and $pCO_2$ monitoring, general cleaning and deproteinizing of all sample pathways, replacement of any fluidics supplies 3) On a bi-annual/annual basis electrodes, valve functions, fluidics functions and all general hardware must be assessed to ensure expiration dates have not been bypassed and replacements can be obtained.

# C. Perfusion System

*Cleaning the system*
At the end of every perfusion, the system was dismantled and every component soaked in Mucasol (BrandTech Scientific) for several hours and then flushed extensively with de-ionized water and allowed to dry. The dialyzer was suctioned dry and then flushed with hydrogen peroxide to remove debris. Setting up each perfusion required reconstitution of the tubing and de-ionized water would be cycled through the perfusion system to remove any particulate matter and wet the dialyzer.

*Calibration of flow*
The system was set up initially as an open system and filled completely with distilled water to remove any air bubbles. The pump would then be calibrated for a constant volumetric flow rate. The water would be pumped out and replaced with perfusate. The system would be allowed to equilibrate before connecting the liver.

# D. Histology Preparation

Perfused livers were fixed in buffered formalin (1:10) Protocol® (containing formaldehyde, sodium phosphate, mono, dibasic, distilled water) obtained from Fisher for various lengths of time (hours, days). Pieces of liver samples were then cut and placed in tissue cassettes that were loaded in the retort of a VIP 2000 Tissue Processor (Miles Scientific) and exposed to the following conditions:

1. Tissues were dehydrated in ethanol baths (70%, 1 bath, 1 hr), (95%, 3 baths, 1 hr each), (100 %, 2 baths, 2 hrs each), cleared in xylenes (2 baths, 2 hrs each), and infiltrated with paraffin wax (4 baths, 2 hrs each). Cassettes were transferred to an embedding console (Tissue-Tek®, Miles/Sakura).

2. Tissues were placed in HistoPrep disposable molds (Fisher) and embedded in paraffin (Paraplast® X-TRA tissue embedding medium) where they were trimmed with a razor blade to expose the tissue. They were then soaked briefly in tap water at room temperature, and sectioned at 4-6 micrometers on a rotary microtome (Leica 820).

3. Sections were floated onto heated distilled water on the surface of Superfrost®*/Plus microscope slides (Fisher) and adhered to the slides using a slide warmer set at 42C.

Sections on slides were manually processed for staining in the following manner:

1. Slides were placed in holders (Tissue-Tek®) and initially de-paraffinized in xylenes (Fisher) (3 baths, 5 min each) and subsequently transferred to ethanols (Fisher) (100% (2baths, 1 min each), 95%, 70%, 50%, 15% (1 bath each, 1 min each) and tap water (3 baths, 5 min total).

2. Slides were immersed in hematoxylin (a nuclear stain) (Modified Harris Hematoxylin with Acetic Acid ) (Fisher) 2 min, washed in tap water (5 baths, total 5min), treated with eosin (cytoplasmic stain) ( Saturated Eosin Y) (Richard-Allan Scientific) (1min 45sec) and quickly exposed to ethanols (95% , 2 baths, 5 dips each; 100%, 2 baths, 10sec & 30 sec each) and placed in xylenes (2 baths, at least 3 min each) before slides were mounted in Permount (Fisher) (2-3 d drops/slide) and glass coverslips (22x 50mm) added.

Slides were places on a slidewarmer for 24-48 hours before being cleaned. They were then examined using a Nikon Eclipse 800 upright compound microscope with brightfield optics. Images were documented using a Spot 2 digital ccd camera (Molecular Dynamics).

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