Hydrodynamics of Aqueous Humor Outflow

by

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Bachelor of Applied Science in Mechanical Engineering
(University of Toronto, 1990)

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Abstract

Aqueous humor (AH) is a clear fluid occupying the anterior and posterior chambers (AC and PC) of the eye, which provides nourishment to the cornea and the lens. A slight positive intraocular pressure (IOP), which helps the eye to maintain its optical properties, is generated due to the resistance to outflow of AH. In primary open angle glaucoma (POAG), changes occur in the outflow pathway which increase the resistance and hence result in elevated IOP. Over time, the elevated IOP can cause damage to the optic nerve resulting in loss of sight. Our goal was to identify the locus of outflow resistance in normal eyes, and thus determine a possible site of pathological change in POAG.

The resistance generated by the hindrance of AH protein molecules during their flow through the glycosaminoglycan (GAG) matrix of the juxtacanalicular tissue (JCT) has been modeled using porous media theory. Actual initial protein concentrations in the outflow pathway were determined from protein assays of perfusate collected during perfusions with protein-free buffer (637 ± 60 μg/ml for bovine; 1976 ± 216 μg/ml for humans). Bulk protein concentrations in the perfusate were found to be consistent with a model of protein diffusion from the ciliary body via the iris root. However, the theoretical resistance generated by the measured protein concentrations and reasonable physiological dimensions for the GAG matrix has been found to be insufficient to account for the experimentally measured values of resistance.

The role that pores of the inner wall endothelium of Schlemm’s canal have in modulating outflow facility has been evaluated using constant flow perfusions. For eyes with a wide range of post-mortem times prior to the start of perfusion, a statistically significant correlation was found between pore density and post-mortem time (P<0.02, n=17) indicating the presence of artefactual influences. No correlation was found with pre-fixation facility (P>0.52) or post-fixation facility (P>0.97). For relatively fresh eyes (t<20 hrs, n=8), no correlation was found with post-mortem time (P>0.22), pre-fixation facility (P>0.18) or post-fixation facility (P>0.31). However, statistically significant correlations were found with volume of fixative perfused (P<0.09), and duration of fixation at pressure (P<0.03). Thus, pores do not appear to influence facility, but are instead passive resistive structures that open in response to a pressure gradient, and can be fixed in the open state.

Thesis Supervisor: Mark Johnson
Title: Principal Research Engineer
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# Table of Contents

1 Introduction ............................................................................................................. 8
   1.1 Gross Anatomy of the Eye ............................................................................. 8
   1.2 Aqueous Humor and The Outflow Pathway .............................................. 10
   1.3 Resistance to Aqueous Outflow and Previous Work .................................. 18
   1.4 Purpose and Goals......................................................................................... 29

2 Citrated Plasma Perfusions ..................................................................................... 32
   2.1 Experimental Methods .................................................................................. 32
   2.2 Experimental Results .................................................................................... 34
   2.3 Discussion and Conclusions ......................................................................... 37

3 Soluble Proteins in the Trabecular Meshwork: Experimental Studies .............. 40
   3.1 Experimental Methods .................................................................................. 41
   3.2 Experimental Results .................................................................................... 47
   3.3 Discussion and Conclusions ......................................................................... 64

4 Soluble Proteins in the Trabecular Meshwork: Modeling Studies ..................... 69
   4.1 Source of Trabecular Meshwork Proteins .................................................... 70
   4.2 Resistance Due To Proteins In The Trabecular Meshwork .......................... 86
   4.3 Summary and Conclusions ......................................................................... 97

5 Pore Density and Its Relationship to Aqueous Outflow Facility ....................... 101
   5.1 Experimental Methods .................................................................................. 102
   5.2 Experimental Results .................................................................................... 105
   5.3 Discussion and Conclusions ......................................................................... 115

6 Conclusions and Future Work ............................................................................... 119
   6.1 Discussion and Conclusions From Previous Chapters ............................... 119
   6.2 Recommendations for Future Work............................................................. 124

Appendix A: Common Experimental Procedures...................................................... 127

Appendix B: Summary of Experimental Data ......................................................... 135

References ............................................................................................................... 170
# List of Figures

**Figure 1.1:** Cross-section of the human eye ................................................................. 9
**Figure 1.2:** Cross-section of the trabecular meshwork ................................................. 13
**Figure 1.3:** Bovine Outflow System ............................................................................... 14
**Figure 1.4:** SEM micrograph of the luminal side of the inner wall endothelium ............. 17
**Figure 1.5:** (a) Bill and Svedbergh model; (b) Moseley et al. model ............................ 22
**Figure 2.1:** Typical citrated plasma experiment ............................................................. 35
**Figure 2.2:** Typical facility vs. volume curve for perfusions with DBG, DBG with 2% sodium citrate, and 15% citrated plasma .......................................................... 37
**Figure 3.1:** Eye-funnel assembly for protein experiments .............................................. 43
**Figure 3.2:** Typical protein concentration versus volume perfused for a bovine eye .... 48
**Figure 3.3:** Protein Concentration vs. Volume (Bovine) ............................................... 49
**Figure 3.4:** Typical data from human protein experiments .......................................... 50
**Figure 3.5:** Protein Concentration vs. Volume (Human) ................................................ 52
**Figure 3.6:** Outflow Facility vs. Volume Perfused (Bovine) .......................................... 54
**Figure 3.7:** Outflow Facility vs. Volume Perfused (Human) ........................................... 55
**Figure 3.8:** Outflow Resistance vs. Protein Concentration (Bovine) ............................ 57
**Figure 3.9:** Outflow Resistance vs. Protein Concentration (Human) ............................. 58
**Figure 3.10:** Initial outflow resistance vs. initial protein concentration (human) ........ 59
**Figure 3.11:** Protein concentration vs. volume for 15% serum perfusions .................. 62
**Figure 4.1:** Flow across the protein depot ..................................................................... 73
**Figure 4.2:** Average Nusselt number for flow between parallel plates:
adiabatic upper plate and isothermal lower plate ......................................................... 79
**Figure 5.1:** Pore density vs. pre-fixation facility for all normal eyes
(n=17, P>0.52) .................................................................................................................. 106
**Figure 5.2:** Pore density vs. post-fixation facility for all normal eyes
(n=17, P>0.97) .................................................................................................................. 107
**Figure 5.3:** Pore density vs. post-mortem time (all normal eyes, n=17, P<0.02) ......... 107
**Figure 5.4:** Pore density vs. volume perfused (t<20 hrs; n=8, P<0.09) ......................... 109
**Figure 5.5:** Pore density vs. duration of fixation (t<20 hrs; n=8, P<0.03) .................... 110
**Figure 5.6:** Pore density vs. post-fixation facility (Allingham data) ............................ 113
**Figure 5.7:** Pore density vs. volume perfused (Allingham data) ................................. 113
**Figure A.3.1:** Schematic of Perfusion System (One side) ............................................ 129
**Figure A.3.2:** Photograph of calf eyes being perfused (protein experiment) ............... 130
**Figure A.3.3:** Photograph of the perfusion apparatus .................................................... 130
**Figure B.1.1:** 15% citrated plasma vs. buffer (09Sept93) ............................................. 135
**Figure B.1.2:** 15% citrated plasma vs. buffer (21Sept93) ............................................ 136
**Figure B.1.3:** 15% citrated plasma vs. buffer (23Sept93) ............................................ 136
Figure B.1.4: 15% citrated plasma vs. buffer (30Nov93) .................................................. 137
Figure B.1.5: 15% citrated plasma vs. citrated buffer (01Feb94) ........................................ 137
Figure B.2.1: Human Protein Data - 20Oct94#0 ................................................................. 139
Figure B.2.2: Human Protein Data - 20Oct94#1 ................................................................. 140
Figure B.2.3: Human Protein Data - 27Jan95#0 ................................................................. 141
Figure B.2.4: Human Protein Data - 01Feb95#0 ................................................................. 142
Figure B.2.5: Human Protein Data - 01Feb95#1 ................................................................. 143
Figure B.2.6: Human Protein Data - 02Feb95#0 ................................................................. 144
Figure B.2.7: Human Protein Data - 02Feb95#1 ................................................................. 145
Figure B.2.8: Human Protein Data - 24Feb95#0 ................................................................. 146
Figure B.2.9: Human Protein Data - 24Feb95#1 ................................................................. 147
Figure B.2.10: Bovine Protein Data - 17Mar94#0 ................................................................. 148
Figure B.2.11: Bovine Protein Data - 17Mar94#1 ................................................................. 149
Figure B.2.12: Bovine Protein Data - 31Mar94#0 ................................................................. 150
Figure B.2.13: Bovine Protein Data - 31Mar94#1 ................................................................. 151
Figure B.2.14: Bovine Protein Data - 17May94#0 ............................................................... 152
Figure B.2.15: Bovine Protein Data - 17May94#1 ............................................................... 153
Figure B.2.16: Bovine Protein Data - 24May94#0 ............................................................... 154
Figure B.2.17: Bovine Protein Data - 24May94#1 ............................................................... 155
Figure B.2.18: Bovine Protein Data - 02Jun94#0 ................................................................. 156
Figure B.2.19: Bovine Protein Data - 02Jun94#1 ................................................................. 157
Figure B.2.20: Bovine Protein Data - 21Jun94#0 ................................................................. 158
Figure B.2.21: Bovine Protein Data - 21Jun94#1 ................................................................. 159
Figure B.2.22: Bovine Protein Data - 21Jul94#0 ................................................................. 160
Figure B.2.23: Bovine Protein Data - 21Jul94#1 ................................................................. 161
Figure B.2.24: Bovine Protein Data - 23Aug94#0 ............................................................... 162
Figure B.2.25: Bovine Protein Data - 08Sep94#1 ............................................................... 163
Figure B.2.26: Sulphated GAG concentration vs. volume perfused (31Mar94) ............... 164
Figure B.2.27: Sulphated GAG concentration vs. volume perfused (17May94) .............. 165
Figure B.2.28: Sulphated GAG concentration vs. volume perfused (24Mayr94) ............ 165
Figure B.2.29: Sulphated GAG concentration vs. volume perfused (31May94) ............. 166
Figure B.2.30: Sulphated GAG concentration vs. volume perfused (02Jun94) .............. 166
List of Tables

Table 4.1: Reynold’s number calculations within the flow channel .................................. 76
Table 4.2: Reservoir Opening Size Calculations ................................................................. 80
Table 4.3: Protein Depot Length Scale Calculation ............................................................. 83
Table 4.4: Diffusion Coefficient Calculation ...................................................................... 85
Table B.1: Data From Pore Study - Part 1 ........................................................................ 167
Table B.2: Data From Pore Study - Part 2 .......................................................................... 168
Table B.3: Unpublished Data From Allingham ................................................................... 169
Chapter 1

Introduction

Primary Open Angle Glaucoma (POAG) is a disease of the eye characterized by an elevated intraocular pressure (IOP). This increase in IOP manifests itself, over time, as a progressive degeneration of the optic nerve, culminating in vision impairment and eventually blindness if left untreated.

The increase in intraocular pressure seen in POAG results from an increased resistance to the outflow of aqueous humor. The goal of this thesis was to localize the main site of resistance in normal eyes, and thus infer a possible site of pathological change in glaucomatous eyes.

1.1 Gross Anatomy of the Eye

The mammalian eye (Figure 1.1) is a roughly spherical orb consisting of three tunics: the fibrous coat, the uveal tract, and the retina [Tripathi and Tripathi, 1984]. The optical properties of the eye are maintained by these coats and the contents of the chambers they form.

The outermost tunic is the fibrous coat which consists of the sclera and the cornea. The sclera is a white, viscoelastic outer coating of the eye. Joined anteriorly with the sclera is the cornea, which consists of a transparent, avascular tissue covered with an epithelium on the outside surface, and an endothelial layer on the inside surface. The region at which the sclera joins to the cornea is called the limbus, and is of considerable interest in aqueous outflow and this thesis.

The uveal tract forms the pigmented vascular tunic of the eye consisting of the choroid, the ciliary body, and the iris. The choroid is a soft, brown, highly vascularized layer of tissue extending from the optic nerve to the ora serrata, where the retina comes to an end.
Anterior to the ora serrata, the vascular coat continues forward to form the ciliary body. Extending from the ciliary body into the posterior chamber are numerous processes which are responsible for production of aqueous humor fluid. Zonular fibers, which secure the lens, are also attached to the ciliary body. On the exterior side of the ciliary body (i.e., towards the sclera) is the ciliary muscle, that executes the accommodative process of the lens by adjusting the tension of the zonular fibers.

The innermost tunic is the retina, a neuroepithelial layer consisting of all the tissues derived from the optic vesicle [Tripathi and Tripathi, 1984]. This includes the nervous tissue of the sensory retina, as well as the epithelial linings of the ciliary body and the iris.

**Figure 1.1:** Cross-section of the human eye [Tripathi and Tripathi, 1984]

The tunics of the eye, along with the lens and iris, form several chambers: the anterior and posterior chambers, and the vitreous body. These chambers and their contents allow the eye to maintain a shape conducive to optical imaging.
The anterior chamber is a roughly spherical section defined anteriorly by the cornea, and posteriorly by the iris. Connected directly with the anterior chamber through the pupillary opening of the iris is the smaller posterior chamber, bounded by the posterior side of the iris and the lens. Both of these chambers are filled with the clear fluid aqueous humor, which maintains nourishment to the cornea and lens.

The vitreous body occupies by far the largest portion of the volume of the eye. It is defined as the space bounded by the retina and the ciliary body posteriorly, and the vitreous hyaloid face anteriorly. It is filled with a clear viscoelastic gel which helps to maintain the shape and mechanical properties of the eye. Although transport does occur through the vitreous [Balazs and Denlinger, 1984], in this thesis we will not be concerned with the dynamics of flow in this region under the assumption that the vitreous has minimal impact on the IOP.

1.2 Aqueous Humor and The Outflow Pathway

The primary purpose of aqueous humor is to supply oxygen and nourishment to the avascular tissues of the cornea and the lens. Aqueous humor is produced in the ciliary processes, passes through the posterior chamber, and then flows through the pupil into the anterior chamber. Fluid in the anterior chamber exits through the angle of the eye (defined by the junction of the cornea and the iris), passing through the trabecular meshwork, and into a highly elongated vessel called Schlemm’s canal (or the angular aqueous plexus in bovine eyes). From Schlemm’s canal, aqueous humor flows into the collector channels which in turn flow into the aqueous veins and anastomose with the episcleral veins, returning the fluid to the vascular system.
This section describes the physiology of the aqueous humor outflow system. In this thesis, both human and bovine eyes were used in experiments and for modeling. Significant differences between the human and bovine systems are noted where applicable.

**Aqueous Humor Production**

Aqueous humor is produced in the ciliary body by a combination of secretion and ultrafiltration [Bill, 1975]. Extending from the ciliary body are a series of fin-like protrusions, called the ciliary processes, through which aqueous humor enters the posterior chamber. In the bovine, the ciliary processes extend to the posterior side of the iris, increasing the area from which fluid can be secreted. The ciliary body is covered with a continuous double layer epithelium, which extends radially inward to the pupil, covering the ciliary processes and the posterior side of the iris. A plasma filtrate is supplied to the ciliary stroma from fenestrated capillaries, which moves across the epithelium by active secretion (from an actively maintained osmotic gradient), as well as by ultrafiltration, into the posterior chamber.

Plasma solutes, such as urea, ethanol and amino acids, cross the epithelial layer by a variety of mechanisms, both active and passive. For large macromolecules such as proteins, however, the tight junctions of the epithelial layer represent an impermeable blood-aqueous barrier. Substantial evidence exists to show that proteins are added to the aqueous humor in the anterior chamber via diffusion from the anterior side of the iris [Freddo et al., 1990; Barsotti et al., 1992], and not into the posterior chamber as previously believed. In particular, in human eyes, protein seems to enter the anterior chamber from the root of the iris just prior to entry into the uveal and trabecular meshworks. In bovine eyes, protein enters the anterior chamber from the entire anterior surface of the iris.

One of the implications of the diffusional protein pathway is that the bulk protein concentrations in anterior chamber aqueous humor may be significantly different than in the
outflow pathway. Normal human aqueous humor has been found to have a protein concentration of about 0.1 mg/ml [Tripathi et al., 1989], while bovine aqueous has been found to have about 0.5 mg/ml [Pavao et al., 1989]. Thus, protein concentrations of aqueous humor are typically less than 1% of plasma protein concentrations (approximately 60 mg/ml) [Cole, 1984; Barsotti, 1990]. One of the goals of this thesis was to determine the true protein concentrations of the fluid passing through the outflow pathway.

**Trabecular Meshwork**

*Human:*

After aqueous humor passes out of the anterior chamber, it flows through the trabecular meshwork (Figure 1.2), defined as the portion of the aqueous outflow pathway between the anterior chamber and Schlemm's canal. The meshwork can be further subdivided into the uveal and corneo-scleral meshworks, and the juxtacanalicular tissue (JCT).

The innermost region is the uveal meshwork, which consists of cord-like structures separated by pores ranging in size from 25 to 75 μm. The corneo-scleral meshwork consists of a series of flat lamellae (collagenous sheets with an endothelial covering), stacked on top of each other, connected by intra-lamellae pores offset from each other. Fluid-filled spaces between the lamellae ranging from 0 to 8 μm in size [Tripathi and Tripathi, 1984], are maintained by beams or trabeculae. Individual corneo-scleral sheets range from 5 to 12 μm in thickness, and intra-lamellae pores range in size from 2 to 20 μm [Tripathi and Tripathi, 1984]. The total number of corneo-scleral layers in humans ranges from 8 to 15, spanning a total distance of 120 to 150 μm in thickness [McMenamin et al., 1986].

Each of the lamellae are attached to the line of Schwalbe (which marks the transition from trabecular to corneal endothelium) on one end, and the tip of the ciliary muscle on the other [Tripathi and Tripathi, 1984]. An increase in IOP causes the sheets of the meshwork to open (like a sponge absorbing fluid), while hypotonic conditions in the anterior
chamber will result in a decrease in the fluid-filled spaces as the layers collapse upon one another [Johnstone and Grant, 1973]. This action is analogous to a one-way valve which prevents the reflux of blood from Schlemm’s canal into the anterior chamber. However, the outflow resistance does not decrease with increasing IOP and the resultant opening of the spaces in the trabecular meshwork [Johnstone and Grant, 1973]. Instead, the resistance increases as the IOP increases, likely due to collapse of Schlemm’s canal (Section 1.3) [Moses, 1977].

The innermost region of the trabecular meshwork is the JCT, which lies between the innermost layer of the corneo-scleral meshwork and the inner wall of Schlemm’s canal. It varies in thickness from 2 to 20 μm in humans, and is formed by 2 to 5 loosely organized mesothelial cell layers [Tripathi and Tripathi, 1984]. The extracellular space includes both impermeable regions which exclude fluid flow (characterized by the presence of collagen, elastin, and fibrillar material), permeable regions which allow flow (characterized by the presence of glycosaminoglycans and proteoglycans), and possibly open spaces which allow unhindered flow [Ethier et al., 1986].

![Diagram of the trabecular meshwork](image)

| C  | cornea       |
| SL | sclera      |
| TM | trabecular meshwork |
| SC | Schlemm's canal |
| IP | iridial process |
| I  | iris        |
| CB | ciliary body |
| SS | scleral spur |

**Figure 1.2:** Cross-section of the trabecular meshwork [Tripathi and Tripathi, 1984]
Bovine:

In the bovine outflow system (Figure 1.3), aqueous humor exits the anterior chamber through a region of relatively large spaces known as the spaces of Fontana. This region is much larger than the reticular meshwork, with fluid flowing through only the anterior portion [Johnson et al., 1990]. Fluid then passes through the reticular meshwork, which represents a less organized version of the trabecular meshwork [Grierson et al., 1985]. A region analogous to the JCT is difficult to distinguish, but can be defined as the space between the endothelial lining of the angular aqueous plexus (equivalent to Schlemm's canal) and the first beam-like structure of the reticular meshwork [Johnson et al., 1990]. Johnson et al. [1990] found the thickness of the reticular meshwork to be about 50 μm, and the thickness of the 'JCT' to be about 15 μm.

![Diagram of Bovine Outflow System](image)

**Figure 1.3:** Bovine Outflow System [Johnson et al., 1990]
Schlemm’s Canal

In humans, the canal runs circumferentially around the eye within the limbus, and is approximately elliptical in cross-section, with a major axis diameter ranging from 200 to 500 μm, and a minor axis diameter ranging from 10 to 25 μm [Tripathi and Tripathi, 1984].

In the bovine (and other lower mammals), there is no single vessel that collects aqueous humor. Instead there are a series of channels that circumscribe the limbus, dividing and anastomosing. The total combined width of the vessels is about 450 μm [Johnson et al., 1990]. Tripathi [1974] has suggested using the general term angular aqueous plexus to describe the outflow apparatus of both primates and sub-primate mammals. However, in this thesis we are not particularly concerned with flows within the canal (for reasons described below), and thus will use the term Schlemm’s canal and angular aqueous plexus interchangeably.

Aqueous humor passes into Schlemm’s canal through the inner wall (adjacent to the JCT) which consists of a continuous endothelial layer attached to a discontinuous basal lamina, surrounded by connective tissue. The basal lamina is incomplete, and only partially separates the endothelial cells from the adjacent JCT [Tripathi and Tripathi, 1984]. The endothelial cells themselves are elongated in the axial (circumferential) direction of the canal measuring, in humans, an average of 100-150 μm in length and 4-7 μm in width [Allingham et al., 1992].

Inner Wall Vacuoles:

The luminal (interior of the canal) side of the inner wall, when fixed at pressure, is characterized by giant vacuoles protruding from the endothelial layer [Garron et al., 1958; Holmberg, 1959; Bill and Svedbergh, 1972]. These structures appear as bulges on the surface of the luminal side of the inner wall of Schlemm’s canal in SEM micrographs (Figure
1.4), and appear as outpouchings of the endothelium in TEM micrographs. Giant vacuoles are typically about 10 μm long, and 3-5 μm wide. Tripathi [1974] has suggested that vacuoles are transient structures which serve a pinocytotic role in moving fluid and particulates across the inner wall. However, little evidence exists to support this view, and it is more likely that vacuole formation occurs as a result of the pressure drop across the inner wall endothelium [Johnstone, 1979]. Vacuoles appear to persist for less than 5 minutes after the removal of flow and the resultant pressure drop [Van Buskirk and Grant, 1974]. In addition, when fixation occurs at atmospheric pressure, no vacuoles are seen in the inner wall endothelium [Johnstone and Grant, 1973].

There has been some suggestion that the vacuoles of the inner wall are completely artefactual. Shabo et al. [1973] fixed in vivo the eyes of anesthetized monkeys via retrograde episcleral perfusion. They found that the incidence of giant vacuoles was extremely low compared to other methods of fixation, such as immersion after enucleation. The density of giant vacuoles appeared to increase dramatically as a function of the post-mortem time prior to fixation. Grierson and Johnson [1981] also found an increase in vacuoles as a function of post-mortem time. However, they suggested that there may be two types of vacuoles: physiologic “giant” vacuoles, and artefactual “post-mortem” vacuoles. While “post-mortem” vacuoles appeared to increase with post-mortem time prior to fixation (reaching a peak at about 4 hours), the density of “giant” vacuoles (usually associated with pores) seemed to decrease with post-mortem time. This is consistent with the notion that vacuoles are formed in response to a transmural pressure gradient, as a result of flow
across the inner wall, and will disappear with the removal of flow. However, the physiologic nature of vacuoles remains an open issue.

**Figure 1.4:** SEM micrograph of the luminal side of the inner wall endothelium

*Inner Wall Pores:*

Associated with giant vacuoles, but also occurring in other areas where the endothelium is thin, are pores in the inner wall (Figure 1.4). These pores represent transmural pathways through which aqueous humor passes from the JCT into the lumen of Schlemm’s canal. Recent evidence using freeze-fracture studies [Bhatt et al., 1995] suggests that the pores are openings or dilations of the tight-junctions of the endothelium. As with the giant vacuoles, pores are believed to open in response to a transmural pressure difference. Inner wall pores range in size from 0.1 μm to 3 μm (or 5 μm in exceptional cases), and have an average size of approximately 1 μm [Bill and Svedbergh, 1972].

*Collector Channels and Aqueous Veins:*

The outer wall (i.e., adjacent to the sclera) of Schlemm’s canal is characterized by the presence of openings (20 μm to 90 μm in diameter) to approximately 25 to 35 endothel-
lium lined vessels, called collector channels [Tripathi and Tripathi, 1984], through which aqueous humor exits. The entrances to the collector channels are often surrounded by septae, possibly to prevent collapse of the canal and occlusion of the collector channel [Johnson, 1981]. The collector channels empty into the aqueous veins, which anastomose with the episcleral veins, and hence return the aqueous humor to the vascular system, from which it was ultimately derived.

**Unconventional Outflow Pathway**

A secondary outflow pathway also exists. In this pathway, aqueous humor enters the uveal meshwork, then passes into the interstitial spaces of the ciliary muscle. From there, it flows into the suprachoroidal space, and filters out through both the vortex veins and the sclera [Bill, 1975; Bill et al., 1980; Cole, 1984]. However, this secondary outflow pathway accounts for only about 5-20% of total aqueous outflow in humans [Bill, 1975], and will not be considered further in this thesis.

1.3 Resistance to Aqueous Outflow and Previous Work

Resistance to outflow of aqueous humor generates a slight positive intraocular pressure (IOP) in the anterior chamber which helps the cornea (and the entire eye) to maintain an optically correct shape. Numerous studies have been performed to attempt to identify the locus of this resistance, and in this section we will briefly review the relevant work by previous authors in this area.

**Intraocular Pressure**

The steady state IOP is influenced by three factors: 1) the rate of production; 2) the resistance to outflow; and 3) the episcleral venous pressure. We may express this relation as:

\[ IOP = P_e + R \cdot Q \]  

(1.1)
where $P_e$ is the episcleral venous pressure, $Q$ is the flowrate of aqueous humor out of the anterior chamber, and $R$ is the resistance to aqueous outflow. Typical values for humans are $\text{IOP} = 15 \text{ mmHg}$, $P_e = 7 \text{ mmHg}$, and $Q = 2 \mu\text{l/min}$, giving a resistance of $4 \text{ mmHg}/(\mu\text{l/min})$. A more common measure of the properties of the outflow system is the facility, $C$, defined as the inverse of the fluid resistance. Note that under transient conditions, the IOP varies as a complex function of the material properties of the eye [Johnson, 1981], and cannot be determined from equation (1.1).

**Role of the Uveal and Trabecular Meshworks, and JCT**

The spaces of the uveal and trabecular meshworks are generally considered too large to generate significant flow resistance. McEwen [1958] first applied Poiseuille’s law to the eye and found that the total outflow facility could be accounted for by a single pore 12 $\mu$m in diameter and 100 $\mu$m in length, which is on the same order of magnitude as each of the multitude of spaces typically found in the meshwork. This finding was consistent with the work of Grant [1955], who found that removal of the uveal meshwork had little effect on the outflow facility.

Ethier et al. [1986] performed extensive modeling of the JCT in an attempt to determine its role in generating outflow resistance. Transmission electron micrographs of the JCT were examined in order to categorize the area ratio (and hence volume ratio) of three types of material: 1) **solids**, including all electron dense materials, such as elastin, collagen, and amorphous fibrillar material; 2) **amorphous material**, made up of fine fibrillar material; and 3) **open spaces**, including all electron transparent spaces (i.e., free of solids or amorphous material).

Utilizing porous media theory (Section 4.2), Ethier et al. [1986] developed two models for the flow of aqueous through the JCT. Model A considered that cells, solids and amorphous material were impermeable, while the open spaces were free of any obstructions to
fluid flow. Model B considered that cells and solids were impermeable, while the amorphous material and electron transparent open spaces were assumed to be filled with a glycosaminoglycan (GAG) gel. For model A, the calculated resistance for normal and POAG eyes was found to be two orders of magnitude less than the measured resistance. This strongly suggested that, if the JCT generated significant outflow resistance, then the electron transparent spaces were actually filled with GAGs or other materials not normally visualized in transmission electron micrographs. Using model B, GAG gel concentrations required to generate the measured resistances were estimated based on morphological dimensions of the JCT. For JCT thicknesses between 10 and 25 μm, gel concentrations were predicted to be between 1.1 to 6.5 mg/ml of void space. This was consistent with measured levels of bulk GAG concentrations. The primary concern with this model is the assumption of uniform gel distribution. The existence of preferential flow channels through relatively gel-free zones would significantly alter the results.

**Role of the Inner Wall Endothelium**

The inner wall endothelium of Schlemm’s canal has been extensively studied as a possible site of outflow resistance. Bill and Svedbergh [1972] performed a painstaking SEM study of the human inner wall to determine its role in generating outflow resistance. A total of about 23,000 endothelial cells were found in the inner wall, with an average luminal surface area of about 480 μm² each. In addition, a total of about 20,000 pores were found, giving an average pore density of about 1840 pores/mm². Pore diameters ranged from less than 0.3 μm, up to about 3 μm. Assuming that: 1) all the pores found were actual fluid pathways; 2) the giant vacuoles associated with the pores were also part of the fluid pathway; and 3) the visible portion of the pore was the narrowest portion of the fluid pathway, they constructed a fluid mechanical model of the pore-invagination unit (pore along with giant vacuole) which is depicted below in Figure 1.5a.
Flow through an aperture is described by Sampson's law [Happel and Brenner, 1965]:

$$\Delta P = \frac{3\mu}{r^3} Q$$

(1.2)

where $\mu$ is the fluid viscosity, $Q$ is the volume flowrate, and $r$ is the radius of the aperture. Comparing this to Poiseuille's equation for flow in circular tubes,

$$\Delta P = \frac{8\mu L}{\pi r^4} Q$$

(1.3)

where $L$ is the length of the channel, an apparent length for the aperture can be calculated:

$$L_{app} = 1.18r$$

(1.4)

Adding this to the actual pore length, $L_0$, gave an effective length, $L_{eff} = L_0 + L_{app}$, which could be substituted back into Poiseuille's equation to give the pressure drop across a pore. Bill and Svedbergh concluded, based on this simple model, that the pores of the inner wall endothelium accounted for at most 10% of the total measured flow resistance in humans.

Moseley et al. [1983] re-examined the modeling of the pore-vacuole unit in an attempt to determine if a more complex model (more closely matching the geometry of the pore-vacuole unit) would account for a larger proportion of the total outflow resistance than was present in the Poiseuille flow model. The pore was thus modeled as a "Venturi tube", with the relation:

$$\Delta P = \frac{3Q\mu}{R^3 (1 + 2\cos a) (1 - \cos a)^2}$$

(1.5)
where \( a \) is the angle of convergence (see Figure 1.5b).

\[ L_0 = 0.3 \, \mu m \]

\[ 0.1 - 3 \, \mu m \]

(a)  

(b)

**Figure 1.5:** (a) Bill and Svedbergh model; (b) Moseley et al. model

While noting that their pore counts were somewhat lower than those found by other researchers, Moseley et al. [1983] attributed this to difference in counting methods instead of differences in perfusion techniques. Using these lower pore densities, they calculated the total resistance due to the inner wall endothelium to be at most 24\% of the total outflow resistance in monkeys. While this was somewhat higher than the resistance calculated by Bill and Svedbergh [1972] for humans, it is still a minor fraction of the total resistance. In addition, this resistance was determined using eyes perfused at 8 mmHg. At higher pressures (15 mmHg), a higher pore density was found, and the resistance was calculated to be 17 times less, even though measured outflow resistance actually increases somewhat going from 8 to 15 mmHg perfusion pressure.

**Interaction Between the JCT and Inner Wall**

Despite the low theoretical resistance of the pores, there has been some evidence of a correlation between pore density and outflow facility. Moseley et al. [1983] examined monkey eyes treated with pilocarpine and found that there was an increase in pore density
as well as facility. Hamanaka and Bill [1987] perfused monkey eyes with the chelating agent Na$_2$EDTA, and found that there was an increase in facility associated with the formation of artificial openings in the inner wall.

Recent theoretical work [Johnson et al., 1992] has indicated the possibility that hydrodynamic interactions between the pores of the inner wall and the JCT may result in much higher resistances than indicated in the models of Bill and Svedbergh [1972] or Moseley et al. [1983]. If the JCT is modeled as a porous medium obeying Darcy's Law, and the inner wall endothelium is modeled as a low porosity, impermeable membrane, then the flow across the JCT is not uniform as is implicitly assumed in previous models [i.e., Ethier et al., 1986]. Instead, fluid is preferentially "funneled" through the region of the JCT in the direct vicinity of a pore, reducing the effective cross-sectional area of the aqueous humor outflow pathway.

By numerically solving Darcy's law (Section 4.2) for normalized pressures in the vicinity of the pore, Johnson et al. [1992] were able to calculate a "resistance enhancement factor" defined as the ratio of resistance in the JCT with funneling to the resistance in the JCT without funneling. Using parameters typical for the human eye, an enhancement factor of approximately 30 was found. The implication of this is that, contrary to the conclusions from previous models of the inner wall [Bill and Svedbergh, 1972; Moseley et al., 1983], the pores density of the endothelial lining may be an important controlling parameter for total aqueous outflow resistance.

Limitations of this model include the assumption of pore diameter as the controlling parameter of the "funneling effect". This does not take into account the effect of the giant vacuoles often associated with pores. If the regions of the giant vacuoles are free of gel (matrix), then the "pore diameter" used in the "funneling" calculations should be the much larger vacuole diameter. This would greatly diminish the funneling effect. Also, the model
does not account for variability in the JCT. Preferential flow through regions of gel-free open space would also serve to diminish the funneling effect.

Support for the funneling model was provided by the work of Allingham et al. [1992]. In a series of constant pressure perfusions and fixations on 6 normal eyes and 4 POAG eyes, outflow facility was measured along with the pore density, pore diameter, and bulge (giant vacuoles or endothelial cell nuclei) density. A statistically significant relation (P<0.001) was found between pore density and the measured pre-fixation facility. For the normal eyes alone, a statistically significant relation (P<0.02) was also found, but for the POAG eyes alone, significance was not obtained (P<0.27). No statistically significant relationship was found between pore diameter or bulge density and the pre-fixation facility.

A major concern about study by Allingham et al. [1992] was the protocol of constant pressure perfusions. It is commonly believed that pores of the inner wall endothelium open in response to transmural pressure difference (i.e., the pressure difference between the JCT and the luminal side of the inner wall). Thus, if the pores are modeled as passive fluid resistors, then the pore density is simply a function of flowrate, which may be controlled by resistances elsewhere in the eye. For constant pressure perfusions, the pore density would be a function of facility (for constant pressure), and not vice versa. One of the goals of this thesis was to resolve this causality dilemma.

**Role of Schlemm's Canal**

To determine the role of Schlemm's canal itself in generating resistance, Johnson and Kamm [1983] modeled the canal as a porous walled, compliant tube, held open by the trabecular meshwork, modeled as linear springs. The tube was allowed to vary in shape in response to the pressure drop across the inner wall endothelium. Flow in the canal was assumed to be quasi one-dimensional in the circumferential direction (i.e., along the axis
of the canal). Flow resistance within the canal was controlled by the spacing between the inner and outer walls, which was determined by the difference between the IOP and the luminal pressure. Numerical simulations (with the canal geometry and dimensions, flow resistance of the inner wall, and inner wall compliance as the model parameters) indicated that significant resistance is not found in Schlemm's canal under normal physiologic conditions.

With increasing IOP, pressure on the inner wall results in collapse of the lumen, increasing the resistance of the canal and overall outflow resistance. Non-physiologic conditions, such as anterior chamber deepening and lens depression, may reduce effects that offset the increase in resistance [Van Buskirk, 1976; Van Buskirk, 1982]. The likely mechanism behind these effects is tension on the meshwork which may aid in keeping the canal open. Pilocarpine, which induces contraction of the ciliary muscle [Grierson et al., 1978] may reduce resistance in a similar manner.

However, the contribution of the canal to the overall outflow resistance is minor until the canal was almost completely collapsed, with the septae surrounding the collector channels preventing complete collapse. Even in the state of maximum collapse, the increase in resistance is much less than that measured in glaucomatous eyes. Thus, Johnson and Kamm [1983] identified the inner wall region as a more likely site of significant flow resistance, and resistance change in POAG.

**Role of the Collector Channels and Aqueous Veins**

The dimensions of the collector channels in humans are on the order of tens of microns, ranging from 20 to 90 μm at the canal, but narrowing as they anastomose with the aqueous veins [Tripathi and Tripathi, 1984]. The flow resistance of these channels, as calculated by Poiseuille's law, is not a significant contributor to the overall outflow resistance. However, Rosenquist et al. [1989] found that total trabeculotomy (eliminating the
outflow resistance of the trabecular meshwork, the inner wall of Schlemm's canal, and Schlemm's canal itself) resulted in a reduction of 71% of resistance at an IOP of 25 mmHg, and a reduction of 49% at an IOP of 7 mmHg. Thus, a surprisingly large portion (up to about 50%) of the total outflow resistance must lie in the collector channels and aqueous veins. Rosenquist et al. [1989] suggested two possible explanations: 1) the vessels were collapsed under physiologic conditions (greatly increasing the resistance above the theoretical value) and the morphological measurements of the channel dimensions were artefactual; or 2) the apparently open vessels were actually gel-filled. Grant [1963], however, found that the resistance of the collector channels and aqueous veins was not altered in glaucomatous eyes, and thus we will not explore this result further in this thesis.

**Role of Proteins in the Trabecular Meshwork**

The use of the bovine model (and other non-human eyes) in perfusion studies has always been complicated by the phenomenon of "washout", which is not present in humans [Erickson-Lamy et al., 1990]. As the eyes are perfused with saline (or other aqueous humor substitutes), the outflow facility steadily increases as an approximately linear function of the volume of fluid perfused [Johnson et al., 1991]. The washout rate could be described as a single parameter, \(dC/dV\), where \(C\) is the facility in [mmHg/(\(\mu\)l/min)] and \(V\) is the volume perfused in [ml]. Clearly, this is an artefactual effect since a steady state facility must be maintained in vivo. If washout were to occur naturally, the aqueous outflow system would soon have no resistance, and no intraocular pressure would be developed.

The washout phenomenon is consistent with the hypothesis of resistance causing macromolecules, such as GAGs or aqueous proteins, washing out of the aqueous outflow pathway. There is evidence that the washout of GAGs is minimal [Knepper et al., 1984; Johnson et al., 1993], leaving the tantalizing possibility that aqueous humor proteins may
be washing out, and hence increasing facility. In vivo, these proteins would be continuously replenished so that a steady state resistance is maintained. However, in perfusion experiments with enucleated eyes, resistance causing proteins would be washed out, and the cessation of blood circulation would prevent their replenishment. Also, since perfusion experiments using live animals must use constant flow conditions, the total flowrate is higher than the physiologic flowrate of aqueous humor. This would result in the removal of resistance causing substances at a higher rate than could be replenished, causing washout even under in vivo conditions.

Recent evidence of the role of aqueous humor proteins in generating outflow resistance was provided by Johnson et al. [1993]. By perfusing a series of bovine eyes with solutions of buffer containing 0 to 15% bovine serum, it was found that the rate of washout could be decreased, with the washout phenomenon essentially eliminated at 15% serum in buffer. As well, modeling studies [Kim et al., 1991] have confirmed that proteins (albumin in particular) flowing through a GAG matrix can generate resistance comparable to that caused by the matrix itself. Johnson et al. [1993] found that a 15% bovine serum solution flowing through a 0.5 mg/ml GAG gel layer 100 μm thick could generate flow resistance (along with the matrix resistance itself) equivalent to measured values. Curiously though, the bulk concentration of proteins in anterior chamber aqueous humor is only about 1% that of serum, much less than that required to prevent washout. However, as noted in Section 1.2, much of the protein in the aqueous humor enters the anterior chamber via diffusion from the root of the iris. This raised the possibility that protein concentrations in the outflow pathway are much higher than in the anterior chamber, and possibly high enough to explain the measured outflow facility and the washout effect.

Further evidence for this idea was recently provided by Kee et al. [1995], who investigated the effects of perfusing serum solutions in monkey eyes. They found that the wash-
out effect was significantly reduced with the perfusion of 5% serum solutions, consistent with the results of Johnson et al. [1993].

Preliminary investigations by our group into the effect of various plasma proteins on outflow resistance has been contradictory. Plasma proteins are a complex mixture composed of approximately 78% albumin, 21% globulins (alpha, beta, and gamma), and 1% fibrinogen, expressed as percentages of the total number of protein molecules [Guyton, 1991]. By assuming that the proteins passing through the trabecular meshwork behaved the same as 100% albumin, Johnson et al. [1993] were able to account for the both the normal measured flow resistance, and its change with washout. However, our laboratory has conducted several experiments in which the perfusion fluid contained albumin concentrations equivalent to 15% of the total plasma protein content. These experiments did not indicate a decrease in the washout rate with pure albumin [Ritter, 1993], further complicating the role of proteins in generating resistance.

Summary

The spaces of the trabecular meshwork [McEwen, 1958], and even the smaller spaces of the JCT [Ethier et al., 1986] were found to be too large to generate significant resistance. The pores of the inner wall endothelium of Schlemm's canal are too numerous to generate the experimentally measured outflow resistance [Bill and Svedbergh, 1972; Moseley et al., 1983]. Schlemm's canal itself may generate significant resistance when near complete collapse, but under normal conditions (uncollapsed) the canal generates negligible resistance [Johnson and Kamm, 1983]. Finally, the collector channels and aqueous veins may be responsible for a significant portion of the total resistance [Rosenquist et al., 1989], but the resistance of these regions are not significantly affected in glaucomatous eyes [Grant, 1963].
This leaves two possible sites of significant resistance in the outflow system which may also result in facility changes in POAG:

1) The JCT may be filled with an extracellular matrix gel through which fluid must flow [Ethier et al., 1986]; resistance may be generated by the matrix itself and also by aqueous humor proteins flowing through the matrix [Johnson et al., 1993].

2) The pores of the inner wall endothelium may modulate outflow resistance by enhancing the resistance of the JCT matrix through a funneling effect [Johnson et al., 1992].

1.4 Purpose and Goals

The broad goal of this thesis was to determine the principal site of aqueous outflow resistance in both human and bovine eyes. Based on the worked performed by previous researchers (reviewed above), potential resistance sites and mechanisms were identified. In this thesis, we investigated the contribution of several different mechanisms to overall outflow resistance, observing the differences between human and non-human eyes. Since humans are the only species definitely known to be afflicted with primary open angle glaucoma (with the possible exception of a rare form in the beagle; [Whitley et al., 1980; Peiffer et al., 1980; Gelatt et al., 1981]), identifying the differences in the mechanisms of outflow resistance between humans and other species may help us to identify the site of pathogenesis in POAG.

This thesis focuses on the role of proteins in generating outflow resistance within the JCT matrix, and its possible relationship to washout in non-human eyes. Johnson et al. [1993] were able to eliminate washout in the bovine eye by perfusion with 15% serum. However, serum proteins are not identical to aqueous humor proteins [Pavao et al., 1989], and there is some evidence to indicate that the resistance generated by serum is not due to
bulk protein concentrations [Ritter, '993]. Thus, the first step in determining the role of proteins was to evaluate if there were components peculiar to serum (possibly clotting by-products) responsible for eliminating the washout effect.

The washout effect in non-human eyes represents a complicating factor in the use of animal models for outflow studies. While Johnson et al. [1993] found one method of eliminating washout, a non-physiologic perfusion fluid was required (15% serum). Instead of providing an accurate model for the human eye, the use of 15% serum solutions in animal studies may add an extra degree of complexity to the interpretation of data and the extrapolation of results to humans. Thus, in addition to identifying a possible mechanism for the resistance change in glaucoma, characterization of the role of proteins in generating outflow resistance may help to provide a more accurate animal model for future perfusion studies.

The lack of washout demonstrated by human eyes suggests that the mechanism of outflow resistance may be completely different than that of bovine and other non-primate eyes. Alternately, the premise that aqueous humor proteins are responsible for a large portion of the observed outflow resistance may be extended to humans, suggesting the existence of a very large protein depot that does not deplete appreciably over the course of a perfusion experiment. While the dependence of facility on volume perfused has been investigated [Johnson et al, 1991], the volume dependence of perfusate protein concentration in human and non-human eyes has not been well characterized, preventing the further investigation of the importance of this mechanism. Determination of the relationship between protein concentration in the trabecular meshwork as a function of volume of fluid perfused is therefore required to determine the importance of proteins in generating resistance in the outflow system.
Finally, the role of aqueous humor proteins in generating resistance may be affected by resistance enhancing factors. If the effective cross-sectional area available for flow is significantly reduced due to the "funneling effect" [Johnson et al., 1992], then the resistance generated by aqueous proteins will be significantly increased. The pore density of the inner wall would thus be an important factor in modulating outflow resistance, and would manifest itself as a correlation between pore density and facility. As mentioned above, Allingham et al. [1992] found a correlation between outflow facility and pore density, but were not able to determine a causal relationship from constant pressure relationships. In order to resolve this critical causality issue, constant flow perfusions would need to be performed.

Specific Goals

The specific goals of this thesis are thus summarized as follows:

1. To determine if the elimination of the washout effect in bovine eyes with 15% serum solution is due to a bulk protein concentration, or due to a component peculiar to serum

2. To determine the concentration of soluble proteins in the trabecular meshwork and their role in generating outflow resistance

3. To determine the role of inner wall pores in controlling outflow resistance (to validate the results of Allingham et al. [1991] using constant flow perfusions)

Chapters 2 to 4 involve goals 1 and 2, while Chapter 5 describes our pursuit of goal 3.
Chapter 2

Citrated Plasma Perfusions

As discussed in the previous chapter, the washout effect in bovine eyes was effectively eliminated when eyes were perfused with 15% serum [Johnson, et al., 1993]. A set of experiments was conducted as part of the attempt to identify the proteins responsible for preventing washout. Specifically, we wished to confirm that serum proteins themselves were responsible for reducing washout, and not by-products of the clotting cascade produced during the production of serum from whole blood. To this end, we wished to perfuse bovine eyes with plasma solutions that had not undergone thrombosis.

2.1 Experimental Methods

Selection of Perfusion Fluid

Whole blood is composed of a cellular component (erythrocytes, leukocytes, and platelets) comprising 40-50% of the total blood volume, and a fluid component, plasma. Plasma is composed of approximately 90% water, 6-8% plasma proteins, and small quantities of nutrients, wastes, gases, and electrolytes. The main plasma proteins are: 1) albumin, which is used as a carrier for other substances, and comprises about 80% of the total plasma protein content; 2) globulins (alpha, beta, and gamma), which have numerous roles including clotting factors, antibodies, and transport; and 3) fibrinogen, a precursor to fibrin which forms the matrix of a blood clot. Serum is produced from whole blood by initiating the clotting cascade, which occurs spontaneously if blood is allowed to sit for a long period of time, or exposed to air or a non-endothelialized surface. Fibrinogen is changed from its precursor form into fibrin, creating a network known as a thrombus. Removal of the thrombus along with blood cells and other materials trapped in the network leaves a
fluid similar to plasma, without the clotting factors. This fluid is serum, and differs from plasma in its inability to undergo thrombosis.

The main difficulty in perfusing plasma through the aqueous outflow pathway is the possibility of thrombosis. The outflow pathway of the eye is kept free of initiators for the clotting cascade by the blood-aqueous barrier, preventing thrombosis from occurring. Thus, even though fibrinogen is present within aqueous humor, it is not converted into fibrin within the eye. However, clotting initiators are generated in plasma when it is collected, and would cause thrombosis to occur if they were introduced into the eye. Thus, in order for plasma to be used in a perfusion solution, an anticoagulant needed to be added to interrupt the clotting cascade.

Our choices for anticoagulants were limited. Although the glycosaminoglycan (GAG) heparin is widely and safely used in clinical applications, GAGs have been suspected as a source of outflow resistance, particularly in the JCT [Barany and Woodin, 1955; Ethier et al., 1986]. Thus, it was felt that the use of GAGs in the perfusion solution could artificially increase the outflow resistance, greatly increasing the difficulty in interpreting the effect of the plasma proteins. Thus, the chelating agent sodium citrate was selected, both for its minimal expected effect on outflow resistance, and its wide use in laboratory applications. However, unexpected ancillary effects were found with the use of sodium citrate, which are discussed in Section 2.3.

**Preparation of Citrated Plasma**

Bovine whole blood was collected from a local abattoir (Arena & Son’s, Hopkington, MA) and mixed with a 3.8 wt% solution of sodium citrate (Mallinckrodt AR, St. Louis, MO) in a 7:1 ratio at the time of collection and transported on ice. Within 24 hours of collection, whole blood was centrifuged (Model HN Centrifuge, International Equipment Co., Needham Hts, MA) for 30 minutes at 2000 rpm to separate the plasma from the blood.
cells. Plasma was pipetted off and ultracentrifuged on a Beckman L2-65B Ultracentrifuge (Beckman Instruments, Co., Palo Alto, CA) for 35 minutes at 35,000 rpm. The supernatant was separated from the pellet and frozen for future use. Ultracentrifuged plasma was diluted to a 15% solution using Dulbecco's phosphate buffered saline (Life Technologies, Grand Island, NY) with 5.5 mM glucose added (Mallinckrodt AR, St. Louis, MO).

For some of the experiments, citrated plasma was obtained prepared and frozen from Lampire Biological Laboratories (Philadelphia, PA). The concentration of sodium citrate used was the same, and the method of preparation was similar to the citrated plasma prepared in-house.

**Experimental Protocol**

Bovine eyes (n=9 pairs) were perfused at a constant pressure of 10 mmHg (Appendix A). A solution of 15% citrated plasma was used on one eye, while Dulbecco's phosphate buffered saline with 5.5 mM glucose (DBG) was used on the contralateral eye as a control. Perfusion experiments were continued for 4 to 5 hours.

**2.2 Experimental Results**

A total of nine experiments were run in which 15% citrated plasma in Dulbecco's phosphate buffered saline with 5.5 mM glucose (DBG) was the perfusion fluid.

For the first 4 experiments, the contralateral eye was used as a control perfused with plain DBG. The washout rate for the experimental eyes (n=4) was found to be 0.23 ± 0.11 (mmHg/μl/min)/ml while the washout rate for the control eyes (n=4) was found to be 0.20 ± 0.09 (mmHg/μl/min)/ml. A two-sided Student's t-test [Box et al., 1978; Draper, 1981] was performed and the difference was not found to be statistically significant (P>0.3, n=4). The results from a typical experiment are shown in Figure 2.1.
Figure 2.1: Typical citrated plasma experiment

While only a small number of eyes were treated with this protocol, the results were very surprising. Johnson et al. [1993] found that perfusions with 15% serum effectively eliminated the washout effect. However, unlike 15% serum solutions, our solutions of 15% citrated plasma appeared to have no effect on the rate of washout.

Further experiments using this protocol met with limited success as the citrated plasma solution was found to undergo thrombosis in the anterior chamber, and thus, meaningful data could not be collected. Sodium citrate is a chelating agent that exerts its anticoagulant effect in part by binding calcium ions which are necessary in the clotting cascade. We hypothesized that the anticoagulant abilities of the citrate were being overwhelmed by its dilution in a calcium-rich medium such as Dulbecco’s phosphate buffered saline (DPBS) or aqueous humor.

To test this hypothesis, a series of experiments were run in which citrated plasma prepared using the standard protocol was diluted to various concentrations with ordinary DPBS, and DPBS with 0 to 3.8% sodium citrate added. The same dilutions were per-
formed using normal saline. It was found that 15% citrated plasma in DPBS was approximately the threshold at which the sodium citrate ability was overloaded and clotting occurred. All concentrations below 15% clotted quickly, and none of the control solutions clotted. Since the investigation of the thrombosis mechanism was beyond the scope of this thesis, these experiments were not carried any further. However, as a result of our investigation into the anticoagulant mechanism of sodium citrate, it was concluded that perfusion experiments could not be continued with the initial protocol.

A new protocol was used in which DBG with 2% sodium citrate was used as the dilution medium for the citrated plasma. The experimental eye was again perfused with 15% citrated plasma solution, while the control eye was perfused with DBG with 2 to 3.8% sodium citrate. Again, the washout rates from both the experimental and control eyes were very similar with this new protocol, with $dc/dV=0.27$ (mmHg/μl/min)/ml for both eyes. This washout rate was again found to be higher than the value for plain DGB perfusions. Typical curves are shown in Figure 2.2, where the washout rate is given by the linear portion of the curve fit. For these examples, the washout rates, in (μl/min/mmHg)/ml, were found to be:

$$0.275 \pm 0.002 \text{ (DBG)}$$

$$0.630 \pm 0.006 \text{ (DBG with 2% sodium citrate)}$$

$$0.514 \pm 0.004 \text{ (15% citrated plasma)}$$

Complete summaries of all the experimental data are provided in Appendix B.
Figure 2.2: Typical facility vs. volume curve for perfusions with DBG, DBG with 2% sodium citrate, and 15% citrated plasma

2.3 Discussion and Conclusions

Although we were unable to find a statistically significant difference between DBG and 15% citrated plasma (or citrated DBG) perfusions, these experiments were not continued. The effect of sodium citrate on facility produced a confounding factor which made it impossible to discern the effects of the plasma proteins. While plasma proteins flowing through the outflow system would be expected to increase resistance in the same manner that serum proteins do [Johnson et al., 1993], this effect was overwhelmed by the resistance decreasing effect of the sodium citrate in the perfusion fluid. Based on other work in this thesis, along with the work of previous researchers, we can speculate about the facility altering mechanisms of both plasma proteins and sodium citrate.

Sodium citrate is a chelating agent which is widely used as a laboratory anticoagulant. One of its modes of action is to bind to free calcium ions which are required for the clot-

1 We thank Dr. David Epstein of the Duke University Medical School for aiding in our interpretation of this data.
ting cascade. If the citrated plasma solution is diluted by a calcium-rich medium, the anticoagulant effect of the citrate is overwhelmed, and the clotting cascade can occur. In order to prevent clotting of the perfusion fluid in the anterior chamber, an excess of sodium citrate needed to be added to the solution.

Unfortunately for these experiments, calcium is a vital component in the binding of the tight junctions. The result of the introduction of a massive amount of sodium citrate is the removal of calcium from the tight junctions between the endothelial cells of the inner wall of Schlemm's canal. Bill et al. [1980] and Hamanaka and Bill [1987] investigated the effect of other chelating agents (Na₂EDTA), and suggested that the opening of junctions leads to a large increase in facility. Since morphology was not performed on the eyes perfused with citrated plasma, we can only assume that the same morphological changes occur as with Na₂EDTA. Thus, even though we would expect the perfusion of 15% plasma solutions to have the same facility reducing effect as 15% serum solutions, the formation of new openings in the inner wall appears to negate this effect by increasing facility.

If the junctions of the inner wall do open in the presence of a chelator, and there are no other physiological changes which significantly affect facility, then the implication is that the outflow facility is affected by the size and number of opening in the inner wall. In the normal physiologic case, these openings would be the pores of the inner wall endothelium. In Chapter 5, we describe our investigation of the possibility that the number of pores in the inner wall endothelium is an important determinant of overall outflow facility.

In the next chapter, we describe our investigations of the soluble protein concentrations in the trabecular meshwork, and their role in generating outflow resistance. However, our goal in this chapter of determining if a clotting by-product was responsible for the elimination of washout in 15% serum perfusions in bovine eyes [Johnson et al., 1993],
remains unaccomplished. Unless we are able to find an anticoagulant that does not affect outflow facility, it is unlikely that this goal can be achieved through perfusions of plasma solutions. A broader goal worthwhile of pursuit is determining the specific serum protein(s) responsible for elimination of the washout effect in bovine eyes. The "brute force" method would be to perfuse bovine eyes with solutions of individual serum components (or combinations of serum components) until we identify those that can eliminate washout. Realistically, though, we must wait for the development of a more elegant technique due to the wide range of possible serum components which must be investigated.
Chapter 3

Soluble Proteins in the Trabecular Meshwork: Experimental Studies

Our goal here was to determine the actual concentration of proteins in the fluid passing through the trabecular meshwork, and its possible relationship to washout in bovine eyes [Johnson et al., 1991] and the lack of washout in human eyes [Erickson-Lamy et al., 1990]. As discussed in Chapter 1, perfusion of bovine eyes with a solution of 15% serum essentially eliminated the washout effect [Johnson et al., 1993]. While this is significantly higher than the protein concentration of anterior chamber aqueous humor (about 1% of serum), the actual protein concentration of the fluid passing through the trabecular meshwork was unknown. The results of Freddo et al. [1990] and Barsotti et al. [1992] suggested that diffusion from the root of the iris could significantly raise protein levels in the fluid passing through the trabecular meshwork. If proteins were responsible for the measured resistance and the washout effect, we would expect that the initial protein concentration in the fluid passing through the trabecular meshwork of an enucleated eye would be approximately 15% of serum, and decrease during experimental perfusions with protein-free media as fluid is perfused through the outflow system, depleting the protein source. In bovine eyes, the protein concentration was expected drop significantly over the course of an experiment, increasing the outflow facility. In human eyes, which do not exhibit washout, protein concentration was expected to remain essentially constant.

To determine the dependence of soluble protein concentration in fluid passing through the bovine and human trabecular meshworks upon volume of protein-free media perfused, a series of perfusion experiments were conducted using protein-free buffer solutions. Since the perfusion fluid replaces the normal flow of aqueous humor, it follows the same
outflow pathway, and eventually flows into the episcleral veins. However, since the eyes are enucleated, fluid which has passed through the outflow system, called perfusate, does not return to the vascular system. Instead, it reaches the surface of the eye through the severed blood vessels where it can be collected and assayed for soluble protein concentration. It was assumed that the protein concentration of the perfusate would correspond to the soluble protein concentration within the trabecular meshwork. Correlations for outflow facility versus volume perfused, and outflow resistance versus protein concentration of the perfusate were also examined.

3.1 Experimental Methods

Description of Experiments

A series of experiments (n=16 eyes) were run in which the bovine eyes were perfused with Dulbecco's phosphate buffered saline with 5.5 mM glucose (DBG). Anterior chambers were exchanged with DBG to ensure that the fluid entering the outflow pathway was protein-free. Thus, the proteins found in the perfusate correspond to those added during the passage of the buffer through the aqueous outflow system. This simplified the interpretation of the data, as well as the modeling of the protein source (Chapter 4).

A small number of bovine eyes (n=2) were perfused with 15% serum in DBG to determine if protein accumulation was occurring within the trabecular meshwork that could explain the elimination of the washout effect found by Johnson et al. [1993]. This phenomenon was expected to manifest itself as a decrease in protein concentration from the perfusion fluid to the perfusate. The contralateral eyes in these experiments were perfused with plain DBG as controls.

In addition to protein concentration, we were interested in determining the concentration of glycosaminoglycans (GAGs) in the perfusate in order to determine its role in wash-
out. As discussed in Chapter 1, GAGs have been identified as a possible source of outflow resistance in the eye [Ethier et al., 1986]. Although there is evidence to indicate that the washout of GAGs is minimal [Knepper et al., 1984; Johnson et al., 1993], quantitation of GAG concentrations in the fluid passing through the trabecular meshwork has not been performed over long perfusion periods. Thus, for a subset of the bovine eyes (n=5 pairs) used above, the perfusate was assayed for concentration of sulphated GAGs, including keratan sulphate, heparan sulphate, and hybrid dermatan sulphate-chondroitin sulphate [Knepper et al., 1984]. Since DBG is GAG-free as well as protein-free, perfusate GAG concentrations correspond to amounts added to buffer in the outflow system.

Similar studies were carried out in human eyes (n=10 eyes), although the perfusions were only performed with DBG. Our hypothesis in these experiments was that the lack of washout in human eyes was due to very slow depletion of the protein source. Perfusion of human eyes with serum solutions were not performed since the additional protein would simply add an extra degree of complexity to interpretation of the data. Also, perfusate from human eyes were not assayed for sulphated GAGs.

One pair of bovine eyes was stored under moist chamber conditions in the refrigerator for 24 hours in order to mimic the post-mortem time experienced by human eyes. For logistical reasons (Appendix A), human eyes were not utilized until up to 30 hours after death, while bovine eyes were typically used within 6 hours of death. This experiment was performed to ensure that any differences between the protein concentration of bovine and human perfusates were due to inherent physiologic differences, and not simply due to different post-mortem conditions.

**Experimental Protocol**

The protocol followed in these studies was based on the method of Johnson et al. [1990]. Common experimental procedures concerning procurement of tissue and perfu-
sion techniques are detailed in Appendix A. After eyes were received, they were cleaned of extraocular fat and muscle; the conjunctiva was carefully and completely removed. The eyes were then soaked in isotonic saline at 4 °C for 1-2 hours to remove soluble proteins from the surface of the eye.

A suture was tied around the optic nerve and pulled through the spout of a funnel, and the end of the funnel was sealed with a rubber stopper. This is shown diagrammatically in Figure 3.1.

![Diagram of eye-funnel assembly](image)

**Figure 3.1**: Eye-funnel assembly for protein experiments

The eye-funnel assembly was then placed in a 50 ml beaker and placed in a water bath at 34 °C.

Each eye had a perfusion needle and an exchange needle inserted as described in Appendix A. The eyes were then covered with a heavier-than-water silicone oil (Dow Corning Fluid #710, Dow Corning Corp., USA). DBG or 15% serum in DBG was perfused from the syringe pumps at a constant pressure of 10 mmHg for 6 to 10 hours. As the perfusate exited the aqueous outflow system, it collected in pools at the top of the silicone
oil layer. The perfusate could easily be distinguished from the silicone oil due to the different indices of refraction, even when small amounts of perfusate were present. The perfusate could then be preferentially pipetted off (5 3/4 inch disposable Pasteur pipet, VWR Scientific, Boston, MA) due to the high viscosity of the silicone oil. Perfusate from the first 30 minutes of perfusion was discarded since the perfusate had to pass through the episcleral veins to reach the surface of the eye, and was likely contaminated with blood. The first 30 minutes of perfusion acted to "flush" out the system with fresh perfusion fluid.

Samples of perfusate were taken every 30 minutes for bovine, and every 60 to 90 minutes for human. Samples of perfusate were frozen until protein assays could be performed. After each sample point, the syringe pump was paused, and the anterior chambers (AC) of the eyes were exchanged with fresh perfusion fluid to ensure that the fluid entering the outflow pathway had essentially zero protein content. The exchange was performed by setting the perfusion reservoir to 2 cmH$_2$O above the set point pressure, and setting the exchange reservoir to 2 cmH$_2$O below the set point pressure. Both lines were then opened, allowing the fluid of the AC to be rapidly exchanged, while keeping the average intraocular pressure approximately constant at the set point pressure. Approximately 3 ml was exchanged for bovine eyes, and 1 ml was exchanged for human eyes.

In these experiments, samples of fluid from the anterior chamber were required in order to determine how much protein was added to the fluid within the AC (this was expected to be minimal as a result of the AC exchanges). Two methods were possible. If periodic samples (at the same time as the perfusate samples) were needed, a third infusion needle could be inserted into the AC prior to the start of the experiment, ensuring that the tip was placed in the AC and not the posterior chamber. The same precautions were taken with this needle as with the perfusion and exchange needles. The tubing was cut short to minimize the fluid trapped in the tubing, and a clamp was used to close the line when not
in use. Opening the line just before the exchange allowed a small sample of fluid to flow out of the AC due to the positive IOP. Since protein concentrations in the AC samples were relatively low and constant, only a single AC sample was taken during later experiment. With this method, a syringe with needle was inserted into the AC at the end of the experiment, and a sample was removed.

**Protein Assays**

Protein assays were performed using the colorimetric Bio-Rad Protein Assay (Bio-Rad Chemical Division, Richmond, CA), based on the method of Bradford [1976]. The Bio-Rad protein assay provides a linear relationship between protein concentration and wavelength for protein concentrations between 2 μg/ml and 20 μg/ml using the microassay procedure (from Bio-Rad Protein Assay instruction manual). Calibration standards were produced using lyophilized bovine serum albumin (Sigma Chemical Co., St. Louis, MO) for bovine eyes and lyophilized human albumin (Sigma Chemical Co., St. Louis, MO) for human eyes. Standards were produced on the day of the protein assay, and calibrations were performed immediately prior to the assay.

For each assay, 200 μl of reagent was combined with 800 μl of sample in a “UV Grade” disposable semi-micro cuvette (VWR Scientific, Boston, MA). Perfusate samples were thawed and gently mixed to ensure uniformity of the sample. Perfusate samples were diluted with distilled water when protein concentrations were outside of the linear calibration range. Contents of the cuvettes were mixed by turning several times, and readings were taken on the spectrophotometer (Model UV160U, Shimadzu Corp., Kyoto, Japan) within 10 to 45 minutes of mixing.

**Sulphated GAGs Assay**

For the perfusate samples assayed for sulphated GAG concentration, the method of Farndale et al. [1986] was followed. This technique is based on the shift in the spectral
absorbance maximum (to 525 nm) of 1,9-dimethylmethylene blue (DMB) when binding to sulphated GAGs occurs. The assay was calibrated for sulphated GAG concentrations from 1 μg/ml to 20 μg/ml using dermatan sulphate ("Super Special Grade", Seikagaku America Inc., Rockville, MD). Standards were produced on the day of the assay, and calibrations were performed immediately prior to the sample measurements.

The DMB reagent was prepared by dissolving 16 mg DMB (Aldrich Chemical Company, Milwaukee, WI) in 1 L water containing 3.04 g glycine, 2.37 g NaCl, and 95 ml 0.1M HCl. The reagent was placed in a brown glass bottle and stored at room temperature. For each measurement, 200 μl of sample solution was mixed with 1 ml of DMB reagent in a "UV Grade" disposable semi-micro cuvette (VWR Scientific, Boston, MA). Since the sulphated GAG-DMB complex starts to aggregate and eventually precipitate, the intensity reading at 525 nm is unstable [Farndale et al., 1986]. Thus, measurements were taken on the spectrophotometer within 10 seconds of mixing the two components.

**Statistical Methods**

The data from the experiments was analyzed using two different methods. In the first method (pooled data method), the data from all the relevant experiments was pooled prior to regression analysis. The method of pooling data was suitable when inter-eye variability was not important [Feldman, 1988]. In these cases, we were interested in the changes of the dependent variable for the entire sample (instead of any individual eye) over the experimental range of the independent variable.

In the second statistical method, the data for individual specimens were fitted to a model using least squares regression analysis, and variance analysis was performed on these calculated parameters. Significance levels were determined by treating the model parameters as raw data, and calculating the significance of the mean for each parameter. This is commonly referred to as the NIH method, in reference to the standard practice at
the National Institutes of Health [Feldman, 1988]. This method was more suitable when
individual specimen variability was considered to be important.

All results are shown along with standard errors.

3.2 Experimental Results
The protein and GAG assay results from the bovine and human perfusate samples are
summarized below, along with the statistical analyses for correlations between: (1) protein
concentration and volume perfused; (2) outflow facility and volume perfused; (3) outflow
resistance and volume perfused; (4) outflow resistance and protein concentration; and (5)
GAG concentration and volume perfused.

Protein Concentration vs. Volume Perfused Correlations

Bovine Eyes (DBG Perfusions):

A total of n=9 pairs of bovine eyes were used in these experiments, of which n=16
eyes were perfused with DBG (7 pairs, plus 2 single eyes), and 2 were perfused with 15% serum in DBG. Figure 3.2 shows perfusate protein concentration data from a typical DBG perfusion experiment, plotted against volume perfused.

For the eyes perfused with DBG, protein concentrations were correlated with volume perfused based on the assumption of a finite protein source being depleted. This produces a protein concentration that decays as a function of the volume of fluid, \( V_p \), following an equation of the form:

\[
c_p = c_i e^{-\left(\frac{V_p}{V_0}\right)}
\]  
(3.1)

where \( V_0 \) is the “washout volume constant”, and \( c_i \) is the initial protein concentration of the perfusate.
Figure 3.2: Typical protein concentration versus volume perfused for a bovine eye

Using the pooled data method, the parameters for equation (3.1) were calculated using a least squares regression (n=133 data points, R=0.665):

\[ c_i = 595 \pm 29 \mu g/ml \quad (P<0.0001) \]
\[ V_0 = 8.61 \pm 0.98 \text{ ml} \quad (P<0.0001) \]

where \( V_0 \) represents the amount of fluid which needs to be perfused to decrease the concentration by a factor \( 1/e \).

Using the NIH method, the data from each eye perfused with DBG was fitted to equation (3.1) using a least squares regression. Variance analysis of the equation parameters produced the following results (n=16 eyes):

\[ c_i = 637 \pm 60 \mu g/ml \quad (P<0.0001) \]
\[ V_0 = 10.03 \pm 1.80 \text{ ml} \quad (P<0.0001) \]

These results are essentially the same as those obtained by the pooled data method, indi-
cating that inter-specimen variations were not important for this correlation. The curve from both methods is plotted below (Figure 3.3) along with the pooled protein data from the bovine eyes.

![Graph showing Protein Concentration vs. Volume (Bovine)](image)

**Figure 3.3: Protein Concentration vs. Volume (Bovine)**

*Human Eyes:*

A total of n=10 human eyes were perfused with DBG and the perfusate analyzed for protein content. Data from a typical experiment is shown in Figure 3.4, plotted against volume of buffer perfused. In our analysis, data from one eye was discarded as an outlier, as the protein concentration was much higher than the other eyes, and concentration was found to increase as a function of volume perfused.
Protein concentration in human eyes was assumed to have the same volume dependence as bovine eyes. The data was fitted using a least squares regression to an equation with the form of (3.1). Using the pooled data method, the parameters for initial protein concentration, $c_i$, and volume constant, $V_0$, were found to be (9 eyes, n=65 data points, R=0.659):

$$c_i = 2270 \pm 140 \ \mu g/ml \ (P<0.0001)$$

$$V_0 = 1.992 \pm 0.334 \ ml \ (P<0.0001)$$

Using the NIH method, the data from each human eye was fitted to equation (3.1) using a least squares regression. Variance analysis of the equation parameters produced the following results (n=9 eyes):

$$c_i = 1976 \pm 216 \ \mu g/ml \ (P<0.0001)$$

$$V_0 = 5.732 \pm 2.155 \ ml \ (P<0.03)$$
While the initial concentration using the NIH method was approximately the same as with the pooled data method, the volume constants were very different. Given these results, two questions concerning their validity arise. First, since only about 1 ml of fluid is perfused in a typical experiment with human eyes, we must question whether or not $V_0$ significantly larger than the experimental range is valid (especially with the NIH method which has a larger volume constant). It is useful to note in this context that 6 of the 9 specimens exhibited statistically significant volume constants ($P<0.05$), ranging from 1.106 ml to 21.181 ml, while 3 of the specimens showed no statistically significant protein decay. This suggests that the washout of proteins, as characterized by $V_0$, does occur (and is not simply an artefact of the statistical analysis method), but at widely differing rates in different eyes.

If washout of proteins in human eyes did occur, then our next concern was determining which statistical method provided a better estimate of the volume constant. The larger $V_0$ calculated using the NIH method suggested that the protein concentration decayed much more slowly in human eyes than was implied by the pooled data method. However, the volume constant produced by the NIH method was simply a mean value produced by weighting the results of all the specimens equally. While this reflects the high degree of variability in human eyes, manifested as a larger error (and higher $P$-value) than in the pooled data method, large $V_0$ values which are not statistically significant for an individual specimen may have been overweighted producing an unrealistically large volume constant. In contrast, the pooled data method does not recognize any of the differences between individual specimens, producing a very low $P$-value, which does not properly represent the variability in the data. Also, since specimens with low perfusion volumes also tend to have higher protein concentrations, an unrealistically low $V_0$ may have been
produced. For these reasons (and others given below), we tend to trust the results of the NIH method more than the results of the pooled data method.

The curves from both methods are plotted below (Figure 3.5) along with the pooled protein data from the human eyes. Data points from different eyes are represented by different symbols in order to emphasize the protein concentration variations for individual eyes.

![Graph](image)

**Figure 3.5: Protein Concentration vs. Volume (Human)**

**Facility vs. Volume Perfused Correlations (Washout)**

**Bovine Eyes:**

The increase in facility as a function of volume during perfusions with DBG is the defining characteristic of the washout phenomenon. Outflow facility, $C$, can be expressed as a function of volume perfused, in the form [Johnson et al., 1991]:

$$C(V_p) = a_0 + a_1 V_p + a_2 V_p^2$$  \hspace{1cm} (3.2)

where $a_0$ represents the initial facility, and $a_1$ represents the first order approximation for
the washout rate, \( dC/dV \), and \( a_2 \) is a constant. Transient data from the first 0.5 ml of volume perfused were not used in the regression analyses.

Using the pooled data method, the combined data from all the bovine eyes perfused with DBG were fitted to equation (3.2). The parameters were calculated as \((n=16\) eyes, \(R=0.80)\):

\[
a_0 = 1.414 \pm 0.013 \, \mu l/min/mmHg \ (P<0.0001)
\]
\[
a_1 = 0.3050 \pm 0.0054 \, (\mu l/min/mmHg)/ml \ (P<0.0001)
\]
\[
a_2 = -0.0033 \pm 0.0005 \, (\mu l/min/mmHg)/ml^2 \ (P<0.0001)
\]

Using the NIH method to analyze the data, the polynomial coefficients for each eye were calculated, and regression analysis was performed on the calculated parameters \((n=16\) eyes):

\[
a_0 = 1.53 \pm 0.11 \, \mu l/min/mmHg \ (P<0.0001)
\]
\[
a_1 = 0.341 \pm 0.030 \, (\mu l/min/mmHg)/ml \ (P<0.0001)
\]
\[
a_2 = -0.0198 \pm 0.0083 \, (\mu l/min/mmHg)/ml^2 \ (P<0.05)
\]

The mean values for initial facility \((a_0)\) and washout rate \((a_1)\) were in good agreement with the values found by Johnson et al. [1991] \((a_0 = 1.3 \pm 0.10 \, (\mu l/min/mmHg)\) and \(a_1 = 0.34 \pm 0.033 \, (\mu l/min/mmHg)/ml\). In addition the results from both the pooled data method and the NIH method were similar, again indicating that inter-specimen differences were not very important for bovine eyes. The curves from both methods are shown below (Figure 3.6), along with the pooled data.
Figure 3.6: Outflow Facility vs. Volume Perfused (Bovine)

While the curve fit from the NIH method accurately indicates a decreasing slope as the volume increases, extrapolation of the facility curve beyond a perfusion volume of 10 ml is not valid since the polynomial curve fit indicates that facility decreases beyond this point. However, most perfusion experiments were not carried out this far. Thus, the curve fit using the NIH method does accurately represent the results for a typical volume range for a perfusion experiment.

*Human Eyes:*

The data for facility and volume from human eyes perfused with DBG were correlated using an equation of the form of (3.2). Outflow facility data collected during the first 0.1 ml of fluid perfused was not used in the correlations in order to eliminate the transient effects.

As with the bovine eyes, the pooled data method was used first. The data from the same eyes was used in this correlation as was used for the protein concentration versus volume perfused correlation. The calculated parameters were (n=9 eyes, R=0.742):
\[ a_0 = 0.214 \pm 0.033 \ \mu l/min/mmHg \ (P<0.0001) \]
\[ a_1 = 0.119 \pm 0.065 \ (\mu l/min/mmHg)/ml \ (P<0.05) \]
\[ a_2 = 0.033\pm0.025 \ (\mu l/min/mmHg)/ml^2 \ (P>0.05) \]

As with the bovine eyes, regression analysis was also performed using the NIH method for the human eyes (n=9 eyes):

\[ a_0 = 0.311 \pm 0.046 \ \mu l/min/mmHg \ (P<0.0001) \]
\[ a_1 = 0.026 \pm 0.057 \ (\mu l/min/mmHg)/ml \ (P>0.30) \]
\[ a_2 = 0.009\pm0.043 \ (\mu l/min/mmHg)/ml^2 \ (P>0.40) \]

**Figure 3.7: Outflow Facility vs. Volume Perfused (Human)**

The results for the outflow facility vs. volume perfused correlations demonstrate the potential drawback of the pooled data method. Even though human eyes have not been found to wash out [Erickson-Lamy et al., 1990], pooling the data together indicates that the washout rate, \( a_1 \), is statistically significantly different than zero. The NIH method, on
the other hand, indicates the lack of measurable washout in human eyes, consistent with
the literature. Both curves are shown in Figure 3.7, along with the combined data.

**Outflow Resistance vs. Protein Concentration Correlation**

For both the bovine and human eyes, relationships were determined for outflow facil-
ity versus protein concentration in the form:

\[
\frac{1}{C} = b_1 + b_2 c_p
\]  

(3.3)

where \(b_1\) and \(b_2\) are constants, \(c_p\) is the protein concentration, and \(1/C\) represents the out-
flow resistance. The model upon which this correlation is based is described in section
4.2.

**Bovine Eyes:**

A linear regression of the resistance and protein concentration data from the bovine
eyes perfused with plain DBG (n=16 eyes), using the pooled data method, produced the
following parameter values (n=131 data points, R=0.59):

\[
b_1 = 0.210 \pm 0.031 \text{ mmHg/\mu l/min (P<0.0001)}
\]

\[
b_2 = 6.44 \times 10^{-4} \pm 7.8 \times 10^{-5} \text{ (mmHg/\mu l/min)/(\mu g/ml) (P<0.0001)}
\]

Re-analysis of the same data using the NIH method produced similar results (n=16 eyes):

\[
b_1 = 0.220 \pm 0.046 \text{ mmHg/\mu l/min (P<0.0001)}
\]

\[
b_2 = 6.52 \times 10^{-4} \pm 9.1 \times 10^{-5} \text{ (mmHg/\mu l/min)/(\mu g/ml) (P<0.0001)}
\]

The least squares curves from both methods are shown below, along with the combined
data from the bovine DBG perfusions.
**Figure 3.8:** Outflow Resistance vs. Protein Concentration (Bovine)

*Human Eyes:*

For human eyes (n=9 eyes), regression analysis using the pooled data method yields the following parameters (n=65 data points, R=0.773):

\[ b_1 = -0.124 \pm 0.124 \text{ mmHg/µl/min (P}>0.10) \]

\[ b_2 = 2.17 \times 10^{-3} \pm 0.22 \times 10^{-3} \text{ (mmHg/µl/min)/(µg/ml) (P}<0.0001) \]

Re-analysis of the same data using the NIH method produced the following results (n=9 eyes):

\[ b_1 = 1.84 \pm 0.58 \text{ mmHg/µl/min (P}<0.01) \]

\[ b_2 = 9.12 \times 10^{-4} \pm 37.36 \times 10^{-4} \text{ (mmHg/µl/min)/(µg/ml) (P}>0.40) \]

While the pooled data method indicates a correlation between outflow resistance and protein concentration, the NIH method does not indicate a statistically significant relationship. Both curves are plotted below (Figure 3.9) along with the combined human data.
**Figure 3.9:** Outflow Resistance vs. Protein Concentration (Human)

The lack of a correlation using the NIH method is consistent with the lack of washout in human eyes. However, the existence of a correlation using the pooled data method suggested the possibility of a correlation between initial outflow resistance and initial protein concentration. The initial values for protein concentration were found by fitting the data from each eye to equation (3.1). The initial values for outflow resistance were found from the outflow resistance versus volume perfused relationship found below. A marginally significant relationship was then found fitting the initial condition data values to equation (3.3); the results are shown below (n=9 eyes, R=0.588):

\[ b_1 = 1.10 \pm 1.75 \text{ mmHg/}\mu\text{l/min (P}>0.54) \]

\[ b_2 = 1.62 \times 10^{-3} \pm 0.84 \times 10^{-3} \text{ (mmHg/}\mu\text{l/min)/(}\mu\text{g/ml (P}<0.097) \]
Figure 3.10: Initial outflow resistance vs. initial protein concentration (human)

Since the relationship is only marginally significant, it is worthwhile to note the meaning of the correlation coefficient, R. We can interpret $R^2$ as the fraction of the total variation of outflow resistance that can be accounted for by the relationship with protein concentration [Freund and Walpole, 1987]. Thus, for $R^2 = 0.345$, 65.5% of the variation in outflow resistance is due to factors other than the change in protein concentration. The importance of the correlation is therefore somewhat suspect.

**Outflow Resistance vs. Volume Perfused Correlation**

The above analysis of the washout effect assumed a polynomial model for the relationship between outflow facility and volume of fluid perfused. An alternate method of examining washout is by using the a model for the outflow resistance, $R$, given by equation (3.3). Since the protein concentration is given by equation (3.1), substitution into equation (3.3) produces an expression for the outflow resistance in terms of the volume of fluid perfused, $V_p$: 
\[ R = b_1 + b_2 \frac{c_i}{V_0} \]  \hspace{1cm} (3.4)

where \(c_i\) is the initial concentration, and \(V_0\) is the washout volume constant.

In the above analyses of protein concentration vs. volume perfused, and outflow facility vs. volume perfused, we found that the pooled data method did not satisfactorily represent the human data. Thus, only the results of the NIH method are presented here.

**Bovine Eyes:**

For the n=16 bovine eyes perfused with plain DBG, the following results were obtained:

\[ b_1 = 0.285 \pm 0.055 \text{ mmHg/\mu l/min (P<0.0002)} \]

\[ b_2 = 0.585 \pm 0.121 \text{ mmHg/\mu l/min (P<0.0003)} \]

\[ V_0 = 3.503 \pm 0.742 \text{ ml (P<0.0001)} \]

A statistically significant washout volume constant was found, as would be expected from the washout effect. However, the value of \(V_0\) calculated above for the resistance vs. volume perfused correlation (\(V_0 = 10.03 \pm 1.80 \text{ ml}\)) is significantly smaller than the value calculated here for the protein concentration vs. volume perfused correlation (P<0.005). This indicates that the protein concentration and the resistance decay at different rates with respect to the volume of fluid perfused.

**Human Eyes:**

Statistically significant results could not be found using the data from human eyes. This is not surprising since the change in resistance (like the change in facility) is very small, and a statistically significant variation would be difficult to detect. For individual eyes, only 3 of the 9 specimens exhibited a statistically significant volume constant.
Other Protein Experiments With Bovine Eyes

15% Serum Perfusions:

For the n=2 single bovine eyes perfused with 15% serum in DBG, protein concentration was found to increase slightly as a function of volume, instead of decrease. Thus, instead of fitting the data to the exponential decay equation (3.1), a linear model was used of the form:

\[ c = c_1 + c_2 V \] (3.5)

where \( c \) is the bulk protein concentration of the perfusate, \( V \) is the volume perfused, and \( c_1 \) and \( c_2 \) are constants. The following equation parameters were found for the 2 eyes using the NIH method:

\[ c_1 = 8616 \pm 132 \text{ \mu g/ml (P<0.01)} \]
\[ c_2 = 207 \pm 34 \text{ (\mu g/ml)/ml (P<0.103)} \]

where protein concentration was found to be a marginally significant function of volume perfused (P<0.103). The protein concentrations of the 15% serum solution itself was found to be 9408 ± 506 \( \mu \text{g/ml} \), which is not significantly different than the initial concentration of the perfusate, represented by \( c_1 \) (P>0.28). While this was not surprising based on the very small sample size, it is consistent with the qualitative observation that the protein concentration of the perfusate is approximately the same as the 15% serum solution. Thus, no protein accumulation from the 15% serum solution appears to be occurring within the outflow pathway. The results of the curve fit are shown below in Figure 3.11.
Figure 3.11: Protein concentration vs. volume for 15% serum perfusions

Extended Post-Mortem Time Experiment:

For the bovine eyes (n=1 pair) stored under moist chamber conditions for 24 hours, no qualitative change was noticed between the protein concentration of the perfusate compared with eyes with shorter post-mortem times. Using the NIH method, the data from this experiment was fitted to equation (3.1) to find the protein concentration versus volume perfused correlation (n=2 eyes):

\[ c_i = 385 \pm 72 \ \mu g/ml \]
\[ V_0 = 17.35 \pm 6.62 \ \text{ml} \]

This is roughly comparable to the results from the bovine eyes with the shorter post-mortem times given above, and very different than the results from human eyes that had similar post-mortem times. These results were not used in the modeling of the protein depot (Chapter 4).
Bovine GAG Data

The perfusate of n=10 of bovine eyes were assayed for sulphated GAG concentration. The mean GAG concentration of the perfusate was found to be $2.85 \pm 0.08$ µg/ml (n=82 data points), while the mean GAG concentration of samples from the AC was found to be $1.55 \pm 0.06$ µg/ml (n=33 data points). No statistically significant relationship was found between sulphated GAG concentration and volume of fluid perfused (P>0.30, R=0.06).

The source of the GAGs in the perfusate is of some concern, since a high concentration of GAGs within the trabecular meshwork could significantly affect outflow facility. While the levels of GAGs found in the perfusate were consistent with the normal physiologic levels of hyaluronic acid found in aqueous humor [Laurent, 1981], a statistically significant difference was found between the GAG concentration of the perfusate, and the GAG concentration of AC samples (P<0.001). This raised the possibility that additional GAGs were being added to the fluid stream within the trabecular meshwork. Based on a typical perfusion volume of 10 ml, and the morphological dimensions given in Chapter 1, our results suggest that the GAG concentration in the trabecular meshwork would need to be on the order of $\sim 10^2$ mg/ml in order to generate the difference between GAG concentrations in the AC samples and perfusate samples. However, we must remember that the we have exchanged the anterior chambers with buffer, so the fluid will not be in an equilibrium state with the fluid of the posterior chamber (PC). If GAGs are being added to the buffer in the PC from the vitreous, and fluid flows preferentially from the PC to the outflow pathway, the GAG concentration in the perfusate would be higher than the bulk concentration in the AC. Thus, the GAGs in the perfusate are probably not due to washout from the trabecular meshwork, consistent with the findings of previous researchers [Knepper et al., 1984]. In the modeling studies of Chapter 4, we assume a constant GAG concentration within the trabecular meshwork.
3.3 Discussion and Conclusions

*Statistical Methods and Washout:*

The correlations produced by the two different statistical analysis methods were approximately the same for the bovine data. However, for the human data, radically different conclusions could be reached depending on the method of analysis used. This could be clearly seen by examining the polynomial model for outflow facility versus volume perfused correlations. The use of the pooled data method suggests that human eyes wash out (as represented by the linear coefficient for the model), while the use of the NIH method suggests that this is not the case, consistent with the results of Erickson-Lamy et al. [1990]. In contrast, both analysis methods indicate a washout rate in bovine eyes consistent with the results of Johnson et al. [1991]. One possible explanation for the existence of discrepancies between the statistical methods used to analyze human data (and the lack of discrepancies in the analysis of the bovine data) is that human eyes show greater variability than bovine eyes. The use of the pooled data method would therefore not be suitable for the analysis of the human data.

*Results from Bovine Eyes:*

The apparent exponential decay of protein concentration as a function of volume of buffer perfused is highly suggestive of a finite protein source being depleted in bovine. This is consistent with the findings of Freddo et al. [1990] and Barsotti et al. [1992] which suggested that protein levels in the trabecular meshwork could be elevated due to diffusion from the root of the iris. If we examine the volume constants ($V_0$) from the NIH method, we find that for a typical experiment with bovine eyes in which about 10 ml of fluid is perfused, the protein concentration would be expected to drop to about 35% of its initial value. This is consistent with the notion that washout is a result of the depletion of resistance causing aqueous humor proteins.
Additional support for this idea comes from the existence of a statistically significant relationship between outflow resistance and protein concentration. This result was expected for bovine eyes as a consequence of the washout effect. As protein concentration decreased over the course of the experiment, the resistance was expected to decrease as well, producing the observed correlation.

Unfortunately, the results of the correlation between outflow resistance and volume perfused do not support the hypothesis of resistance causing aqueous humor proteins depleting and causing resistance. A statistically significant volume constant was found for bovine eyes, as was expected from an exponential model of washout (as opposed to the polynomial model for facility used above). However, comparison with the volume constant from the protein concentration vs. volume perfused correlation indicates a statistically significant difference. This suggests that the decay of protein concentration and the decay of outflow resistance with respect to volume perfused may be governed by different mechanisms.

In addition, we had expected the protein concentration in the trabecular meshwork to be approximately equal to the 15% serum required by Johnson et al. [1993] to prevent washout. This would correspond to the initial perfusate concentration determined from the protein concentration versus volume perfused correlation. Instead, the initial perfusate protein concentration was found to be only about 1% serum, or about equal to normal anterior chamber (AC) aqueous humor protein concentration. As this represents the amount of protein added to the buffer in the outflow system, the actual concentration of fluid entering the outflow system in vivo may be slightly higher. However, it would still be an order of magnitude less than the expected equivalent to 15% serum.

One potential explanation for these results is the possibility that rather than outflow facility being controlled by the protein content of the fluid passing through the trabecular
meshwork, the protein concentration simply may be a function of the time history of out-
flow facility. Over the course of a perfusion experiment, the protein source would become
depleted, leading to a decreased protein concentration in the perfusate. In addition, if the
outflow facility is increased, the flowrate is also increased, leading to a further decrease of
the bulk concentration of protein in the perfusate. Thus, rather than protein level determin-
ing outflow facility, instead facility controls flowrate which determines protein level. The
outflow facility would be controlled by a resistance causing mechanism other than the
bulk level of aqueous humor proteins.

Results from Human Eyes:

For the human data, we also found a correlation between protein concentration and
volume perfused, even though human eyes do not appear to wash out based on the polyno-
mial outflow facility model. This initially appears to contradict the notion that facility is
controlled by soluble protein concentration in the fluid passing through the trabecular
meshwork, since we would expect the resistance to decrease along with bulk protein con-
centration. However, if we examine the volume constant produced from the analysis using
the NIH method (which is more reliable for human data) we find that for a typical experi-
ment in which about 1 ml of fluid is perfused, the protein concentration would only be
expected to drop to about 85% of its initial value. Thus, if facility is controlled by aqueous
humor protein concentration, it would not be surprising that little change in facility is
noticed during a typical perfusion experiment. This would be particularly true for rela-
tively short experiments, such as those performed by Erickson-Lamy et al. [1990]. It is
possible that human eyes do wash out, but the change in facility occurs over a much
longer time period than the bovine eyes. This would also explain the lack of a correlation
between outflow resistance and volume perfused.
In addition, the perfusate protein concentration in human eyes was found to be higher than in bovine eyes (about 4 times higher), with approximately 4% of the serum protein concentration. Since humans have a normal AC aqueous humor protein concentration of about 120 μg/ml [Tripathi et al., 1989], this suggests that protein levels in the trabecular meshwork are about 18 times the AC level. While this is still 4 times less protein than the level required by Johnson et al. [1993] to prevent washout in bovine eyes, it is approximately the same as the 5% serum required by Kee et al. [1995] to significantly reduce washout in monkey eyes (however, recall that human eyes do not washout whereas monkey eyes do). Thus, the bulk protein concentration of the perfusate from human eyes may be high enough to generate significant outflow resistance, and the rate of depletion may be slow enough to prevent detection of washout.

For the outflow resistance vs. protein concentration correlation, human eyes demonstrated no statistically significant relationship using the NIH method. This would appear to contradict the hypothesis that outflow facility is controlled by aqueous humor proteins, and would seem to support the results of the polynomial outflow facility model that shows a lack of washout in human eyes. However, a marginally significant correlation was found between initial outflow resistance and initial protein concentration, consistent with our hypothesis. Given this result, along with the other results from the human eyes, further investigation of the resistance caused by aqueous humor proteins was warranted.

Need for Modeling Studies:

We have suggested the possibility that in bovine eyes, protein concentration is a function of facility and not vice versa. If proteins were being removed from a finite source, then over the course of a perfusion experiment, the protein source would become depleted. The protein concentration of the perfusate would consequently be expected to decrease over the course of the experiment and furthermore, would depend on flowrate and thereby
depend on outflow facility. In the next chapter, we present our modeling studies in which we investigated the consistency our experimental data with the idea of a finite depleting protein depot.

For human eyes, we found that the protein concentration in the fluid passing through the trabecular meshwork may be sufficiently high, and the depletion rate sufficiently slow, to explain the lack of washout found by previous researchers [Erickson-Lamy et al., 1990]. Therefore, in the next chapter we also report on our modeling studies of the theoretical outflow resistance generated by the measured levels of aqueous humor proteins. Both of the modeling studies were performed for human and bovine eyes.
Chapter 4

Soluble Proteins in the Trabecular Meshwork: Modeling Studies

In Chapter 3, we suggested the possibility that outflow resistance was not controlled by the bulk concentration of aqueous humor proteins. Instead, resistance may have been determined by an unrelated mechanism, and bulk protein concentration may have simply been a function of the flowrate (dictated by the resistance), and the perfusion history. We would expect this dependence if the soluble proteins in the perfusate were originating from a finite protein depot. Differences in the initial protein concentration of the perfusate would be determined by different initial flowrates, while decreasing protein concentration over the course of the experiments would result from the depletion of the protein depot.

In the previous chapter, it was found that the protein concentration of the perfusate from both bovine and human eyes decayed as an exponential function of volume of buffer perfused. Utilizing this result in the first of two modeling studies described in this chapter, we compared the experimentally measured protein levels with theoretical models of protein diffusion in order to determine if our data was consistent with the concept of a finite protein depot that depleted significantly over the course of an experiment.

In the experimental studies described in the previous chapter, we also found that the results from the bovine eyes were inconsistent with the idea that outflow facility was controlled by bulk protein concentration of fluid passing through the trabecular meshwork. The initial protein concentration of the perfusate from bovine eyes was only equivalent to approximately 1% serum. If aqueous humor outflow facility were controlled by bulk protein concentration, we would expect that the soluble protein concentration of the fluid passing through the trabecular meshwork would be approximately the same as the 15%
serum used by Johnson et al. [1993] to prevent washout. Also, the volume constant from
the outflow resistance vs. volume perfused and protein concentration vs. volume perfused
correlations were significantly different, indicating that outflow resistance and protein
concentration may be controlled by different mechanisms. However, for human eyes, no
washout was detected, and the protein concentration was found to decrease very slowly.
Thus, unlike the results for bovine eyes, it was not clear if the results for human eyes were
inconsistent with the hypothesis of aqueous humor proteins controlling outflow facility.

In order to further investigate the possibility that outflow facility was controlled by
bulk protein concentration in the fluid passing through the trabecular meshwork in human
eyes (and to verify that this was not the case in bovine eyes), we performed a second mod-
eling study. We compared the correlations between outflow resistance and protein concen-
tration found in the previous chapter with an existing theoretical model of albumin flow
through a hyaluronic acid matrix [Kim et al., 1991]. Utilizing this model we determined if
the measured outflow resistance could be accounted for by the bulk protein concentration
in the perfusate, using reasonable physiologic parameters. If the parameters calculated by
the model were not physiologically realistic, this would provide further evidence that pro-
tein concentration was controlled by facility, and not vice versa.

4.1 Source of Trabecular Meshwork Proteins

Recent evidence has indicated that a substantial portion of the proteins in aqueous
humor are added by diffusion from the ciliary body through the anterior side of the iris
root [Freddo et al., 1990; Barsotti et al., 1992]. One implication of this finding is that the
aqueous humor entering the trabecular meshwork may be of substantially higher protein
concentration than the aqueous humor of the anterior chamber. The experimental results
from human eyes supported this concept, while the experimental results for bovine eyes
indicated that protein concentrations in the trabecular meshwork were similar to protein concentrations in the anterior chamber. However, both the human and bovine results indicated the existence of a protein depot in the anterior segment, since perfusions were performed with protein-free buffer.

In this section, we developed a conceptual model of our protein source and calculated the model parameters that would be required to produce the experimentally measured protein concentrations in the perfusate. Our goal in this study was to determine if the experimentally measured protein levels were consistent with known physiological parameters in the outflow pathway. Specifically, we wished to identify a protein source, and determine if its geometric characteristics were consistent with the bulk perfusate protein concentration being determined by facility, and not vice versa.

Four measured variables were utilized in our analysis: (1) initial perfusate protein concentration, $c_i$; (2) volume scale to remove protein, $V_0$, found from the protein concentration vs. volume perfused correlations; (3) typical flowrate during perfusions, $Q$; and (4) total protein removed during experiments. The values from the NIH analysis method were used since they were more appropriate for human eyes, and provided essentially the same results as the pooled data method for bovine eyes. Using these values as inputs, we estimated three parameters for the protein depot, and compared them with the corresponding physiologic parameters to check for consistency: (i) length of the portion of the depot exposed to flow, $L$; (ii) length scale for the entire protein depot itself, $H$; and (iii) diffusivity of albumin in the protein depot, $D_r$. Based on these dimensions and proximity to the outflow pathway, we were able to identify the ciliary body itself as the probable protein source. In addition, the diffusion coefficient for proteins in the depot ($D_r$) was calculated based on the experimental protein flux rates. Comparison with known diffusion coefficients of proteins in other tissues gave us another measure of the validity of our model.
Idealized Model of the Protein Source

The first step in the theoretical analysis of the experimental data was the development of a protein reservoir model, based on the morphology of the bovine and human outflow systems given in Chapter 1. As aqueous humor flows from the anterior chamber into the outflow pathway, it enters a narrowing channel defined anteriorly by the cornea, and posteriorly by the iris. In the bovine, this leads to the spaces of Fontana, while in the human the pathway simply narrows until it reaches the trabecular meshwork itself. During its passage through this channel, aqueous humor passes by several potential protein depots, including the ciliary body, and in the bovine, the spaces of Fontana. In our analysis, we determined if the experimental data was consistent with these structures as the sources of protein in the perfusate.

In order to simplify the analysis we have idealized aqueous humor outflow as fluid flow between two flat plates, approximated by the ciliary cleft in the bovine, and the cornea and iris root immediately adjacent to the trabecular meshwork in the human (Figure 4.1). The top plate, representing the cornea, is assumed to be essentially impermeable to soluble proteins. The bottom plate represents the surface of the protein depot, and is assumed to allow diffusion of soluble protein from the depot into the fluid stream. The size of the bottom plate \( L \) approximates the distance that the fluid flows over the actual protein source, which is likely different than the depth of the depot \( H \). For example, in human eyes, the fluid stream would be expected to only come in contact with the ciliary body over the short distance of the iris root. It is useful to emphasize here that we have assumed that fluid flows over the protein depot, and not through it. This could be the case whether the fluid follows a preferential path through the anterior side of the spaces of Fontana, or flows over the iris root prior to entering the trabecular meshwork. The flow chan-
nel represented by these regions prior to the trabecular meshwork were assumed to be free of GAGs or any other flow obstructions.

Figure 4.1: Flow across the protein depot

The approximate dimensions for our model were determined from morphological studies available in the literature. For bovine eyes, the circumference of the depot was assumed to be approximately the same as the circumference of the iris, \( w = 5.5 \pm 0.5 \text{ cm} \) (assuming 10% variability in the size of bovine calf eyes) and, the average height of the flow channel was assumed to be roughly the same as the total width of the angular aqueous plexus, giving \( a = 450 \pm 150 \text{ \( \mu \)m} \) [Johnson et al., 1990]. For human eyes, the cir-
Cumferential length of the depot was assumed to be approximately the same as the trabecular meshwork circumference, giving \( w = 3.1 \pm 0.3 \) cm [Ethier et al., 1986], assuming 10% variability in the size of adult human eyes. The average channel height was assumed to be approximately equal to the width of the major axis of Schlemm’s canal, giving \( a = 350 \pm 150 \) \( \mu \)m [Tripathi and Tripathi, 1984]. The dimensions of the opening in the posterior plate, \( L \), were determined from the initial protein concentration, flowrate, and mass transfer coefficient (given below).

The protein depot itself was idealized as a semi-infinite medium, which depletes radially (r-direction in Figure 4.1) from the exposed surface of the posterior plate. In addition, the rate of protein flux was assumed to be limited only by the surface area available for mass transfer and the diffusion coefficient of the medium (diffusion dominated process). Thus, the decrease in protein flux out of the reservoir during the course of a perfusion experiment was assumed to be due to a local depletion of the medium. The reservoir was further assumed to be large enough so that the concentration beyond the local depletion zone is the same as the initial concentration.

**Protein Source Opening Size**

We first determined the size of the posterior plate that would be required to deliver the initial measured protein concentrations in the perfusate.

**Perfusate Protein Concentration:**

The mass flux per unit area, \( \dot{m}^\prime \prime (t) \), out of the reservoir can be determined from the from the convection equation at the surface of the reservoir:

\[
\dot{m}^\prime \prime (t) = \overline{h_L} (c_s (t) - c_\infty)
\]

where, \( \overline{h_L} \) is the average mass transfer coefficient between the reservoir and the fluid stream over the length of the opening \( L \), \( c_s \) is the surface concentration of the protein
source, and \( c_{\infty} \) is the protein concentration in the free stream fluid. The fluid flowing over the depot was assumed to be protein free (i.e., negligible amounts of protein are added in the anterior chamber prior to passing over the depot), so \( c_{\infty} = 0 \ \mu\text{g/ml} \). Also, the initial \( (t = 0) \) protein concentration in the depot was assumed to be the same as ciliary stroma, which Barsotti [1990] gives as approximately 70% of blood plasma protein concentrations, or an initial concentration of \( c_0 = 42.0 \times 10^3 \pm 10\% \ \mu\text{g/ml} \) (assuming 10% error).

The total mass flux out of the reservoir is calculated as \( \dot{m}(t) = \dot{m}''(t) wL \), where \( w \) is the circumference of the depot, and \( L \) is the length of the opening. The initial bulk protein concentration of the perfusate, \( c_i \), is thus given by:

\[
c_i = \frac{\dot{m}(t = 0)}{Q_i} = \frac{wL \cdot h_L}{Q_i} \cdot c_0
\]  

(4.2)

where \( Q_i \) is the initial volume flowrate of fluid. Rearranging to find \( L \) gives:

\[
L = \frac{c_i \cdot Q_i}{c_0 \cdot w h_L}
\]  

(4.3)

**Flow Regime (Calculation of Reynold’s Number):**

In order to determine the mass transfer coefficient required for equation (4.3), it was first necessary to determine the flow regime in the channel formed by the parallel plates. Using the approximate dimensions for the model given above, along with typical initial volume flowrates of within the aqueous humor outflow system, we were able to determine Reynold’s numbers for our model. For flow between parallel plates, the Reynold’s number is given by:

\[
Re_{d_h} = \left( \frac{Q_i}{wa} \right) \left( \frac{d_h}{v} \right)
\]  

(4.4)
where $Q_i$ is the initial volume flowrate of the perfusion fluid, $wa = \text{(circumference } \times \text{ channel height)}$ is the cross-sectional area available for flow, and $v = 7.46 \times 10^{-7} \text{ m}^2/\text{s}$ [Johnson et al., 1993] is the kinematic viscosity of the saline vehicle at 34 °C. For flow between parallel plates, the hydraulic diameter, $d_h$, is twice the plate spacing ($d_h = 2a$).

Using typical flowrates from the experimental data, and the approximate channel dimensions gives the following results:

<table>
<thead>
<tr>
<th></th>
<th>Human</th>
<th>Bovine</th>
</tr>
</thead>
<tbody>
<tr>
<td>$Q_i$</td>
<td>$3.0 \pm 1.0 \mu l/min$</td>
<td>$15 \pm 5 \mu l/min$</td>
</tr>
<tr>
<td>$w$</td>
<td>$3.1 \pm 0.3 \text{ cm}$</td>
<td>$5.5 \pm 0.5 \text{ cm}$</td>
</tr>
<tr>
<td>$a$</td>
<td>$350 \pm 150 \mu m$</td>
<td>$450 \pm 150 \mu m$</td>
</tr>
<tr>
<td>$Re_{d_h}$</td>
<td>$0.0043 \pm 0.0015$</td>
<td>$0.0122 \pm 0.0042$</td>
</tr>
</tbody>
</table>

Table 4.1: Reynold's number calculations within the flow channel

Note that the cumulative fractional errors (here and elsewhere in this chapter) were calculated as the square root of the sum of the squares of the individual fractional errors. Actual errors in the calculated values were then determined from the fractional errors.

The low Reynold's numbers (Stokes flow regime) allows us to assume a fully developed velocity profile within the channel. Thus, in our calculations of the coefficient of mass transfer between the protein source and the fluid stream, we only needed to consider the development of the concentration boundary layer.

**Calculation of Mass Transfer Coefficient**

The average mass transfer coefficient, $h_L$, between the protein depot and the fluid stream is determined by the thickness of the velocity and concentration boundary layers within the flow channel. With a fully developed velocity profile, the concentration boundary layer is governed by the general mass convection equation (1-D velocity profile):
\[ u \frac{\partial c}{\partial x} = D_0 \left( \frac{\partial^2 c}{\partial x^2} + \frac{\partial^2 c}{\partial y^2} \right) \] (4.5)

where \( x \) and \( y \) are the directions shown in Figure 4.1. The solution to this equation is given in terms of an average Sherwood number, defined as:

\[ \overline{Sh}_{d_h} = \frac{d_h \overline{h_L}}{D_0} \] (4.6)

where \( d_h \) is the hydraulic diameter, and \( D_0 \) is the mass diffusion coefficient of protein in water. It is useful to note here that the diffusion coefficient is specified for the flow channel prior to entering the trabecular meshwork, and not for the protein depot. Only the boundary conditions for the problem are determined by the protein depot.

A mathematically equivalent problem is the case of heat transfer to a hydrodynamically fully developed flow between two parallel plates, governed by the heat transfer equation (1-D velocity profile) where \( \alpha \) (thermal diffusivity) replaces \( D_0 \) in equation (4.5). Equivalent boundary conditions are selected: instead of the impermeable plate represented by the cornea, we assume an adiabatic upper plate; instead of the uniform concentration protein depot, we assume a uniform temperature lower plate. The solution to this problem is given by the dimensionless average Nusselt number, defined as:

\[ \overline{Nu}_{d_h} = \frac{d_h \overline{h_{T,L}}}{k} \] (4.7)

where \( \overline{h_{T,L}} \) is the heat transfer coefficient between the plates and the fluid, and \( k \) is the thermal conductivity of the fluid.

Shah and London [1978] determined the solution for this problem in terms of the non-dimensionalized development length \( 2/G_z = x^+ \). The Graetz number, \( G_z \), is defined as:
\[ G_z = Re_{d_h} Pr \left( \frac{d_h}{L} \right) \tag{4.8} \]

where \( Pr = \nu/\alpha \) is the Prandtl number, \( \nu \) is the kinematic viscosity of the fluid, and the hydraulic diameter \( d_h = 2a \). We can also define a non-dimensional development length for the mass transfer problem as \( x^+ = 2/Gz_m \). The Graetz number for mass transfer problems, \( Gz_m \), is defined as:

\[ Gz_m = Re_{d_h} Sc \left( \frac{d_h}{L} \right) \tag{4.9} \]

where \( Sc = \nu/D_0 \) is the Schmidt number. When the non-dimensional development lengths for the heat and mass transfer problems are equal, the solutions will be equal as well so that \( \overline{Nu}_{d_h} = \overline{Sh}_{d_h} \). Thus, we can solve for the mass transfer problem by analogy to the heat transfer problem. The solution is presented graphically in Figure 4.2.

**Calculation of Opening Size:**

The calculation of the depot surface length exposed to fluid flow, \( L \), was an iterative process. First, the length was roughly estimated from morphological dimensions and substituted into equation (4.9) to find the Graetz number. The hydraulic diameter, \( d_h = 2a \), was calculated for bovine and human eyes using the model parameters given above. The Reynolds’s number had already been found in the above calculations. To calculate an approximate Schmidt number, we used the kinematic viscosity of the saline vehicle given above, and the diffusion coefficient of protein (modeled as albumin) in water \( D_0 = 6.0 \times 10^{-11} \text{ m}^2/\text{s} \) [Laurent et al., 1961] to find, \( Sc = 12.34 \times 10^3 \).

The non-dimensional development length, \( x^+ \), was then calculated, and the average Nusselt number, \( \overline{Nu}_{d_h} \), was determined from Figure 4.2. Equating the Nusselt number to the Sherwood number, the mass transfer coefficient could then be calculated from equa-
tion (4.6), and substitution into equation (4.3) providing an improved estimate of $L$ based on the initial perfusate protein concentration ($c_i$) and typical flowrates ($Q$) from the experiments. The new value of $L$ was then used to calculate a new $x^+$ from equation (4.9), using the same input parameters as with the initial guess. This iterative process was continued until a convergent value of $L$ was found. The relevant parameters, along with the final values for $L$ are given below in Table 4.2.

![Average Nusselt Number vs. $x^+$](image)

**Figure 4.2:** Average Nusselt number for flow between parallel plates: adiabatic upper plate and isothermal lower plate [adapted from Shah and London, 1978]

For bovine eyes, the mean length scale of the portion of the depot exposed to flow, $L$, was roughly comparable to (although less than) the width of the spaces of Fontana (approximately 450 μm [Johnson et al., 1990]). For human eyes, the result compared well with the width of the portion of the ciliary body exposed to fluid flow (0.1 mm) in owl monkeys [Barsotti et al., 1992]. We were able to use this comparison as a consistency
check since the anterior segments of human and monkey eyes are similar in size and physiology [Tripathi, 1974]. Thus, the results for both the bovine and human eyes were consistent with the idea that protein enters the fluid stream from a depot (the ciliary body) just before passing into the trabecular meshwork.

<table>
<thead>
<tr>
<th>Human</th>
<th>Bovine</th>
</tr>
</thead>
<tbody>
<tr>
<td>$Re_{d_h} = 0.0043 ± 0.0018$</td>
<td>$Re_{d_h} = 0.0122 ± 0.0052$</td>
</tr>
<tr>
<td>$Q_i = 3.0 ± 1.0 \ \mu l/min$</td>
<td>$Q_i = 15 ± 5 \ \mu l/min$</td>
</tr>
<tr>
<td>$w = 3.1 ± 0.3 \ \text{cm}$</td>
<td>$w = 5.5 ± 0.5 \ \text{cm}$</td>
</tr>
<tr>
<td>$a = 350 ± 150 \ \mu m$</td>
<td>$a = 450 ± 150 \ \mu m$</td>
</tr>
<tr>
<td>$c_i = 1976 ± 216 \ \mu g/ml$</td>
<td>$c_i = 637 ± 60 \ \mu g/ml$</td>
</tr>
<tr>
<td>$\bar{Sh}_{d_h} = 11.6 ± 2.3$</td>
<td>$\bar{Sh}_{d_h} = 8.64 ± 1.73$</td>
</tr>
<tr>
<td>$L = 167 ± 71 \ \mu m$</td>
<td>$L = 114 ± 48 \ \mu m$</td>
</tr>
</tbody>
</table>

Table 4.2: Reservoir Opening Size Calculations

Examining Figure 4.2, we note that in our flow regime ($x^* \sim 0.01; \overline{Nu}_{d_h} \sim O(10)$), the results for $L$ are relatively insensitive to changes in the dimensionless development length (which is affected by the Reynold's number, Schmidt number, and channel height). Also, from equation (4.3) we see that $L$ is directly proportional to the experimental determined value for $c_i$. However, for an order of magnitude change in $x^*$, the Nusselt number only changes by approximately a factor of 2. Over the course of a typical perfusion experiment, the flowrate in bovine eyes will change by a factor of 2 to 3 due to washout. Facility in human eyes does not change appreciably during an experiment, but initial flowrate can vary by a factor of 2 to 3 between specimens. An important implication of this result is that, for our flow regime, the Sherwood number is found to vary only slightly with the flowrate variations. The mass transfer coefficient will therefore only vary slightly, and the bulk protein concentration of the perfusate will vary directly with the flowrate. This result
adds support to the idea that protein concentration is simply a function of facility and the
time history of volume perfused.

**Protein Depot Length Scale Analysis**

A second test of consistency between the experimental data of the previous chapter
and the model of protein entering the perfused fluid from the ciliary body was an analysis
of the depot dimensions that would be required to deliver protein at the measured deple-
tion rates. From the experimental data, we were able to determine how much protein
would be collected if the depots were perfused until completely depleted. Using this infor-
mation, the approximate length scales of the depots were calculated.

*Amount of Protein Removed in Experiments:*

In the experimental results of Chapter 3, the protein concentration of the perfusate was
correlated with the volume of fluid perfused using equation (3.1). An estimate of the total
mass of protein removed from the depot during an experiment was determined by integrat-
ing this equation with respect to volume of fluid perfused, \( V_p \):

\[
M_{out} = \int_0^{V_p} c_i \exp \left( -\frac{V_p'}{V_0} \right) dV_p' = c_i V_0 \left( 1 - \exp \left( -\frac{V_p}{V_0} \right) \right) \quad (4.10)
\]

If the perfusion volume is much larger than the volume constant \( V_0 \), the amount of protein
removed during an experiment approaches a limit given by:

\[
M_{out} \bigg|_{max} = c_i V_0 \quad (4.11)
\]

This value represents the total capacity of the protein depot.
Amount of Protein Available in the Depot:

An order-of-magnitude estimate of the total protein available in the protein depot was found from scaling analysis. For our conceptual model of a depot depleting radially (r-direction in Figure 4.1) from a small exposed surface, the total protein available scales as follows:

\[
M_{out} \bigg|_{max} \sim c_0 w \left( \frac{\pi}{2} H^2 \right)
\]  

(4.12)

where \( c_0 = 42.0 \times 10^3 \) is the initial protein concentration within the depot [Barsotti, 1990], \( w \) is the circumference of the iris, and \( H \) is the length scale. Rearranging and substituting equation (4.11), we find an expression for the length scale in terms of known parameters:

\[
H \sim \sqrt{\frac{c_i}{c_0} \cdot \frac{2V_0}{\pi w}}
\]  

(4.13)

Length scales for bovine and human eyes were determined using the results from Chapter 3 calculated with the NIH method, and are tabulated below. In both cases, the length scale is consistent with the actual size of the ciliary body, and is thus consistent with our conceptual model of the protein depot. Also, we find that the length scale required to deliver protein at the measured depletion rates is an order of magnitude larger than the length of the surface exposed to flow, \( L \). This is consistent with the model used by Barsotti [1992], and is consistent with our assumption of radial depletion from the surface into the body of the depot. Note that for human eyes, the length scale, \( H \), is more accurately a measure of the depth of depletion instead of the total size of the depot (since significant depletion does not occur). Also, for bovine eyes, protein diffusion into the anterior chamber may occur along the entire surface of the iris, and not just the iris root. However, we assumed that the
iris depleted relatively quickly in comparison with the ciliary body, so our scaling analysis should still be valid.

<table>
<thead>
<tr>
<th>Human</th>
<th>Bovine</th>
</tr>
</thead>
<tbody>
<tr>
<td>( w = 3.1 \pm 0.3 \text{ cm} )</td>
<td>( w = 5.5 \pm 0.5 \text{ cm} )</td>
</tr>
<tr>
<td>( V_0 = 5.73 \pm 2.16 \text{ ml} )</td>
<td>( V_0 = 10.03 \pm 1.80 \text{ ml} )</td>
</tr>
<tr>
<td>( c_i = 1976 \pm 216 \text{ ( \mu \text{g/ml} )} )</td>
<td>( c_i = 637 \pm 60 \text{ ( \mu \text{g/ml} )} )</td>
</tr>
<tr>
<td>( H = 2.35 \pm 0.98 \text{ mm} )</td>
<td>( H = 1.32 \pm 0.33 \text{ mm} )</td>
</tr>
</tbody>
</table>

Table 4.3: Protein Depot Length Scale Calculation

**Diffusion Coefficient Within the Protein Depot**

The final validity check for our model was the comparison of the protein diffusivity required by the reservoir to deliver the experimentally measured protein levels with known protein diffusivities for connective tissue.

For our scaling analysis of the diffusion coefficient, we wanted to find a characteristic length that protein molecules would have to diffuse from the body of the protein depot to the surface exposed to fluid flow. For our case of radial depletion, in which protein molecules diffuse inwards towards the surface opening (instead of radially outwards), modeling of the length scale is difficult to perform explicitly. However, we can model the surface as a region of zero protein concentration which diffuses outwards into the body of the reservoir. A characteristic length scale for diffusion of this zero concentration region comes from consideration of the Brownian motion of the molecules as represented by the random walk model [Probstein, 1994]. In cylindrical coordinates, the probability that a particle will move from radius \( r \) to \( r + dr \) in time \( t \) is given by the Gaussian distribution:
\[ P(r, t) = \frac{1}{2D_r t} \exp \left( -\frac{r^2}{4D_r t} \right) r dr \]  

(4.14)

Since positive and negative displacements are equally probable, the mean displacement will always be zero. A more useful measure is the mean square displacement, defined as:

\[ \langle r^2 \rangle = \int_0^\infty r^2 P(r, t) \, dr = \frac{1}{2D_r t} \int_0^\infty r^3 \exp \left( -\frac{r^2}{4D_r t} \right) \, dr \]  

(4.15)

Evaluating this integral and taking the square root gives us our characteristic length scale:

\[ H \sim \langle r^2 \rangle^\frac{1}{2} = \sqrt{4D_r t} \]  

(4.16)

and rearranging gives us an expression for the diffusion coefficient:

\[ D_r = \frac{H^2}{4t} \]  

(4.17)

Since we were only interested in an order-of-magnitude estimate of the diffusion coefficient as a consistency check, we only needed to determine a characteristic time:

\[ t \sim \frac{V_0}{Q} \]  

(4.18)

where \( V_0 \) is again the volume constant from the experimentally determined relationship between perfusate protein concentration and volume of fluid perfused (Chapter 3), and \( Q \) is a typical flowrate for an experiment. Also, the length scale was taken from the calculations of the previous section. Using parameters from Chapter 3, the diffusion coefficients were calculated for human and bovine eyes. Modeling the protein as albumin, the ratio of the calculated diffusivities to the diffusivity of albumin in water, \( D_r/D_0 \), was then deter-
mired. The diffusivity of albumin in water was determined by Laurent et al., [1961] as 

\[ D_0 = 6.0 \times 10^{-7} \text{ cm}^2/\text{s} \]

<table>
<thead>
<tr>
<th>Human</th>
<th>Bovine</th>
</tr>
</thead>
<tbody>
<tr>
<td>( Q = 3.0 \pm 1.0 \text{ \mu l/min} )</td>
<td>( Q = 30 \pm 10 \text{ \mu l/min} )</td>
</tr>
<tr>
<td>( V_0 = 5.73 \pm 2.16 \text{ ml} )</td>
<td>( V_0 = 10.03 \pm 1.80 \text{ ml} )</td>
</tr>
<tr>
<td>( H = 2.35 \pm 0.69 \text{ mm} )</td>
<td>( H = 1.32 \pm 0.25 \text{ mm} )</td>
</tr>
<tr>
<td>( D_r = 1.21 \times 10^{-7} \pm 0.79 \times 10^{-7} \text{ cm}^2/\text{s} )</td>
<td>( D_r = 2.19 \times 10^{-7} \pm 1.02 \times 10^{-7} \text{ cm}^2/\text{s} )</td>
</tr>
<tr>
<td>( D_r/D_0 = 0.202 \pm 0.132 )</td>
<td>( D_r/D_0 = 0.365 \pm 0.169 )</td>
</tr>
</tbody>
</table>

**Table 4.4: Diffusion Coefficient Calculation**

Although there is significant uncertainty associated with our order-of-magnitude calculations, the mean values for \( D_r/D_0 \) are consistent with the values found in the literature of \( D/D_0 \sim 0.1 \) to 0.25 [Bert and Pearce, 1984]. This provides another indication that our model of the ciliary body as the source of the proteins in the perfusate is consistent with the experimental data.

**Summary**

In this section, we performed several checks for consistency between the experimental data collected in Chapter 3 and our conceptual model of a depleting protein depot within the outflow pathway. The parameters calculated (depot surface length exposed to flow, length scale of depot body, and protein diffusivity in the depot) have added support to the idea that the protein depot arises from the ciliary body. This is consistent with the idea that protein concentration in the perfusate is a function of the history of perfusion, and decreases as the protein depot becomes depleted.
In the analysis of the protein source opening size ($L$), the sensitivity of the mass transfer coefficient to flowrate variations was discussed. It was found that Sherwood number, and hence the mass transfer coefficient, was relatively insensitive to changes in flowrate for the flow regime typically encountered in our experiments. Therefore, bulk protein concentration in the perfusate would vary almost directly with flowrate. This is consistent with our hypothesis that perfusate protein concentration is controlled by facility, and not vice versa.

4.2 Resistance Due To Proteins In The Trabecular Meshwork

In this section we returned to our original hypothesis that aqueous humor proteins generate resistance as they flow through a gel matrix in the JCT (and perhaps elsewhere in the trabecular meshwork), and outflow facility is determined by the aqueous humor protein concentration. From the experimental results of Chapter 3, a statistically significant relationship was found between aqueous outflow resistance and protein concentration in the perfusate, for bovine eyes. However, the initial protein concentration of the perfusate from bovine eyes was much less than the 15% serum solution required by Johnson et al. [1993] to prevent washout. This was much lower than what was expected to be required in order to generate significant outflow resistance. In human eyes, a marginally significant correlation was found between initial outflow resistance and initial protein concentration. In contrast to bovine eyes, however, the initial protein concentration in humans was comparable to the 15% serum used by Johnson et al. [1993], as well as the 5% serum solution used by Kee et al. [1995] to reduce washout in monkey eyes. Protein concentration in human eyes was also found to decay very slowly. Our goal in this section was to determine if the measured protein levels was sufficient to generate the observed outflow resistance in human, and to verify that this was not the case in bovine eyes.
Assuming that resistance is controlled by protein concentration, we used the experimental relationships between outflow resistance and protein concentration to estimate the fraction of the outflow resistance that was due to proteins. Using a model of albumin flow through a hyaluronic acid matrix developed previously [Kim et al., 1991], we estimated the length of the matrix that would be required to generate the observed resistance. In addition, we examined the fraction of the outflow resistance due to the matrix itself.

Porous Media Theory

Aqueous humor proteins may generate resistance as they flow through a gel matrix, which is believed to exist within the JCT [Ethier et al., 1986], and possibly elsewhere in the trabecular meshwork. The matrix can be modeled as a porous medium, and flow through a porous medium is described by Darcy's law. For flow through the aqueous outflow pathway, pressure gradients are assumed to be 1-dimensional [Ethier et al., 1986], and the flow resistance is found to be:

\[ R = \frac{\mu L_m}{KA} \]  

(4.19)

where \( L_m \) is the length of the porous matrix, \( A \) is the cross-sectional area available to flow, and \( \Delta P = RQ \) is the pressure drop across a region with resistance \( R \). From the experiments of Chapter 3, an empirical expression for the resistance as a function of protein concentration was deduced. Our task in modeling the effect of proteins on resistance was to relate the empirical resistance to a theoretical model for resistance, and thus determine the length the matrix, \( L_m \).

Permeability of Proteins in the JCT Matrix

The resistance of a porous medium is characterized by its permeability, \( K \). For our analysis, we needed to determine the permeability of aqueous humor proteins passing
through the extracellular matrix gel of the JCT. Since we were interested in examining the
effect of bulk protein concentrations on resistance we modeled the aqueous humor pro-
teins as behaving like albumin, which comprises approximately 78% of total aqueous
humor proteins in humans and 59% in bovine [Francois et al., 1958]. Albumin within the
aqueous humor will generate additional resistance when their velocity is different than
that of the saline vehicle. For our analysis, we used the model of Kim et al. [1991] who
calculated the permeability of a bovine serum albumin (BSA) solution as it flowed
through a matrix of the glycosaminoglycan hyaluronic acid. For a homogeneously distrib-
uted gel and solute, and using the GAG matrix as a fixed reference frame, the total perme-
ability is determined as:

\[
\frac{1}{K_{net}} = \frac{1}{K_m} + \frac{g(\Phi_m)}{K_a}
\]  

(4.20)

where \( K_m \) is the permeability of the GAG matrix, \( K_a \) is the permeability of a fixed albumin
matrix, and \( g(\Phi_m) \) is a hindrance function that measures the effect of the change in veloc-
ity from a free albumin particle to one that is hindered by the GAG matrix at a solid frac-
tion \( \Phi_m \). Each of these components are described below.

It is useful to note here that the component of permeability due to the albumin will
vary as the protein concentration varies. On the other hand, the portion due to the GAG
matrix is assumed to be constant since the concentration of GAGs in the outflow pathway
was assumed to be essentially constant. When the protein depot is completely depleted in
a perfusion experiment, the outflow resistance will be reduced to the portion due to the
matrix alone.
*Permeability of the Matrix, $K_m$:

We modeled the gel matrix as behaving similar to hyaluronic acid, which is a long chain glycosaminoglycan ubiquitous in the intercellular spaces of connective tissue. Its role in generating resistance to fluid flow has been of considerable general interest for a variety of reasons, such as maintenance of the compressive stiffness of tissues, and regulation of flow of specialized fluids [Levick, 1987]. Our interest lies in the role that hyaluronic acid plays in determining the aqueous outflow resistance of the trabecular meshwork, and in particular the JCT.

Ethier [1986] has reviewed the literature concerning flow through highly porous media and found that the model of Spielman and Goren [1968] best fit the available data. Ethier [1986] developed a simplified approximation for their model, given as:

$$K_m = 0.319a^2\phi^{-1.17} \text{ [cm}^2\text{]}$$ (4.21)

where $a$ is the fiber radius, and $\phi = (1 - \text{porosity})$ is the solid fraction. Using this expression, Ethier et al. [1986] determined the thickness and concentration of the GAG layer required in the JCT to generate the measured resistance. Assuming a resistance of 2.5 mmHg/μl/min ($\Delta P = 15$ mmHg, $Q = 2$ μl/min), a JCT length ranging from 10 to 25 μm, and a porosity ranging from 0.2 to 0.7, required GAG concentrations were calculated. It was also shown that there was significant overlap between actual GAG concentrations (based on bulk concentration measurements, corrected for porosity) and predicted GAG concentrations. This suggested the possibility that the matrix alone could account for the experimentally measured outflow resistance in human eyes. However, the predicted values of GAG concentration spanned an order of magnitude (from 0.5 mg/ml to 5 mg/ml) leaving significant room for interpretation.
From sedimentation studies, Ethier [1986] found the best fit relationship for permeability to be:

\[ K_m = 2.92 \times 10^{-16} c_m^{-1.47} \text{[cm}^2\text{]} \]  \hspace{1cm} (4.22)

where the GAG concentration in [g/ml], \(c_m\), is found from \(c_m = \rho \phi\), and \(\rho\) is the molecule density. The discrepancy between the form of equation (4.22) and equation (4.21) was investigated by Johnson et al. [1987], who found that three separate concentration regimes (dilute, semi-dilute, and homogeneous) produced three different relationships for permeability. The fiber matrix model used by Ethier et al. [1986] was expected to only apply in only the homogeneous regime. The GAG concentrations used in our analysis of the GAG matrix length (calculated below) were also typically lower than those used by Ethier et al. [1986]. Using \(\rho = 1.5\) g/ml for hyaluronic acid [Ethier, 1986] our situation is more accurately described by the empirical sedimentation equation (4.22), which applies to the semi-dilute regime (O(\(\phi\)) \(\sim\) \(10^{-4}\)).

Permeability of Bovine Serum Albumin, \(K_a\):

Kim et al. [1991] have examined the issue of calculating the permeability of a matrix of bovine serum albumin (BSA). Modeling the ellipsoidal albumin particle as a rigid sphere with an equivalent hydrodynamic radius, a semi-empirical relation was found for the permeability of a BSA solution:

\[ K_a = 1.60 \times 10^{-14} c_a^{-1} (1 - 1.348 c_a) \]  \hspace{1cm} (5.1) [cm\(^2\)]

where \(c_a\) is the concentration of albumin in [g/ml]. Assuming that the concentration of protein is relatively low (O(\(c_a\)) \(\sim\) 0.001 g/ml), we can simplify the permeability expression to:

90
\[ K_a = 1.60 \times 10^{-14} c_a^{-1} \text{ [cm}^2\text{]} \]  

(4.24)

The portion of the resistance due to albumin will thus be directly proportional to the concentration.

*Hindrance Function, \( g(\Phi_m) \):*

In a solution of macromolecules such as albumin, a difference in speed between the fluid vehicle (in our case, saline) and the particles will manifest itself as a flow resistance. For a solution in which there are no obstacles to flow, the only motion of the particles relative to the vehicle will be due to the random motions of diffusion. Since diffusion will occur in all directions, there is zero net effect on flow resistance. However, if the solution must flow through a fixed matrix, such as a GAG solution, then the passage of the particles will be hindered, generating additional flow resistance. The hindrance function, \( g(\Phi_m) \) is a measure of the hindrance of the particles in solution relative to the saline vehicle due to the presence of the matrix, and \( \Phi_m \) is the volume fraction of the matrix [Kim et al., 1991].

Laurent and Pietruszkiewicz [1961] and Laurent et al. [1963] determined an empirical relationship between the sedimentation rates of compact macromolecules and GAG concentrations. Expressed as the ratio of the sedimentation speed in GAG solutions, \( S \), and the sedimentation speed in buffer, \( S_0 \), the relationship was shown to be:

\[
\frac{S}{S_0} = A \exp \left( -B c_m^{1/2} \right) \]  

(4.25)

where \( A \) is a constant depending on the solute, \( B \) is a constant depending on the diameter of the particles, and \( c_m \) is the concentration of the particles in [g/ml]. This equation was found to be valid for a wide variety of particles, from albumin to polystyrene latex spheres. For bovine serum albumin, the coefficients were found to be \( A = 1.03 \) and \( B = \)
6.05 [ml/g]^{1/2}. As well, for a few selected substances (including bovine serum albumin) this equation was found to be valid for determining the ratio of hindered and free diffusion coefficients, $D/D_0$. This gives us our definition for the hindrance function, $g(\Phi_m)$:

$$g(\Phi_m) = 1 - \frac{D}{D_0}$$  (4.26)

While this relationship was empirically based, Ogston et al. [1973] were able to show that a stochastic model based on steric hindrance of particles in hyaluronic acid could produce a relationship of the same form. This implies that equation (4.25) does not account for any molecular interactions which may be more complex than steric factors. While this is sufficient to account for the effect of bulk protein concentration, this model cannot account for the interactions of specific proteins.

**Combined Permeability, $K_{net}$:**

Substituting equations (4.22), (4.24), and (4.26) into equation (4.20) gives us the expression for the combined permeability of albumin flowing through a hyaluronic acid matrix:

$$\frac{1}{K_{net}} = \frac{c_m^{1.47}}{2.92 \times 10^{-16}} + \frac{(1 - \frac{D}{D_0})c_a}{1.60 \times 10^{-14}} \text{ [1/cm}^2\text{]}$$  (4.27)

where $c_m$ is the concentration of hyaluronic acid in [g/ml], and $c_a$ is the concentration of albumin in [g/ml].

**Calculation of GAG Matrix Length**

Based on experimental results, Johnson et al. [1993] estimated that the washout effect in bovine eyes reduced the outflow resistance by about half. Assuming that this decrease was due to depletion of the protein depot, the component of the resistance due to proteins
could be estimated. Using the model outlined above, the length of the GAG matrix required to generate half of the total outflow resistance could be estimated based on the protein concentration required to eliminate the washout effect (15% serum).

With the additional data gathered in Chapter 3, we used a more generalized method of modeling the resistance contribution of proteins. This allowed us to model the resistance of proteins in the human eye as well, using the initial outflow resistance vs. initial protein concentration correlation. Substituting equation (4.27) into Darcy's law (in the form of equation (4.19)) gives us and expression for the overall outflow resistance:

$$ R = \frac{1}{K_{net}} \frac{\mu L_m}{A} = \lambda_1 + \lambda_2 \left( 1 - \frac{D}{D_0} \right) c_a \ [g/(cm^4 \text{ sec})] \quad (4.28) $$

where $\lambda_1$ and $\lambda_2$ are constants given by:

$$ \lambda_1 = \frac{\mu L_m}{A} \cdot \frac{c_m^{1.47}}{2.92 \times 10^{-16}} \ [g/(cm^4 \text{ sec})] \quad (4.29) $$

and,

$$ \lambda_2 = \frac{\mu L_m}{A} \left( 1.60 \times 10^{-14} \right)^{-1} \ [1/(cm \text{ sec})] \quad (4.30) $$

with $\mu$ given in poise, $L_m$ in cm, $A$ in cm$^2$, $c_m$ in g/ml, and $c_a$ in g/ml (empirical constants are given in appropriate cgs units). Outflow resistance is thus expressed as a linear function of albumin concentration.

In the previous chapter, a relationship between outflow resistance and protein concentration was found with the same linear form, for both bovine and human eyes:

$$ \frac{1}{C} = b_1 + b_2 c_p \ [g/(cm^4 \text{ sec})] \quad (4.31) $$
Assuming that the aqueous humor proteins can be modeled as albumin, the portion of the resistance due to GAGs, and the portion of the resistance due to proteins in equations (4.28) and (4.31) could be equated to give:

\[ b_1 = \frac{\mu L_m}{A} \cdot \frac{c_m^{1.47}}{2.92 \times 10^{-16}} \text{ [g/(cm}^4 \text{ sec)]} \tag{4.32} \]

\[ b_2 = \frac{\mu L_m}{A} \left(1.60 \times 10^{-14}\right)^{-1} \left(1 - \frac{D}{D_0}\right)^{-1} \text{ [1/(cm sec)]} \tag{4.33} \]

A semi-empirical expression for length of the GAG matrix required to generate the experimentally measured resistance could then be calculated from equation (4.32) to give:

\[ L_m = 2.92 \times 10^{-16} \cdot \frac{b_1 A}{\mu c_m^{1.47}} \text{ [cm]} \tag{4.34} \]

Rearranging equation (4.33) gives an alternate expression for the length of the GAG matrix:

\[ L_m = \left(1 - \frac{D}{D_0}\right)^{\mu} \frac{1.60 \times 10^{-14} A b_2}{\mu} \text{ [cm]} \tag{4.35} \]

where all parameters are in cgs units.

**Human GAG Matrix:**

For the calculation of the matrix length in human eyes, a porosity of \( \phi = 0.5 \) was assumed following Johnson et al. [1993]. Assuming the same cross-sectional area as the JCT, the total matrix area was 0.11 cm\(^2\) [Ethier et al., 1986], and the cross-sectional area available to flow was thus \( A = 0.055 \text{ cm}^2 \). The viscosity of the saline vehicle at 34 °C was
\( \mu = 0.74 \) centipoise [Johnson et al., 1993]. The coefficient \( b_2 \) was taken from the correlation between initial outflow resistance and initial protein concentration:

\[
b_2 = 1.296 \times 10^{11} \pm 0.672 \times 10^{11} \ [1/(\text{cm sec})]
\]

Substitution into equation (4.35) produced an expression for the matrix length:

\[
L_m = \frac{154.1 \pm 79.9}{\left(1 - \frac{D}{D_0}\right)} \ [\mu\text{m}] \tag{4.36}
\]

Substitution of the ratio of protein diffusivity in the matrix to diffusivity in water into the above expression gave us an estimate for the matrix length. Values for \( D/D_0 \) were estimated from the typical values for connective tissue, which are about 0.25 [Bert and Pearce, 1984]. Substituting this value into the above equation, we found a GAG matrix length of approximately \( 205 \pm 106 \) \( \mu\text{m} \). While this was much longer than the JCT length of 25 \( \mu\text{m} \) [Ethier et al., 1986], it was about the same order of magnitude as the length of the entire trabecular meshwork [McMenamin et al., 1986]. Thus, if the entire trabecular meshwork were filled with a GAG gel, we would not be able to rule out the possibility that the relationship between outflow resistance and protein concentration was due to the bulk protein concentration and the hindrance mechanism described above.

However, a statistically significant value for the coefficient \( b_1 \) was not found for human eyes (so we were unable to calculate the matrix length based on equation (4.34)). This suggests that the portion of the outflow resistance due to the matrix alone was not significant, which would be the case if the GAG matrix layer were very dilute, or preferential flow channels through the matrix existed. The implication of this result is that the ratio \( D/D_0 \) may be very close to unity, and the GAG matrix length would have to be much
longer than the trabecular meshwork itself in order for aqueous humor proteins to generate the experimentally observed resistance.

**Bovine GAG Matrix:**

For the calculation of the matrix length in bovine eyes, a porosity of $\phi = 0.5$ was assumed again. Assuming a matrix area equivalent to the JCT area of 0.25 cm$^2$ [Johnson et al., 1990], the cross-sectional available to flow was thus $A = 0.125$ cm$^2$. The viscosity of the saline vehicle at 34 °C again was $\mu = 0.74$ centipoise [Johnson et al., 1993]. The coefficient $b_2$ from the NIH method was used, and the results are summarized below:

$$b_2 = 5.216 \times 10^{10} \pm 0.730 \times 10^{10} \text{ [1/(cm sec)]}$$

$$L_m = \frac{141.0 \pm 19.7 \text{ [\mu m]}}{\left(1 - \frac{D}{D_0}\right)} \quad (4.37)$$

As with human eyes, the protein diffusivity was estimated to be the same as connective tissue giving $D/D_0 = 0.25$ [Bert and Pearce, 1984]. Using this value, the required GAG matrix length was found to be the same order-of-magnitude (within the range of error) as the entire bovine trabecular meshwork length of about 50 µm [Johnson et al., 1990]. Thus, as with the human eyes, we could not rule out the possibility that aqueous humor proteins control outflow facility, based on this relationship alone.

However, for the bovine eyes, a statistically significant value was also found for the coefficient $b_1$, where:

$$b_1 = 1.760 \times 10^7 \pm 0.368 \times 10^7 \text{ [(cm}^4 \text{ sec)/g]}$$

Using equation (4.34), we were able to find a second expression for the matrix length:
\[
L_m = \frac{8.68 \times 10^{-4} \pm 1.81 \times 10^{-4}}{c_m^{1.47}} \text{ [\mu m]}
\]

which was necessarily consistent with the expression for length given by equation (4.37), since they are not independent equations. As well, the ratio \(D/D_0\) for albumin could be calculated from equation (4.25):

\[
\frac{D}{D_0} = 1.03 e^{-6.05c_m^{0.5}}
\]

Substituting into equation (4.37), and equating with (4.38), gave an expression which could be solved for the GAG matrix concentration, \(c_m\):

\[
\frac{141.0 \pm 19.7}{1 - 1.03 e^{-6.05c_m^{0.5}}} = \frac{8.68 \times 10^{-4} \pm 1.81 \times 10^{-4}}{c_m^{1.47}}
\]

Solving equation (4.40), a GAG concentration of \(c_m = 0.017 \pm 0.006 \text{ mg/ml}\) was found. Substitution back into (4.39) gave a value of \(D/D_0\) approaching unity, and substitution into equation (4.38) gave \(L_m = 8579 \pm 4817 \text{ \mu m}\). Thus, in order for the results from the outflow resistance vs. protein concentration to be self-consistent, a matrix length far longer than the entire trabecular meshwork would be required. Thus, our model is not consistent with the physiology of the bovine eye, and outflow resistance is likely not due to the resistance generated by the steric hindrance of aqueous humor proteins.

4.3 Summary and Conclusions

Our goal in this chapter was to help clarify some of the results from our protein experiments of the previous chapter. In particular, we wanted to examine more closely the relationship between outflow resistance and perfusate protein concentration. We did this by
exploring the possibility that protein concentration was a function of outflow resistance (and hence flowrate), and the time history of the perfusion experiment. We also determined if the relationship could be explained using an existing model of protein hindrance and reasonable physiological parameters.

The source of proteins entering the fluid passing into the trabecular meshwork was modeled as a reservoir with a small opening exposed to the aqueous humor flow, depleting due to protein diffusion into the stream. The reservoir was assumed to be the ciliary body. The experimental data was then analyzed using this model to check for consistency with physiologic parameters. All three of our criteria (opening size, depot length scale, and protein diffusivity in the depot) indicated that the data was consistent with our conceptual model. This analysis confirmed the possibility that protein concentration could simply be a function of the time history of the experiment. As the protein depot became depleted over the course of the experiment, the bulk protein concentration of the perfusate decreased as well.

In our calculation of the mass transfer coefficient, we found that for our flow regime, the mass flux rate would vary only slightly with changing flowrates (within the range typically seen in our experiments). Bulk protein concentration in the perfusate thus appears to be a function of the volume flowrate of fluid during the perfusion. This implies that the high initial protein concentration of the perfusate from human eyes, compared with bovine eyes, may simply be due to a lower flowrate. This would also explain the correlation between initial outflow resistance and the initial protein concentration in human eyes.

Modeling of the resistance generated by aqueous humor proteins indicated that the relationship between outflow resistance and protein concentration (in bovine eyes) could not be explained using our protein hindrance model. An unrealistic matrix length would be required in order to generate the resistance measured. It is interesting to note that this
contradicts the conclusions of Johnson et al. [1993]. However, it was assumed in that study that protein levels in the trabecular meshwork were approximately equal to 15% serum, since perfusion with 15% serum solutions essentially eliminates washout. The experimental results used in our modeling study indicated that actual protein concentrations were far lower (about 1% serum). Thus, it is not surprising that the use of the same model of protein hindrance could not explain the correlation between outflow resistance and protein concentration.

For human eyes, the lack of a significant outflow resistance component due to the matrix alone suggested that the GAG concentration was very low. Assuming that this was the case, the protein hindrance model required a matrix length longer than the entire trabecular meshwork in order to generate the measured outflow resistance. A more likely explanation, based on our analysis of the mass transfer coefficient, was presented above. Our results suggest that outflow resistance is controlled by some mechanism other than the one examined here, and initial protein concentration variations between specimens are just a consequence of flowrate differences.

While the work presented so far has suggested explanations for the observed experimental results, the mechanism behind outflow resistance (and washout in bovine) has not be elucidated. One possibility is that instead of the bulk protein concentrations being responsible for controlling outflow resistance, it may be controlled by a single protein or combination of proteins. Its mechanism for generating resistance would likely involve molecular interactions more complex than the simple steric hindrance investigated here. If this component depleted at a rate different than the rate of depletion of the bulk proteins, this concept would be consistent with the experimental results.

Previous research has provided evidence supporting the idea of a specific resistance causing aqueous humor protein. Johnson et al. [1986] investigated the blockage of
microporous filters with pore sizes (0.2 μm) similar to the inner wall. They found that blockage occurred during perfusions with aqueous humor, but not with serum solutions with the same bulk protein concentration. The cause of blockage was apparently due to two aqueous components, which they described as "bricks and mortar". Ethier et al. [1989] determined the "bricks" to be albumin, while the "mortar" was some macromolecule which binds hydrophobically to the filter surface as well as to the albumin particles. Thus, the resistance across the filter face was found to be dependent on protein concentration, but required the presence of a key component of aqueous humor to bind them together. This was consistent with the results of Ritter [1993] who found that albumin alone, in the same concentration as 15% serum, could not prevent washout. Also, Pavao et al. [1989] identified two components of aqueous humor that were either not present in serum, or were present in much lower quantities. If these components represent the "mortar" molecules, then serum must be perfused in much higher bulk concentrations than the perfusate from our protein studies in order to generate the same resistance. This would explain why the bulk soluble protein concentration of bovine perfusate was much lower than 15% serum.

Another possible explanation for the experimental results of Chapter 3 is that fluid funneling [Johnson et al., 1992] through the JCT, toward the pores of the inner wall of Schlemm's canal, enhances the resistance generated by the proteins. The "baseline" facility would then be governed by the inner wall pores, but variations in bulk protein concentration of the aqueous humor could result in significant changes in facility, including the washout effect. In the next chapter, we report on our investigations of the relationship between pores of the inner wall and aqueous outflow facility.
Chapter 5

Pore Density and Its Relationship to Aqueous Outflow Facility

In Chapter 1, we discussed the possibility that hydrodynamic interactions between the JCT and the pores of the inner wall could greatly enhance the outflow resistance of the JCT. Johnson et al. [1992] numerically modeled this region of the outflow system and found that fluid may be preferentially "funneled" through the JCT towards the pores. This would reduce the cross-sectional area available for flow within the JCT, enhancing the outflow resistance. Thus, even though the pores themselves would generate little resistance [Bill and Svedbergh, 1972; Moseley et al., 1983], they may be able to modulate the outflow resistance through the funneling effect. However, several limitations of the model were noted, including the possible existence of preferential flow channels within the JCT, and the possibility that fluid was funneled to the much larger vacuoles, instead of pores. Either of these situations would greatly reduce the resistance enhancement factor of the funneling effect.

Recent work by Allingham et al. [1992] found a linear relationship between pore density and aqueous outflow facility, providing support for the funneling model. However, a major concern about this study was the use of constant pressure perfusions (with changing flowrates). It is widely believed that the pores open in response to a transmural pressure difference generated by fluid flow across the inner wall [Grierson and Lee, 1978]. Thus, Allingham et al. [1992] left open the possibility that pores opened passively in response to changing flowrates, and the resistances were controlled elsewhere in the eye. Pore density would therefore be a function of facility, and not vice versa. Our goal was to verify the results of Allingham et al. [1992] using constant flow perfusions.
5.1 Experimental Methods

In this study, a series of experiments were performed in which eyes were perfused at a constant flowrate, fixed at the pressure achieved during the constant flow perfusion, and examined using SEM for inner wall pore density. The perfusion flowrate was selected to match the normal aqueous humor flowrate in humans of 2 μl/min [Cole, 1974]. Also, the experimental protocol was designed to ensure that the pressure within the anterior chamber did not vary significantly from the desired pressure during the fixation process. A total of n=17 (9 pairs, minus one eye) normal human eyes were utilized. A single set (n=2) of glaucomatous eyes was used, and a modified experimental protocol was used for one of the pair.

Experimental Protocol

Perfusion Procedure:

Each eye had a perfusion needle and an exchange needle inserted as described in Appendix A, with the associated fluid reservoirs attached. After perfusing from reservoir for approximately 10 minutes at 10 mmHg to reduce the filling time, the eyes were perfused at the constant flowrate of 2 μl/min from syringe pumps for 60 to 90 minutes, or until a steady state pressure was achieved. The eyes were then fixed at the constant pressure produced under constant flow conditions. Since fixation reduces facility, this change to constant pressure perfusions meant that the initial 2 μl/min flowrate would only occur at the start of fixation. However, a variation in flowrate was preferable to the high non-physiologic pressures that would occur with constant flow if the facility was significantly reduced by the fixation process. Also, the effect of fixation on facility was roughly equal for all the eyes, so the post-fixation flowrates were comparable.
Fixation Procedure:

The first step in the fixation process required exchanging the anterior chambers with modified Karnovsky's fixative (2.5% glutaraldehyde, 2.0% paraformaldehyde in phosphate buffer, pH 7.3 - see Appendix A). The exchange was performed by placing the perfusion reservoir 2 cmH₂O above the steady state pressure level, and placing the exchange reservoir 2 cmH₂O below the steady state pressure level. This ensured that the average pressure in the eye was never more than 2 cmH₂O (about 1.5 mmHg) away from the desired pressure, and thus reduced the possibility of pressure dependent changes occurring during fixation. Both reservoir lines were then opened to allow rapid exchange of the anterior chamber. Approximately 2 ml of fluid needed to be exchanged to thoroughly flush the anterior chamber as well as the fluid lines. A small amount (approximately 10 mg/100 ml of fixative, although the exact amount was not critical) of fluorescein sodium salt (Sigma Chemical Co., St. Louis, MO) was added to help visualize the fixative and ensure that the anterior chamber was well exchanged.

After the AC exchange, the eyes were perfused from the syringe pumps at the steady state pressure achieved during the constant flow portion of the experiment. Since the flow lines were also filled with Karnovsky's fluid, perfusion from the pumps resulted in the desired constant pressure fixation, while allowing continuous facility measurements to be recorded. In order to investigate the effect of duration and volume of fixative perfused, the constant pressure perfusion of fixative was continued for a minimum of 60 minutes (to ensure complete fixation of the outflow pathway), up to 700 minutes on some eyes. After completion of the constant pressure fixation, the eyes were cut open well behind the limbus and immersed in fixative for at least 12 hours.
Pore Counting Procedure

Pore counts were performed by F. Coloma and Prof. C.R. Ethier of the Department of Mechanical Engineering, University of Toronto, Toronto, Canada.

After fixation, eyes were prepared for morphology, and pores were counted following the method of Allingham et al. [1992]. The methodology is summarized here. The eyes were bisected along the equator, and the lens was removed from the anterior segment. Two meridional wedges were taken from each quadrant. Schlemm’s canal was opened by placing gentle tension on the ciliary body, which allowed access to the posterior margin of the canal, without damaging the inner wall endothelium. After the canal was opened, samples were taken consisting of iris, ciliary body, the outer trabecular meshwork, and the inner wall endothelium. Specimens were dehydrated with graded alcohols, critical point dried, mounted on stubs, sputter coated, and examined using a Hitachi S-570 SEM (Nissei Sangyo America Ltd., Division of Hitachi, Mountain View, CA).

The inner wall endothelium was photographed at X1800 using Polaroid (Cambridge, MA) 55 film. Montages of photographs were created for a section of each specimen. Unlike Allingham et al. [1992], pores were not counted directly off of the montages. Instead, probable pores were identified and rescanned at higher magnification to verify that the pores were real. Openings in the endothelium were disregarded as artifactual tears if the edges were rough, rather than smooth and regular. Since pores are typically elliptical, the major and minor axes were measured and averaged to give a “diameter”. Length measurements were calibrated using latex microspheres. Whole eye pore density and size distribution was determined by averaging the results from each quadrant.
5.2 Experimental Results

The correlations in these experiments were analyzed using two-sided Student’s t-tests, and mean values are given with standard errors where applicable. Analysis was performed using data for whole eyes only, as quadrant variations have not been found to be statistically significant in other studies [Allingham et al., 1992].

Results of Constant Flow Perfusions

Normal Eyes (Full Data Set):

A total of n=17 normal eyes were perfused and found to have a mean pore density of 1525 ± 224 pores/mm², which was comparable to the 1840 pores/mm² found by Bill and Svedbergh [1972]. Donor age ranged from 5 years to 97 years, with a mean age of 69.3 ± 7.0 years. Mean outflow facility prior to addition of fixative was 0.255 ± 0.054 (μl/min/mmHg), while the mean facility after fixation was 0.144 ± 0.046 (μl/min/mmHg). These values were found to be significantly different than each other (P<0.002).

No statistically significant relationship was found between pore density and either pre-fixation facility (P>0.52), or post-fixation facility (P>0.97). This data is shown in Figure 5.1 and Figure 5.2. Since this result was in contradiction with the findings of Allingham et al. [1992], other experimental and morphological parameters were investigated to determine a possible relationship with pore density.

From Johnson et al. [1992], a relationship between outflow facility and pore density times average pore diameter (ND) would be expected if flow funnelling were significantly enhancing resistance. However, no statistically significant correlation was found between ND and either pre-fixation facility (P>0.64) or post-fixation facility (P>0.23).

Other parameters which were investigated, but did not demonstrate a correlation with pore density included volume of buffer perfused (P>0.51), volume of fixative perfused (P>0.78), duration of fixation (P>0.52), and donor age (P>0.86). However, comparison of
pore density with the post-mortem time to the start of the experiment did reveal a statistically significant relationship between these two variables (P<0.02). Post-mortem times ranged from 8.3 to 34.3 hours, with a mean post-mortem time of 21.1 ± 2.1 hours. Using a linear regression model of the form:

\[ N = \beta_1 + \beta_2 t_{pm} \]  

(5.1)

where \( N \) is the pore density in [pores/mm²], and \( t_{pm} \) is the post-mortem time in hours produced the following parameters:

\[ \beta_1 = 2828 \pm 521 \text{ pores/mm}^2 \]

\[ \beta_2 = -61.60 \pm 23.05 \text{ [pores/mm}^2]/\text{hour} \]

This relationship is shown in Figure 5.3 along with the pore density versus post-mortem time data.

![Figure 5.1: Pore density vs. pre-fixation facility for all normal eyes (n=17, P>0.52)](image)
Figure 5.2: Pore density vs. post-fixation facility for all normal eyes (n=17, P>0.97)

Figure 5.3: Pore density vs. post-mortem time (all normal eyes, n=17, P<0.02)

These results would seem to indicate the presence of alterations in the inner wall pore density as the cells of the endothelium undergo natural post-mortem changes. In an attempt to remove this confounding factor, data from eyes with relatively short post-mor-
tem times (t<20 hours) were isolated and re-analyzed. A least squares fit using equation (5.1) produced a curve similar to that of the full data set,

$$\beta_1 = 3186 \pm 976 \text{ pores/mm}^2$$

$$\beta_2 = -95.79 \pm 70.25 \text{ [pores/mm}^2]/\text{hour}$$

but, the relationship was not statistically significant (P>0.22).

*Normal Eyes (Short Post-Mortem Time):*

A total of n=8 normal eyes were used within 20 hours post-mortem. Actual post-mortem times ranged from 8.3 to 17.8 hours with a mean time of 13.47 ± 1.28 hours. Mean age of donors was 85.2 ± 4.7 years and ranged from 65 to 97 years. With this set of eyes, we again did not find a statistically significant correlation with pore density and either pre-fixation facility (P>0.18) or post-fixation facility (P>0.31), or volume of buffer perfused (P>0.32). In addition, no statistically significant correlation was found between pore density times average pore diameter (ND) and pre-fixation facility (P>0.51) or post-fixation facility (P>0.68). However, marginally significant correlation was found with the volume of fixative perfused at pressure (P<0.09), and a significant correlation was found with the duration of fixation at pressure (P<0.03).

The pore density versus volume data was fit to a linear relationship of the form:

$$N = \alpha_1 + \alpha_2 V_p$$  \hspace{1cm} (5.2)

where $N$ is the pore density in [pores/mm$^2$], and $V_p$ is the volume of fixative perfused in [ml] and the coefficients are:

$$\alpha_1 = 1008 \pm 474 \text{ pores/mm}^2$$

$$\alpha_2 = 5219 \pm 2505 \text{ [pores/mm}^2]/\text{ml}$$
The pore density versus duration data was fit to a linear relationship of the form:

\[ N = \gamma_1 + \gamma_2 t_p \]  

(5.3)

where \( N \) is the pore density in \( \text{[pores/mm}^2\text{]} \), and \( t_p \) is the duration of fixation at pressure in minutes and the coefficients are:

\[ \gamma_1 = 923 \pm 366 \text{ pores/mm}^2 \]
\[ \gamma_2 = 6.08 \pm 2.02 \text{ [pores/mm}^2\text{]/min} \]

These relationships are shown in Figure 5.4 and Figure 5.5, along with the data for eyes with post-mortem time less than 20 hours. These results suggest the possibility of further artefactual influences on the pore density of the inner wall.

\[ \text{Figure 5.4: Pore density vs. volume perfused (t<20 hrs; n=8, P<0.09)} \]
**Figure 5.5**: Pore density vs. duration of fixation (t<20 hrs; n=8, P<0.03)

**POAG Eyes:**

For one of the pair of POAG eyes examined in this study, the pressure produced during the constant flow portion of the experiment exceeded 150 mmHg. However, due to the necessity of exchanging from reservoirs, the range of fixation pressures was limited by what could be achieved hydrostatically. In this case, the maximum pressure achieved during constant flow (steady state was not obtained) exceeded the maximum hydrostatic pressure of 64.5 mmHg possible with the existing experimental setup. The anterior chamber exchange and fixation was therefore performed at the maximum pressure of 64.5 mmHg.

Qualitative results for this eye show deviation from the correlations found above. Final pre-fixation facility was less than 0.013 (µl/min)/mmHg, and post-fixation facility was 0.011 (µl/min)/mmHg. A very low pore density (214 pores/mm²) was found, despite the fact that the post-mortem time was short (t<15 hours), and the volume of fixative perfused was comparable to other eyes (volume = 0.17 ml). However, no conclusions or speculations could be made since no other eyes were fixed using the alternate protocol.
The contralateral eye did not demonstrate any obvious deviations from the results of the normal eyes. It had a pore density of 2366 pores/mm², pre-fixation facility of 0.06 (μl/min)/mmHg, and post-fixation facility of 0.05 (μl/min)/mmHg, placing it well within the data set for normal eyes. However, this data was not included in the above analysis since we were interested in the correlations for normal eyes.

Re-Analysis of the Allingham Data

The findings of the constant flow perfusions were surprising when compared with the findings of Allingham et al. [1992] who found a highly significant correlation between pre-fixation facility and pore density. Using unpublished data from that study (herein called the Allingham data, courtesy of Prof. C.R. Ethier, University of Toronto), the results were re-analyzed in light of our data from the constant flow experiments.

The Allingham data was collected from five pairs of eyes (n=10), with three pairs normal, and two pairs POAG. Average pore density was found to be 1437 ± 173 pores/mm² for normals, and 489 ± 86 pores/mm² for POAG eyes. Post-mortem time ranged from 10 to 22 hours, and no statistically significant correlation was found with pore density (P>0.94). This data was consistent with our result that relatively fresh eyes do not demonstrate a post-mortem change in pore density.

Statistically significant correlations were found with volume of fixative perfused (P<0.02), and well as duration of fixation at pressure (P<0.02). These findings were also consistent with the findings from the constant flow perfusions. We should note that since the volume of fixative perfused is simply the integral of flowrate with respect to time, volume and duration are not independent variables. Thus, the effect of volume and time are coupled, and cannot be separated.

Correlation of pre-fixation facility with pore density yielded the highly significant relationship (P<0.0001) reported by Allingham et al. [1992]. A statistically significant
relationship was also found with post-fixation facility (P<0.02) and is shown in Figure 5.6. This is not surprising since the fixation process reduces the facility roughly by half, and so a good correlation with pre-fixation facility implies a good correlation with post-fixation facility. However, unlike the relationship between pore density and pre-fixation facility, no significant correlation was found with post-fixation facility if only the normal eyes were considered (P>0.25).

The relationships between pore density and outflow facility were not found with our constant flow data, but a possible explanation can be found in the experimental methods used in the Allingham experiments. The pre-fixation facility is defined as:

\[ C = \frac{Q}{\Delta P} \]  

(5.4)

where \( Q \) is the volume flowrate, and \( \Delta P \) is the pressure drop. We also know that the volume of fixative perfused is given approximately by \( V_p = Qt \), for fixation time at pressure, \( t_p \). For the Allingham data, perfusions were performed at constant pressure, and approximately constant fixation periods. Substituting into equation (5.4), and solving for the volume of fixative perfused:

\[ V_p = C\Delta P t_p = kC \]  

(5.5)

where \( k \) is constant. A correlation between facility and pore density is thus equivalent to a correlation between volume of fixative perfused and facility for the Allingham data. The data is shown in Figure 5.7, along with our constant flow data for reference.
Figure 5.6: Pore density vs. post-fixation facility (Allingham data)

Figure 5.7: Pore density vs. volume perfused (Allingham data)
Multivariate Correlations

If flowrate were not important factor in determining pore density, then the results of Allingham et al. [1992] could be explained using the analysis performed above. On the other hand, if flowrate is an important parameter, then the correlation between pore density and facility from the Allingham data could be explained simply as due to flowrate variations. Since our experiments were all performed at the same flowrate, we were not able to evaluate the effect of this parameter on pore density using our data alone. However, by combining the Allingham data with our data from the normal eyes with post-mortem times less than 20 hours to produce a larger data set of relatively fresh eyes (n=18), we could attempt to evaluate the importance of flowrate on pore density. More specifically, we were interested in comparing the effect of flowrate on pore density with the apparent effects of duration and volume perfused on pore density.

We first assumed that volume of fixative perfused (and not duration) is correlated with pore density. Allowing volume of fixative perfused and flowrate to be independent parameters, we assumed a pore density relation of the form:

\[ N = \eta_0 + \eta_1 V_p + \eta_2 Q \]  

(5.6)

where \( V_p \) is the volume of fixative perfused, and \( Q \) is the volume flowrate. A multivariate linear regression analysis produced the constants \( \eta_i \) (P_{overall}<0.002):

\[ \eta_0 = 283 \pm 352 \text{ pores/mm}^2 \]
\[ \eta_1 = 7131 \pm 1629 \text{ (pores/mm}^2)/\text{ml} \text{ (P<0.001)} \]
\[ \eta_2 = 84.9 \pm 117.8 \text{ (pores/mm}^2)/(\mu\text{l/min}) \text{ (P>0.48)} \]

A statistically significant correlation was found with volume, but not with flowrate. This suggested that the effect of volume of fixative perfused was much greater than the effect of
flowrate. This supported our analysis of the Allingham data which suggested that the correlation between facility and pore density was really a correlation with volume perfused.

A similar analysis was performed using duration of fixation (instead of volume) and flowrate versus pore density:

\[ N = \theta_0 + \theta_1 t_p + \theta_2 Q \]  \hspace{1cm} (5.7)

where \( t_p \) is the duration of perfusion, and \( Q \) is again the volume flowrate. A linear regression produced the following coefficients (\( P_{\text{overall}}<0.0001 \)):

\[ \theta_0 = -158 \pm 289 \text{ pores/mm}^2 \]

\[ \theta_1 = 8.14 \pm 1.19 \text{ (pores/mm}^2)/\text{min (P}<10^{-5} \text{)} \]

\[ \theta_2 = 310.6 \pm 88.7 \text{ (pores/mm}^2)/(\mu\text{l/min (P}<0.04 \text{)} \]

In this case, statistically significant correlations were found with both duration and flowrate. In contrast to the previous correlation, this suggests that the flowrate does have a significant effect on pore density, and the results of Allingham et al. [1992] were due both to flowrate variations and to fixation artefacts.

Unfortunately, we cannot determine which relationship is more valid since we cannot separate the effects of duration and volume. These two parameters are not independent variables, so using multivariate correlations would not produce meaningful results. We are therefore unable to determine which explanation for the Allingham data is more valid.

5.3 Discussion and Conclusions

The analysis of the data from our constant flow experiments, along with the re-analysis of unpublished data from Allingham et al. [1992], have indicated the lack of any correlation between pore density and either pre-fixation or post-fixation outflow facility. This suggests that pores are not facility controlling structures, but simply passive resistive ele-
ments of the outflow pathway that open and close in response to changes in flowrate and fixation conditions. This is supported by the results from the multivariate correlations with the combined data set, which suggests that flowrate may have a significant effect on pore density. As well, there is a lack of any correlation between pore density times pore diameter (ND) and outflow facility, in contradiction with the predictions of the funnelling model of Johnson et al. [1992]. It thus appears that the “funnelling effect” is not an important contributor to the generation of outflow resistance.

Post-mortem time has been identified as a likely cause of artefactual changes in the pore density. Previous investigations into post-mortem changes in the inner wall [Shabo et al., 1973] have found that the incidence of giant vacuoles appeared to increase dramatically as a function of the post-mortem time prior to fixation. However, Grierson and Johnson [1981] differentiated between true giant vacuoles and artefactual “post-mortem” vacuoles, suggesting that the density of the former decreased with post-mortem time, while the density of the latter increased. If we assume that pores are only associated with true giant vacuoles, then these findings are consistent with the results from our constant flow perfusions, in which pore density was found to decrease with post-mortem time for eyes used later than 20 hours post-mortem.

The most important parameters likely affecting pore density in typical perfusion experiments is the volume of fixative perfused and/or the duration of fixation at pressure, and possibly flowrate. The perfusion fluid used for fixation was 2.5% glutaraldehyde and 2.0% paraformaldehyde in phosphate buffer, a solution usually considered a “contact fixative”. However, the mode of action of glutaraldehyde is to cross-link proteins by binding to amino side-chains, which leaves the phospholipid bilayer of the endothelial cell membranes unaffected [Kuchel and Ralston, 1988]. The lipids in cell membranes are not fixed until the specimens are post-fixed in osmium tetroxide. As well, proteins within the cells
are not fixed until they come in contact with the cell membrane via diffusion [T. Freddo, personal communication]. The increase in pore density with volume/time of fixation, along with the finding for post-mortem time, allows us to hypothesize about a possible mechanism for pore formation.

The pore density correlations that we have found suggest that pores are passive resistors, with a finite time associated with their creation and closure. Evidence exists to suggest that pores will close quickly after the post-mortem cessation of outflow and the resulting elimination of transmural pressure [Van Buskirk and Grant, 1974]. With the restart of flow during fixation at pressure, pores would begin to open in response to the re-established transmural pressure. In addition, it is possible that the pore formation process is facilitated by a protein that becomes fixed due to its proximity to the cell membrane. A similar process occurs during the formation of coated vesicles in phagocytosis, which is facilitated by the membrane-bound protein clathrin [Albets et al., 1989]. As a result, any pores that are open would be fixed in the open state when exposed to fixative. As volume/time of perfusion with fixative is increased, more pores would have the opportunity to form, and would subsequently be fixed in the open state as soon as they appear. In this manner, a correlation between perfusion volume/time and pore density would be established. After long post-mortem periods (greater than 24 hours), the cells would no longer be functional and thus would not be able to form pores. The perfusion volume/time vs. pore density correlation would therefore not be found with eyes with long post-mortem times.

We also found that the facility decreases approximately by a factor of 2 due to fixation. This change was found to occur rapidly (within approximately 30 minutes of the AC exchange) when anterior chambers were properly exchanged with fixative. For some eyes, the facility decayed over a longer period after the start of constant pressure fixations.
Although the cause of this change was not investigated in this thesis, our results suggest that it is not due to changes in the inner wall pore density. If this were the case, we would expect facility to rise as the fixation at pressure progressed, and the pore density increased.

While there is no correlation between outflow facility and pore density in our data, we cannot rule out the possibility that such a relationship exists in vivo. We have seen that different experimental parameters, including post-mortem time, can significantly affect pore density. There is the possibility that changes occurring immediately after death may affect the pore density dramatically. If this is the case, none of our experiments could provide the true value for pore density under normal physiologic conditions since even the freshest specimen was used over 8 hours post-mortem. Until the pore density in vivo is determined, our conclusions remain slightly suspect. For example, if the physiologic pore density is only 10% of the value measured post-mortem, then the entire normal outflow resistance can be explained by the pores alone. Other post-mortem changes may result in an increase in resistance which masks the effect of increased pore number.

As discussed above, glutaraldehyde does not fix cell membranes, and thus cannot be used to definitively determine pore density. Characterization of the true pore density may require fixation at pressure with osmium tetroxide. Due to the high toxicity of this substance, careful design of experimental protocols and equipment is crucial. Also, due the possibility of post-mortem changes, a definitive determination of pore density will ultimately require in vivo fixation using osmium.
Chapter 6

Conclusions and Future Work

Our goal in this thesis was to characterize the site and mechanism of outflow resistance in normal human and bovine eyes. Our focus was the effect on resistance of aqueous humor proteins flowing through a gel-filled JCT. As well, we have examined the possibility that this resistance may be enhanced by a funneling effect, manifested as a relationship between outflow facility and inner wall pore density. In this chapter, we review the important conclusions from the previous chapters, and discuss the work as a whole. In addition, suggestions for future work are presented.

6.1 Discussion and Conclusions From Previous Chapters

Citrated Plasma Perfusions

Johnson et al. [1993] were able to prevent washout in bovine eyes by perfusing with a solution of 15% serum instead of buffer. However, there was some evidence to indicate that the effect of the serum was not due to the bulk protein concentration [Ritter, 1993], but instead due to the effect of a particular component of serum. Our objective in this portion of the thesis was to determine, by perfusing bovine eyes with 15% citrated plasma solutions, if there was a by-product of the clotting process in serum that prevented washout.

Perfusions with 15% citrated plasma solutions were expected to have the same effect on facility as perfusions with 15% serum solutions. However, the results of our experiments suggested that the outflow facility during perfusions with 15% citrated plasma was not significantly different than during perfusions with buffer. This was likely due to the effect on facility of the chelating agent, sodium citrate. Previous researchers [Bill et al.,
1980; Hamanaka and Bill, 1987] found that perfusion with the chelating agent Na₂EDTA resulted in increased outflow facility. It was suggested that the chelator was removing of calcium from the tight junctions between endothelial cells, creating artefactual openings (pores) in the inner wall, and causing an increase in facility. Thus, the effect of plasma on outflow facility was likely being masked by the effect of the sodium citrate. Little useful information concerning the effect of proteins on outflow facility could be learned from further citrated plasma perfusions.

**Soluble Proteins in the Trabecular Meshwork**

Our goal in this section of the thesis was to evaluate the role of aqueous humor proteins in generating outflow resistance. In particular, we wanted to determine if there was a relationship between protein concentration in the fluid passing through the trabecular meshwork and washout in bovine eyes, and the lack of washout in human eyes. A series of perfusion experiments were run, on human and bovine eyes, in which the perfusate was collected and assayed for protein content.

Statistically significant correlations were found between protein concentration and volume of fluid perfused for both bovine and human eyes. However, the volume constant with respect to typical perfusion volumes during an experiment was much smaller for the bovine than the human. Over the course of a typical experiment, the protein concentration in the bovine perfusate would be expected to drop by about 65%, while the protein concentration in human perfusate would only drop about 15%. This was consistent with notion that proteins control outflow resistance, and washout is caused by the depletion of proteins. The lack of a measurable washout effect in humans could simply be due to the very slight decrease in outflow resistance associated with the slight decrease in protein concentration.
A statistically significant correlation was found between outflow resistance and volume perfused for bovine eyes, based on an exponential model of resistance decay. However, the volume constant was significantly different than the volume constant from the protein concentration vs. volume perfused correlation, suggesting that outflow resistance and protein concentration were governed by different mechanisms (since they decayed at different rates). For human eyes, no statistically significant correlation was found between outflow resistance and volume perfused using the exponential model of resistance decay. This was consistent with lack of washout found using the polynomial model of outflow facility vs. volume perfused, as well as the lack of washout in human eyes found by previous researchers [Erickson-Lamy et al., 1990]. However, this may have been due to the slow rate of decay of protein concentration (and consequently outflow resistance) in human eyes.

A statistically significant correlation was also found between outflow resistance and protein concentration for bovine eyes. For human eyes, a marginally significant correlation was found between initial outflow resistance and initial protein concentration. Both of these findings were consistent with notion that aqueous humor proteins control outflow resistance. However, the initial protein concentration of perfusate in bovine eyes was found to be much less than the 15% serum solution required by Johnson et al. [1993] to prevent washout. This raised the possibility that outflow resistance was not a function of protein concentration; instead protein concentration was a function of facility controlled by some other mechanism within the bovine eye. In contrast, the perfusate protein concentration in human eyes was very close to the 5% serum used by Kee et al. [1995] to significantly reduce washout in monkey eyes. Along with the finding that protein concentration in human eyes decayed much more slowly than in bovine eyes, our results suggested that outflow facility in human and bovine eyes may be governed by different mechanisms.
In order to investigate the possibility that outflow facility was not a function of aqueous humor protein concentration, but instead perfusate protein concentration was a function of flowrate and the time history of the perfusion, a series of modeling studies were performed. A conceptual model was developed of a depleting protein depot from which protein was assumed to be added to the aqueous humor just before entering the trabecular meshwork. The experimental data was found to be consistent with physiologic parameters (including length scales and protein diffusivity) for the ciliary body. This was consistent with the idea that perfusate protein concentration decayed over the course of the perfusion simply due to depletion of the ciliary body protein depot. Also, for our flow regime, the mass transfer coefficient from the depot into the aqueous humor stream was found to be largely independent of the fluid velocity. The bulk protein concentration of the fluid passing through the trabecular meshwork would therefore be determined directly by the volume flowrate of fluid.

In order to further investigate the possibility that bulk protein concentration in aqueous humor does not control outflow facility, we used a model of protein hindrance within a GAG matrix to determine if the measured protein levels could generate the experimentally observed outflow resistance. For both human and bovine eyes, it was found that a matrix longer much longer than the entire trabecular meshwork would be required to generate the measured resistance. Thus, it is unlikely that the bulk protein concentration of the fluid passing through the trabecular meshwork is responsible for controlling overall outflow facility.

**Pore Density and Its Relationship to Aqueous Outflow Facility**

The final aspect of this thesis explored the relationship between inner wall pore density and aqueous humor outflow facility. Allingham et al. [1992] found a statistically significant correlation between pore density and outflow facility using constant pressure
perfusions, which we sought to confirm using constant flow perfusions. The existence of this correlation would suggest the presence of a funneling effect [Johnson et al., 1992] that could signficantly enhance resistance within the JCT. As a result, the resistance effect of protein hindrance within a GAG matrix might also be significantly enhanced, providing a possible alternate explanation for the experimental results found during the study of soluble proteins in the trabecular meshwork. Specifically, changes in the soluble protein concentration of the fluid passing through the trabecular meshwork could alter the outflow resistance, resulting in the washout effect and other correlations found in the soluble protein study of Chapter 3.

However, no correlation was found between pore density and either pre-fixation facility or post-fixation facility, suggesting the lack of a funneling effect. Instead, several factors were found to artefactually affect the pore density. When all the eyes used in the study were considered, a correlation was found between pore density and post-mortem time. However, no correlation was found between pore density and post-mortem time if only eyes with post-mortem times less than 20 hours were considered. This suggested that cell death was occurring about 20 hours after the cessation of flow, and pores could no longer be formed.

When only relatively fresh eyes (post-mortem time less than 20 hours) were considered, further possible artefactual influences on pore density were found. Statistically significant correlations were identified between pore density and volume of fixative perfused, as well as pore density and duration of fixation at pressure. As well, re-examination of the data from Allingham et al. [1992] (which also included relatively fresh eyes) indicated that the results from that study were likely due to a correlation of volume/duration of fixation with pore density, or due to a correlation of flowrate with pore density. The implica-
tion of these findings was that the pore densities measured in typical perfusion experiments were not representative of the pore densities in vivo.

Summary of Conclusions

1. Aqueous humor outflow facility is not controlled by bulk protein concentration, and cannot be accounted for by the simple steric hindrance of protein molecules in a GAG matrix. Instead, facility is likely controlled (or at least affected) by a specific component of aqueous humor, involving a complex protein interaction.

2. Protein concentration in the bovine trabecular meshwork is much lower than the 15% serum required by Johnson et al. [1993] to prevent washout.

3. The lack of washout in human eyes is likely due to a much slower rate of depletion of a specific resistance causing protein than bovine eyes.

4. The pores of the inner wall endothelium of Schlemm's canal are passive resistive elements which do not significantly influence the total facility of the aqueous outflow system, directly or indirectly.

5. Pore density is altered artefactually by parameters not usually controlled in perfusion experiments. The most important of these are volume of fixative perfused and/or duration of fixation at pressure, and post-mortem time to start of perfusion.

6.2 Recommendations for Future Work

Aqueous Humor Proteins

The purpose of the perfusions with citrated plasma was to evaluate the importance of by-products of the clotting cascade in eliminating washout. Unfortunately we have not been able to answer this question, and are thus have not narrowed down the possible components of serum which may be generating outflow resistance. Although it may be possible to attempt using other anticoagulants besides sodium citrate, the widely used GAG
heparin has already been ruled out. Another possibility is the use of coumarins, which act as vitamin K antagonists, and hence interrupt the clotting cascade [Vermeer, 1986]. However, a preliminary study of the effect on facility of coumarins themselves is necessary before they can be used as anticoagulants in perfusions with plasma solutions.

The investigation of the role of soluble proteins in the trabecular meshwork on outflow resistance suggested the possibility that a particular component of aqueous humor might be responsible for controlling outflow resistance. This component might also be present in serum (in lower concentrations), and may be responsible for the elimination of washout in bovine eyes during perfusions with 15% serum solutions [Johnson et al., 1993]. As a broader goal then, the specific resistance generating components of serum solutions will need to be identified, even though they may not be just by-products of the clotting cascade. As well, a more sophisticated model is required in order to determine the true role that proteins play in generating outflow resistance. This model will need to include not only the steric hindrance of proteins by a fixed matrix, but also the interaction of the resistance controlling molecules with other proteins.

**Pores of the Inner Wall Endothelium**

The key question that remains to be answered concerning the inner wall pores is: *What is the pore density under physiologic conditions?* We have found evidence for artefactual effects on the pore density of enucleated eyes. It is possible that the pore density *in vivo* is much less than the pore density of enucleated eyes fixed at pressure with glutaraldehyde. If this is the case, then the entire normal outflow resistance might be explained by the resistance generated by pores. Other post-mortem changes may result in an increase in resistance which masks the effect of increased pore number.

Since glutaraldehyde does not fix cell membranes, characterization of the true pore density may require fixation at pressure with osmium tetroxide. However, due the possi-
bility of post-mortem changes, a definitive determination of pore density will ultimately require \textit{in vivo} fixation using osmium tetroxide solutions.
Appendix A

Common Experimental Procedures

All the experiments performed in this thesis involved the perfusion of enucleated eyes. Procedures which were common to all the experiments are described in this section.

A.1 Procurement of Tissue

Human eyes were obtained from eye banks through the National Disease Research Interchange (NDRI) of Philadelphia, PA. Eyes were transported under moist chamber conditions, in an insulated container, and packed with ice. Human tissue was typically used within 30 hours post-mortem. Eyes were excluded from the studies if they met any of the following criteria: 1) any type of ophthalmic surgery; and 2) eyes from diabetic donors.

Bovine eyes were obtained from 2-4 week old calves from a local abattoir (Arena & Son’s of Hopkington, MA). Eyes were transported in isotonic saline packed in ice, and were typically used within 6 hours of enucleation.

A.2 Cleaning Procedures

The outflow pathway of the eye can be thought of as a filter system which can be blocked by the passage of particulates. Care was required to ensure that all fluids perfused into the eye were as free of contaminants as possible in order to prevent blockage and the resulting artefactual increase in resistance.

Ultraclean water was produced by filtering distilled water through a 0.08 μm Nucleopore polycarbonate membrane (Nucleopore Corp., Pleasanton, CA) to remove any particulates. All glassware, tubing, syringes, valves, and needles were flushed with ultraclean water. The tubing used was sterile, disposable pressure tubing (0.050 in. ID) from Mallinckrodt Anesthesia Products (St. Louis, MO). The valves used were sterile, dispos-
able four-way stopcocks from Mallinckrodt Critical Care (Glen Falls, NY). Cleaned equipment was protected from contamination by covering with Parafilm M (American National Can Co., Greenwich, CT).

All perfusion fluids were sterilized by filtration through a 0.2 μm Costar, cellulose acetate bottle top filter (Costar Scientific Corp., Cambridge, MA). In addition, all perfusion fluids were passed through a 0.2 μm Uniflo cellulose acetate syringe filter (Schleicher & Schuell, Inc., Keene, NH) prior to being infused into the eye.

A.3 General Perfusion Setup

The general setup of the perfusion apparatus is shown schematically in Figure A.3.1 (photographs of the setup are shown in Figure A.3.2 and Figure A.3.3). Pressure tubing and stopcocks were filled with perfusion fluid starting from the stopcock at the syringe pump, and filling downstream towards the eye. At the stopcock closest to the eye, an infusion needle, or cannula, was attached (23 gauge for bovine, and 25 gauge for human, both from Becton Dickinson Vascular Access, Sandy, UT) to one port, and the tubing to the perfusion reservoir attached to the other. Each piece of tubing was added just before it was filled, which helped to minimize the occurrence of air bubbles in the lines. If necessary, an exchange reservoir was set up with tubing running to a second infusion needle of the same gauge. The exchange reservoir was initially filled with a small amount of the perfusion fluid, and clamped off using a tubing clamp prior to use. The perfusion reservoir was filled completely with perfusion fluid. Both the perfusion and exchange reservoirs were adjusted to the experimental heights. The tubing from the pressure transducer was filled with distilled water and connected to the stopcock by the syringe pump. The syringes for the perfusion pumps were filled with perfusion fluid and attached to the appropriate stopcock. Perfusion from the pump used a 10 ml gas-tight syringe for bovine, and a 500 μl gas-tight syringe for humans (both from Hamilton Co., Reno, NV).
Each eye was placed in a 50 ml beaker filled with isotonic saline (Baxter Healthcare Corp., Deerfield, IL). The beakers were partially filled with cotton gauze in order to elevate the eye to just below the top of the beaker, as well as to minimize movement of the eye. (Note that a different procedure was used for the studies of soluble proteins in the trabecular meshwork, Section 3.1). The beaker was then placed in a water bath at 34 °C to approximate physiologic temperatures in the eye. Cold cataracts in bovine were allowed to clear prior to cannulation. The tip of the infusion needle connected to the syringe pump was bent to about a 45° angle. Flow from the perfusion reservoir was started, and the needle was inserted through the cornea close to the limbus, threaded through the pupil, and
Figure A.3.2: Photograph of calf eyes being perfused (protein experiment)

Figure A.3.3: Photograph of the perfusion apparatus
placed in the posterior chamber. The needle was rotated so that the bent tip lifted the iris slightly to allow pressure equilibration between the anterior and posterior chambers. This helped to prevent the phenomenon of anterior chamber deepening which artificially elevates the facility [Moses, 1977]. In addition, care was taken to ensure that the needle was not resting on the lens, as this would pull on the zonular fibers, opening up the meshwork, and again artificially elevating the facility. If the desired position of the needle was not inherently stable (due to torsion of the tubing), it could be held in place by clamping the infusion set just behind the needle using a magnetic Flexbar with an alligator clamp attached (Tom Thumb Flexbar, cat. #55145, Stoelting, Wood Dale, IL).

If AC exchanges were required, flow was started from the exchange reservoir, and the exchange needle was also inserted through the cornea near the limbus, but the tip was left in the anterior chamber. A slight bend to the tip of the needle helped to ensure that the needle was not resting on the lens.

For all experiments, eyes were perfused from the perfusion reservoir at a pressure of 10 mmHg for about 10 minutes prior to the start of facility measurements. This allowed the eyes to “fill” quickly, avoiding the large overshoots that would result in constant pressure perfusions (as the flowrate was ramped up to achieve the steady state pressure), or the long transients (settling times) that would result from constant flow perfusions.

**A.4 Data Acquisition and Control**

The data acquisition and control (DAC) system is similar to that used by Whale [1992]. Instantaneous pressure and flowrate measurements were recorded, allowing continuous calculation and display of outflow facility. We shall give a brief overview of the system, highlighting any differences.
The DAC system is shown in Figure A.3.1. Intraocular pressures were measured using pressure transducers (Microswitch pressure sensor, 142PC05G, Brownell Co., Woburn, MA) connected via tubing to the cannulae. Harvard Apparatus (Harvard Apparatus, Inc., South Natick, MA) model 22 syringe pumps were used to deliver a digitally controlled flowrate, as determined by the control program. The perfusion system was calibrated prior to each experiment.

The data acquisition system is based on the *Perfuser* program [Whale, 1992], running on a Macintosh SE computer. This program controls the flowrate from the syringe pumps, maintaining either a constant pressure or a constant flowrate, as required. Pressure readings from the transducers are recorded, along with time, and flowrate. Pressure signals were digitized using a MacADIOS-8ain A/D converter (GW Instruments, Inc., Somerville, MA) and fed into the Macintosh SE via the SCSI port. The control algorithm is described in detail by Whale [1992].

### A.5 Selection of Perfusion Parameters

**Perfusion Pressure:**

For constant pressure perfusions in both human and bovine eyes, our selection of perfusion pressure has differed from that of other authors. Previous researchers [Grierson and Lee, 1975; Moseley et al., 1983; Epstein and Rohen, 1991; Allingham et al., 1992; Johnson et al., 1993] have tried to match the average human physiologic intraocular pressure, which is about 15 mmHg [Davson, 1990]. However, the primary area of interest is the outflow pathway of the eye, suggesting that a perfusion pressure should be selected to match the pressure drop that occurs between the anterior chamber and the episcleral veins, which is about 5 mmHg. Unfortunately, at very low IOP, the shape of the eye is not maintained, and the outflow characteristics are subject to change due to a flattening of the meshwork [Johnstone & Grant, 1973; Grierson & Lee, 1974]. Neither of these two
extremes ideally mimics physiologic conditions. The approach taken in these studies was to choose a pressure between these two extremes. The perfusion pressure for constant pressure experiments was thus selected to be 10 mmHg.

**Time to Steady State:**

The eye is a complex viscoelastic organ which can be modeled approximately as a fluid capacitor [Johnson, 1981]. Since we were interested in the steady state conditions in our experiments, it was necessary to consider the time required to pass by any significant system transients. Using typical physiologic properties of the human eye, Johnson [1981] found time constants of about 15.1 minutes for constant flow, and 9.6 minutes for constant pressure. Thus, to reach 99% of steady state from zero initial pressure requires about 5 time constants, or 75 minutes for constant flow, and 45 minutes for constant pressure. These values were assumed to be roughly the same for bovine eyes.

**A.6 Preparation of Modified Karnovsky’s Fixative**

Modified Karnovsky’s fluid is a 2.5% glutaraldehyde, 2.0% paraformaldehyde fixative used for the fixation of all tissues in this thesis. Its preparation is detailed here for reference.

**Preparation of Sorenson’s Phosphate Buffer**

Sorenson’s buffer is a physiologically normal (pH = 7.3) phosphate buffer used as the basis of modified Karnovsky’s fluid. Solutions were prepared using ultraclean water.

A) 0.2 M solution of NaH$_2$PO$_4$·H$_2$O (Sodium phosphate monobasic, Mallinckrodt Specialty Chemicals Co., Paris, KY) was prepared using 27.6 g per liter of H$_2$O.

B) 0.2 M solution of Na$_2$HPO$_4$ (Sodium phosphate dibasic anhydrous, Mallinckrodt Specialty Chemicals Co., Paris, KY) was prepared using 28.4 g per liter of H$_2$O.

Solutions were sterilized via filtration through a 0.2 µm Costar, cellulose acetate bottle top filter (Costar Scientific Corp., Cambridge, MA). Each 100 ml of Sorenson’s buffer was
prepared by mixing 23 ml of solution A with 77 ml of solution B.

**Preparation of Fixative**

A 10% paraformaldehyde (purified grade, Fisher Scientific, Pittsburgh, PA) was prepared using the following protocol:

1) 2.0 g of paraformaldehyde was added to 20 ml of H$_2$O.

2) One to two drops of 0.1 M NaOH solution was added as a catalyst for the solvolysis of the paraformaldehyde.

3) The mixture was heated and stirred under a fume hood until the solution was clear (about 30 minutes).

4) The solution was cooled and filter through a general purpose cellulose filter (S&S filter paper, 9 cm, Schleicher & Schuell, Keene, NH).

Modified Karnovsky’s fixative preparation was completed by adding glutaraldehyde (50% solution, EM Grade, Electron Microscopy Sciences, Fort Washington, PA) to the above solutions in the following proportions:

1) 10% paraformaldehyde solution 12.5 ml

2) Sorenson’s buffer 20.0 ml

3) 50% glutaraldehyde 2.5 ml

4) Ultracean H$_2$O 15.0 ml

50.0 ml fixative

The pH of the fixative was checked using a pH meter (Model SA 720, Orion Research Inc., Boston, MA) to ensure that pH = 7.3. The final fixative solution was stored in a refrigerator and used within 24 hours of preparation.
Appendix B

Summary of Experimental Data

The complete results of the experiments are given in this section. Where appropriate, the standard errors are given along with the mean value. Both linear and non-linear regressions were performed in analyzing the data. The computer program Kaleidagraph™ (Version 3.0.1, Abelbeck Software, distributed by Synergy Software, Reading, PA) was used in non-linear curve fitting and regression analysis, while the program SYSTAT (Macintosh Version 5.2, SYSTAT, Inc., Evanston, IL) was used for linear single and multivariate regression analysis.

B.1 Citrated Plasma Perfusions

The outflow facility vs. volume perfused curves from the 15% citrated plasma perfusions are given in this section.

![Graph showing outflow facility vs. volume perfused curves for 15% citrated plasma and DBG.]

**Figure B.1.1:** 15% citrated plasma vs. buffer (09Sept93)
Figure B.1.2: 15% citrated plasma vs. buffer (21Sept93)

Figure B.1.3: 15% citrated plasma vs. buffer (23Sept93)
Figure B.1.4: 15% citrated plasma vs. buffer (30Nov93)

Figure B.1.5: 15% citrated plasma vs. citrated buffer (01Feb94)
B.2 Soluble Proteins in the Trabecular Meshwork
Bovine and Human Protein Data

In this section, the data from the investigations into the role of soluble proteins in the trabecular meshwork on outflow facility (Chapter 3 and Chapter 4) are presented. Since each eye was treated individually (contralateral eye was not used as a control), data is presented for each individual eye. Four sets of data, and the associated best-fit curve used in the analysis of the data, are given below for each bovine and human eyes: (1) Outflow facility vs. volume perfused; (2) Perfusate protein concentration vs. volume perfused; (3) Outflow resistance vs. volume perfused; and (4) Outflow resistance vs. perfusate protein concentration.
Figure B.2.1: Human Protein Data - 20Oct94#0
Figure B.2.2: Human Protein Data - 20Oct94#1
**Figure B.2.3: Human Protein Data - 27Jan95#0**
Figure B.2.4: Human Protein Data - 01Feb95#G
Figure B.2.5: Human Protein Data - 01Feb95#1
Figure B.2.6: Human Protein Data - 02Feb95#0
Figure B.2.7: Human Protein Data - 02Feb95#1
Figure B.2.8: Human Protein Data - 24Feb95#0
Figure B.2.9: Human Protein Data - 24Feb95#1
Figure B.2.10: Bovine Protein Data - 17Mar94#0
Figure B.2.11: Bovine Protein Data - 17Mar94#1
Figure B.2.12: Bovine Protein Data - 31Mar94#0
Figure B.2.13: Bovine Protein Data - 31Mar94#1
Figure B.2.14: Bovine Protein Data - 17May94#0
Figure B.2.15: Bovine Protein Data - 17May94#1
Figure B.2.16: Bovine Protein Data - 24May94#0
Figure B.2.17: Bovine Protein Data - 24May94#1
Figure B.2.18: Bovine Protein Data - 02Jun94#0
Figure B.2.19: Bovine Protein Data - 02Jun94#1
Figure B.2.20: Bovine Protein Data - 21Jun94#0
Figure B.2.21: Bovine Protein Data - 21Jun94#1
Figure B.2.22: Bovine Protein Data - 21Jul94#0
Figure B.2.23: Bovine Protein Data - 21Jul94#1
Figure B.2.24: Bovine Protein Data - 23Aug94#0
**Figure B.2.25: Bovine Protein Data - 08Sep94#1**
Bovine GAG Data

For 10 bovine eyes, the perfusate was assayed for sulphated GAG concentration. This data is presented here as sulphated GAG concentration vs. volume perfused curves.

Figure B.2.26: Sulphated GAG concentration vs. volume perfused (31Mar94)
Figure B.2.27: Sulphated GAG concentration vs. volume perfused (17May94)

Figure B.2.28: Sulphated GAG concentration vs. volume perfused (24May94)
Figure B.2.29: Sulphated GAG concentration vs. volume perfused (31May94)

Figure B.2.30: Sulphated GAG concentration vs. volume perfused (02Jun94)
### B.3 Pores of the Inner Wall of Schlemm’s Canal

Data From Current Study

**Table B.1: Data From Pore Study - Part 1**

<table>
<thead>
<tr>
<th>Eye no.</th>
<th>Donor Age</th>
<th>Pore Density (pores/mm²)</th>
<th>Average Pore Diameter (µm)</th>
<th>Post-Mortem Time (hours)</th>
<th>Steady State Pressure (mmHg)</th>
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</thead>
<tbody>
<tr>
<td>16Mar94#0</td>
<td>86</td>
<td>1316.9</td>
<td>0.62</td>
<td>14.33</td>
<td>43.0</td>
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<td>2829.6</td>
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</tr>
<tr>
<td>06May94#0*</td>
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<td>0.74</td>
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<td>64.5</td>
</tr>
<tr>
<td>06May94#1*</td>
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<td>2366.7</td>
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<td>2391.2</td>
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<td>7.5</td>
</tr>
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</table>

* POAG eyes - not used in calculations
Table B.2: Data From Pore Study - Part 2

<table>
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<th>Eye no.</th>
<th>Pre-Fixation Facility (µl/min)/mmHg</th>
<th>Post-Fixation Facility (µl/min)/mmHg</th>
<th>Duration of Fixation (min)</th>
<th>Fixative Volume Perfused (ml)</th>
<th>Buffer Volume Perfused (ml)</th>
</tr>
</thead>
<tbody>
<tr>
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<td>0.115</td>
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<tr>
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<td>0.138</td>
<td>0.290</td>
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* POAG eyes - not used in calculations
### Table B.3: Unpublished Data From Allingham

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<th>Post-Fixation Facility (µl/min)/mmHg</th>
<th>Duration of Fixation (min)</th>
<th>Fixative Volume Perfused (ml)</th>
<th>Post-Mortem Time (hours)</th>
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References


